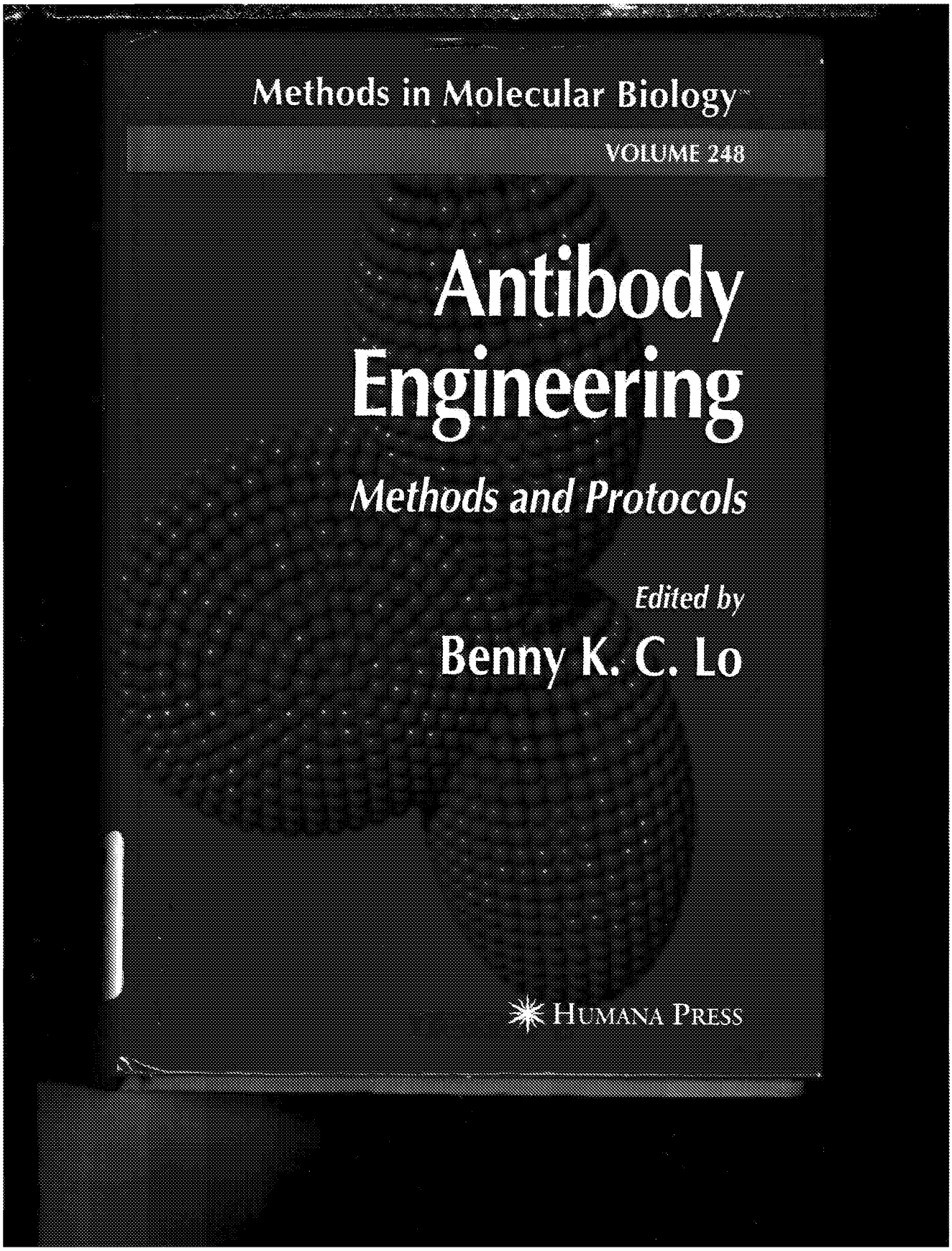


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METHODS IN MOLECULAR BIOLOGY™

Antibody Engineering

Methods and Protocols

Edited by

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Antibody Humanization by CDR Grafting

Benny K. C. Lo

1. Introduction

The goal of antibody humanization is to engineer a monoclonal antibody (MAb) raised in a nonhuman species into one that is less immunogenic when administered to humans. The development of this technology had transformed the stagnant state of antibody therapeutics in the 1980s, when the major obstacle was the human anti-murine antibody (HAMA) response *(1, 2)*. The HAMA response occurred in up to 50% of patients upon the administration of murine hybridoma-derived antibodies *(3)* and severely compromised the safety, efficacy, and biological half-life of these reagents. In addition, murine antibody constant regions are inefficient in directing suitable human immune effector functions for therapeutic effects. Efforts to produce human antibodies by hybridoma technology *(4)* and Epstein-Barr virus (EBV)-mediated B-lymphocyte transformation *(5)* have met with limited success, but their widespread application is hampered by the lack of robust human hybridoma fusion partners and the instability of EBV-transformed clones, respectively (*see* **ref. *6–8*** for review). Consequently, humanization technology was well-placed to exploit the plethora of murine MAbs against a variety of disease targets and turn them into effective clinical reagents.

Two methods were developed in the mid-1980s as attempts to reduce the immunogenicity of murine antibodies: chimerization *(9–11)* and humanization by CDR grafting *(12–14)* (*see* **Fig. 1A**). In mouse/human chimeric antibodies, the immunogenic murine constant domains (66% of total sequence) are replaced by the human counterpart to reduce the likelihood of eliciting the HAMA response. At the same time, intact murine variable domains are preserved to maintain the intrinsic antigen-binding affinity. Effector functions of chimeric antibodies may also be programmed by choosing constant domains of different human immunoglobulin isotypes *(15, 16)*. In the clinic, chimeric anti-

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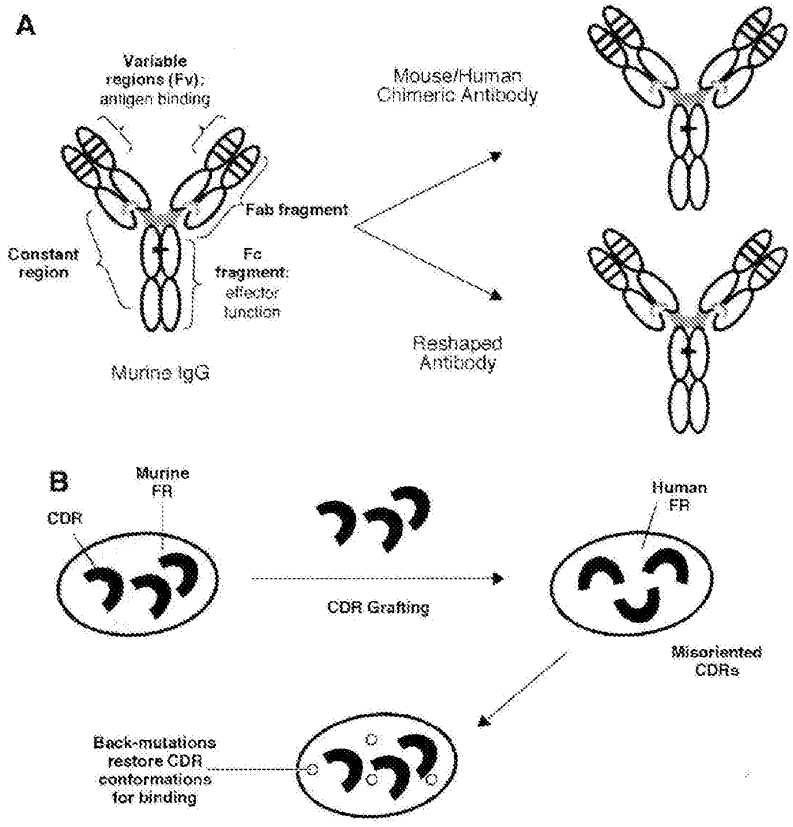


Fig. 1. Antibody humanization and CDR Grafting. **(A)** Mouse/human chimeric antibodies retain the entire Fv regions from the murine antibody (66% human). Reshaped antibodies, on the other hand, only retain the CDRs and a few framework residues from the murine antibody (90–95% human), making them minimally immunogenic. (Shaded ovals: murine sequences; blank ovals: human sequences; black lines across ovals: murine CDRs). **(B)** The transplantation of murine CDRs onto a human framework (FR) often lead to suboptimal orientations of these binding loops. Consequently, critical murine framework residues need to be re-introduced as back-mutations to restore the optimum CDR conformations for antigen binding.

bodies have been successfully used in the treatment of Crohns disease (Remicade®), non-Hodgkin's lymphoma (Rituxan®), and renal transplant rejection (Simulect®). Although the chimerization of some murine antibodies led to the disappearance of the HAMA response, others remained immunogenic *(3)*,

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partly because of the presence of murine epitopes in the Fv. As an alternative approach, Dr. Greg Winter and colleagues at the Medical Research Council, Cambridge pioneered the complementarity-determining region (CDR) grafting technology *(12–14)* in which the antigen-binding specificity of a murine antibody is transferred to a human antibody by replacing the CDR loops and selected variable-region framework residues. The reshaped, humanized antibody only retained the essential binding elements from the murine antibody (5–10% of total sequence) and was predicted to be minimally immunogenic. Indeed, the in vivo tolerance and efficacy of many humanized antibodies have been shown to be more favorable than murine antibodies. Clinical trials of four such antibodies—Hu2PLAP *(17)*, hMN-14 *(18)*, CAMPATH-1H *(19)*, and HuOKT3 *(20)*—demonstrated minimal or no detectable HAMA response after intravenous administration, thus establishing the potential for therapeutic use. A number of humanized antibodies have recently reached the market for diseases such as cancer (Herceptin®, Myelotarg®, Campath®), allograft rejection (Zenapax®) and viral infection (Synagis®) (*see* **ref. *21*** for review). These successes provide further evidence of the power of CDR grafting and antibody-based reagents in complementing traditional chemotherapeutic drugs.

Despite the development of molecular display technologies (Chapters 8 and 9) and transgenic animals (Chapter 10) for the generation of fully human antibodies, CDR grafting remains an attractive and proven strategy for turning well-characterized, highly specific murine antibodies into clinical reagents. The vast amounts of data often available for the parent murine antibody should greatly facilitate the movement of the humanized antibody from the laboratory to the clinic. Furthermore, the grafting of naturally evolved CDRs onto human antibody frameworks eliminates the need for the generation of a completely new human antibody with methods such as in vitro library selection, affinity maturation, or further immunization of transgenic animals.

1.1. Structural Analysis of the Murine Fv Region

The key to a successful CDR grafting experiment lies in the preservation of the murine CDR conformations in the reshaped antibody for antigen binding. This can benefit from a detailed analysis of the structure and sequence of the murine Fv. The antibody Fv region comprises variable domains from the light chain (V_L) and heavy chain (V_H) and confers antibodies with antigen-binding specificity and affinity. The variable domains adopt the immunoglobulin fold in which two antiparallel β-sheet framework scaffolds support three hypervariable CDRs. Structural variation between antibodies is dependent on the length, sequence, conformation, and relative disposition of the CDRs, and the pairing of CDRs from repertoires of V_L and V_H sequences. These parameters create the unique topography of each combining site and form the basis for specific antigen recognition. Although anti-

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gen binding is predominantly regulated by CDR residues, framework residues may also contribute, either through direct antigen contact *(22)* or indirectly through packing with CDR residues. The implication for CDR grafting is that it may be necessary to revert one or more human framework residues to the murine equivalents in the reshaped antibody (so-called framework back-mutations; *see* **Fig. 1B**) to restore the native biomolecular environment for antigen binding *(13)*.

The fact that grafted murine CDRs are able to function on human antibody frameworks is primarily made possible by the highly conserved structure of antibody framework regions and the limited repertoire of backbone conformations in five of the six hypervariable regions. These canonical structures, described by Dr. Cyrus Chothia and colleagues *(23–26)*, were identified for CDRs 1, 2, and 3 of V_L and CDRs 1 and 2 of V_H. Each conformation is dependent on the interactions of a few conserved residues at specific sites, both within the CDRs and the framework regions. Murine CDRs are thus expected to adopt the same backbone conformation when the basic sequence requirements for the corresponding canonical structures are met by the human frameworks. Moreover, the inherent flexibility of the antigen-binding site may accommodate small imperfections from loop grafting in an induced-fit fashion upon antigen binding *(14*, and references herein). Such conformational self-correction mechanisms have been proposed as the basis for binding, even when considerable differences exist between the antigen-free structures of the murine and reshaped binding sites *(27–29)*. On the whole, these properties of the antibody Fv make CDR grafting a versatile process, providing that the key framework requirements are satisfied.

Framework residues that participate in antigen binding can often be identified by inspecting a three-dimensional (3D) model of the murine Fv for potential framework–CDR and framework–antigen interactions. Ideally, an X-ray crystallographic structure of the antigen–Fv complex is used in such an analysis. If this is unavailable, a predicted structure from molecular modeling is usually sufficient. Molecular models can be generated for both the murine and reshaped forms of the Fv to locate critical framework residues and evaluate the suitability of the human acceptor frameworks. Additional analyses are performed by sequence alignment with other antibodies to reveal unique sequence features. These insights into the murine Fv structure are invaluable for the selection of human acceptor frameworks and, if necessary, the introduction of framework back-mutations to optimize antigen binding.

1.2. Choice of Human Acceptor Frameworks

With the blueprint of critical murine framework residues from structural analysis, database searching is next performed to short-list suitable human antibody frameworks to support the murine CDRs. The inclusion of these critical

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framework residues increases the chance of success in producing a functional reshaped antibody. Depending on the framework selection strategy, one or more such residues may be incorporated into the native acceptor sequence or introduced subsequently by framework mutagenesis. The five major framework selection strategies are summarized here:

1. Fixed frameworks: the same set of human acceptor frameworks is used regardless of sequence homology to the murine antibody. This is particularly advantageous when the chosen frameworks are well-characterized in terms of expression levels and availability of solved crystal structures. Early humanization experiments were performed with frameworks from the human myeloma proteins REI *(30)* and NEWM *(31)* for V_L and V_H, respectively *(12–14,32)*.
2. Homology: in this approach, human antibodies sharing maximum sequence homology to the murine antibody are selected to provide the acceptor frameworks *(33–36)*. Generally speaking, the use of homologous human frameworks is least likely to distort CDR conformations in the reshaped antibody. This strategy also minimizes the need for framework mutagenesis. However, one possible drawback is that the use of individual antibody sequences may include somatic hypermutations, thereby creating potentially immunogenic epitopes.
3. Subgroup consensus: the Kabat database *(37)* catalogs antibody sequences into subgroups of different variable-region families. The consensus sequence of each Kabat subgroup is compiled from the most common residue at all the framework positions. In this strategy, consensus from the human subgroups most homologous to the murine antibody is selected for CDR grafting *(38–42)*. The use of consensus frameworks may reduce the likelihood of immunogenicity as somatic hypermutations are minimized except to the extent that consensus sequences are artificial and may themselves contain unnatural elements that are immunogenic.
4. Germline: the most homologous human immunoglobulin germline sequences *(43–45)* are selected as the frameworks. Since germline sequences originate from un-rearranged immunoglobulin genes, such frameworks should therefore be free from idiosyncratic mutations and are minimally immunogenic. With the availability of the complete human immunoglobulin germline gene locus, this strategy is gaining popularity *(46,47)*.
5. Veneering: also known as "variable region resurfacing," veneering has developed from the view that protein epitopes recognized by antibodies mainly reside on the solvent-exposed surface; murine residues buried inside the protein fold are therefore predicted to be minimally antigenic. Subsequently, all buried antibody framework positions are kept murine while solvent-exposed positions are humanized *(48,49)*. This preserves all buried packing residues in the reshaped antibody that are potentially important for CDR conformations and minimizes the likelihood of antigenic response developed against the solvent-exposed regions. It remains to be seen whether the buried murine residues are immunogenic through the display of degraded antibody fragments as major histocompatibility complex (MHC) class II complexes on antigen-presenting cells, thus inducing a T-cell response.

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Human frameworks for V_L and V_H are often selected from two different antibodies to achieve higher homologies for each chain. This is enabled by the conserved V_L/V_H interface that permits chain pairing and the formation of the antigen-binding site between variable domains from different antibodies. It is even possible to maximize homology by the use of hybrid frameworks for each variable domain *(50)*. Furthermore, the exact location of the framework regions can also vary depending on whether "Kabat CDRs" (**ref. *37***; original Kabat definition of antigen-binding loops based on sequence variability) or "Chothia loops" (**ref. *23***; Chothia definition based on structural loops) are used for grafting. Although these strategies have all resulted in functional reshaped antibodies, the nature of the human framework regions is likely to be structurally and immunologically distinct. It should therefore be emphasized that reshaped antibodies represent a *broad* class of CDR-grafted molecules, and the exact strategy chosen may carry significantly different consequences in the immunogenicity of the engineered molecule.

1.3. Introduction and Analysis of Framework Back-Mutations

The use of carefully selected human frameworks in CDR grafting maximizes the probability of retaining antigen-binding affinity in the reshaped antibody. This was well-exemplified by the anti-mucin antibody HMFG1 *(51)* and the anti-CD56 antibody N901 *(52)*, in which the reshaped antibody retains almost full binding affinity to the target antigen immediately after CDR grafting. However, these examples represent the ideal scenarios and are a minority among the many successfully reshaped antibodies. Often, antigen-binding affinity is greatly reduced or abolished after loop grafting *(38,39,53)*, and is restored only after the re-introduction of murine framework residues as back-mutations. The likelihood of producing a functional antibody usually increases with the number of back-mutations made on the CDR-grafted structure. However, one should aim to limit the number of back-mutations to a minimum, thereby keeping the reshaped antibody as "human" as possible and reducing the possibility of immunogenicity.

The identification of critical framework back-mutations truly represents the art of CDR grafting, as it is frequently the most difficult and unpredictable step in the process. Although the many reshaping experiments available in the literature provide a "rough guide" (see the website "Humanization by Design," http://people.cryst.bbk.ac.uk/~ubcg07s for an excellent database of published humanized antibodies), it is not possible to generalize back-mutations. A number of framework positions are known to be critical and buried residues in close proximity to the CDRs and the antigen-binding surface are generally more important than those that are solvent-exposed and distant from the binding site. Different strategies have been developed to derive the crucial combination of back-mutations to restore antigen binding in a timely manner. They mainly involve the stepwise or simultaneous incorporation of one or more back-muta-

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tions in the reshaped variable domains followed by their evaluation on antigen binding. The conventional approach relies on the use of a structural model to guide the introduction of strategic back-mutations. Alternatively, candidates for back-mutations are included in a combinatorial library of variable domains displayed on filamentous bacteriophage *(54,55)*. Variable domain sequences in the library are synthesized so that both the murine and human residues are represented at selected positions for back-mutation. This allows the simultaneous analysis of a much larger pool of mutants. The best combinations of back-mutations are swiftly identified by panning the phage library for antigen binding, followed by sequencing of the clones of highest affinity.

1.4. Summary

The humanization of murine antibodies by CDR grafting is a major protein-engineering task. The transfer of antigen-binding loops from a naturally evolved murine framework to a different, artificially chosen human framework must be performed so that the native loop conformations are retained for antigen binding. Meanwhile, careful consideration should be given during the design process to minimize the immunogenic potential of the humanized antibody by limiting residual murine elements. Additional measures such as specificity determining residues grafting (**ref.** *56*; *see* also Chapter 21), removal of immunogenic epitopes *(57)*, use of constant regions that do not activate effector functions *(58)*, and the generation of immunological tolerance *(59)* may also be taken. It should be noted that immunogenicity is a complex issue; intrinsic factors such as protein sequence and structure are likely to be as important as extrinsic ones (e.g., dosage, route, and schedule of administration, and patient's immune status). Thus, it is very difficult to predict the immunogenicity of a therapeutic antibody accurately. Only favorable results from clinical studies can confirm the success of a humanization experiment.

In this chapter, protocols are described for the CDR grafting of murine antibodies using germline human antibody frameworks and Kabat CDRs (*see* **Fig. 2**). Since many of the procedures involve standard molecular biology manipulations, emphasis is placed on the design strategy; cross-references to other chapters in this volume are provided where appropriate. Finally, the protocols are written purely from an academic perspective; if the reader is interested in developing a humanized antibody for commercial applications, professional advice should be sought because of the numerous patents covering all aspects of this technology.

2. Materials

2.1. Homology Modeling and Framework Selection

1. Silicon Graphics workstation (SGI, CA).
2. Molecular graphics software (e.g., Insight II; Accelrys, CA).

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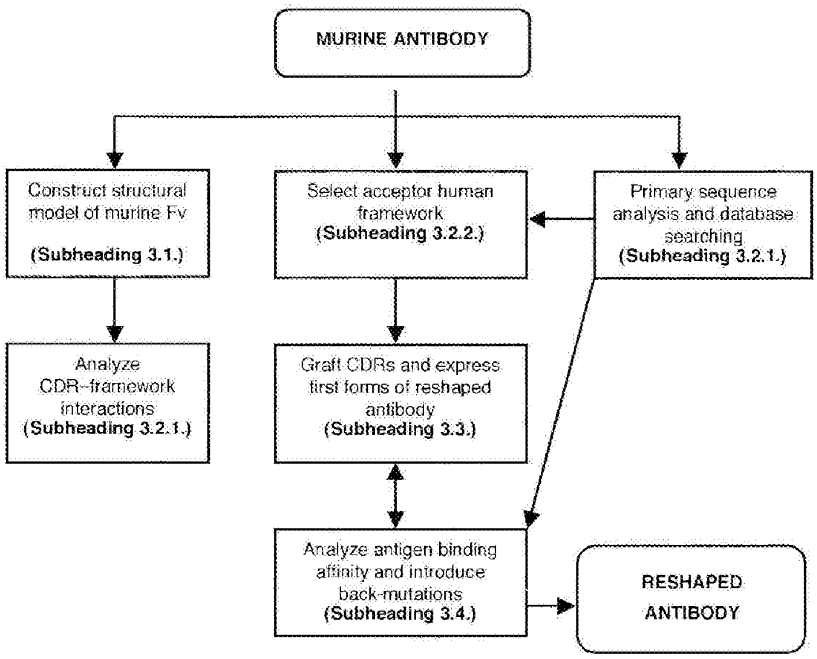


Fig. 2. CDR grafting strategy

3. Homology modeling software:
   a. WAM (**ref. *60***; accessible at http://antibody.bath.ac.uk);
   b. SWISS-MODEL (**ref. *61***; accessible at http://www.expasy.org);
   c. INSIGHT-HOMOLOGY (Accelrys); or
   d. COMPOSER (Tripos, MO).
4. SeqTest (accessible at http://www.bioinf.org.uk/abs/ – under "Sequences").
5. Kabat Database online (**ref. *62***; accessible at http://www.kabatdatabase.com) and printed *(37)* editions (*see* also Chapter 2).
6. Genedoc (**ref. *63***; downloadable from http://www.psc.edu/biomed/genedoc).
7. V-BASE (accessible at http://www.mrc-cpe.cam.ac.uk/vbase).
8. IMGT, the International ImMunoGeneTics Information System (**ref. *64***; accessible at http://imgt.cines.fr; *see* also Chapter 3).

2.2. CDR Grafting and Antibody Expression

1. GCG Wisconsin Package (Accelrys).
2. Thermocycling block for polymerase chain reaction (PCR).
3. PCR reagents: Geneamp 10X PCR buffer with 15 m*M* $MgCl_2$ (Applied Biosystems); AmpliTaq DNA polymerase (Applied Biosystems); 10 m*M* deoxynucleotide

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triphosphate (dNTP) mix (Sigma); high-performance liquid chromatography (HPLC)-purified oligonucleotide primers; sterile, deionized water (H_2O).
4. Low melting-point agarose.
5. Ethidium bromide stock solution 10 mg/mL, store at 4°C.
6. Tris-borate EDTA (TBE) buffer: 90 m*M* Tris-borate, 2 m*M* EDTA in H_2O, autoclave at 121°C for 15 min and store at room temperature.
7. Agarose gel-running apparatus and ultraviolet (UV) transilluminator.
8. Qiaquick gel extraction kit, PCR purification kit, and Qiaprep miniprep kit (Qiagen).
9. TOPO TA cloning kit and One Shot TOP10 chemically competent *E. coli* (Invitrogen).
10. LB-ampicillin agar plates: suspend 40 g of Luria agar (Sigma) in 1 L of H_2O, boil to dissolve, autoclave at 121°C for 15 min, cool to 50°C, add ampicillin to 100 µg/mL, and dispense into Petri dishes. Store at 4°C for up to 3 wk.
11. LB medium: dissolve 25 g of Miller's LB broth (Sigma) in 1 L of H_2O, autoclave at 121°C for 15 min.
12. Restriction endonucleases, T4 DNA ligase, and associated reaction buffers (New England Biolabs).
13. Antibody expression vectors (*see* **Note 1**).
14. Sterile 0.45-µm filters.
15. Sodium azide stock solution 20% (w/v), store at 4°C.

2.3. Binding Analysis and Framework Back-Mutations

1. Nunc Maxisorp 96-well plates (Nalgen Nunc) and adhesive plate sealers.
2. Enzyme-linked immunosorbent assay (ELISA) plate shaker and reader.
3. Phosphate-buffered saline (PBS): 8 m*M* Na_2HPO_4, 1 m*M* KH_2PO_4, 160 m*M* NaCl, 3 m*M* KCl, pH 7.3, autoclaved.
4. PBST: PBS containing 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20).
5. Blocking buffer: PBS containing 1% (w/v) bovine serum albumin (BSA).
6. ELISA buffer: PBS containing 0.2% (w/v) BSA and 0.02% (v/v) Tween 20.
7. Human serum IgG1/κ immunoglobulins (Sigma).
8. Goat anti-human IgG (Fc-specific), affinity-purified (Jackson Immunoresearch).
9. Goat anti-human κ-chain horseradish peroxidase (HRP) conjugate, affinity-purified (Sigma).
10. Goat anti-human IgG γ-chain HRP conjugate (Sigma).
11. TMB substrate solution (Pierce).
12. 1 *M* H_2SO_4.

3. Methods

3.1. Homology Modeling of the Murine Variable Region

The highly homologous nature of antibody Fv makes it convenient to build molecular models using homology modeling methods *(65,66)*. A number of well-established antibody modeling approaches based on homology *(67–69)* and conformational searching *(70–72)* algorithms have been described (*see* **Note 2**).

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Computer software incorporating these methods is also available (e.g., WAM, SWISS-MODEL, INSIGHT-HOMOLOGY, and COMPOSER; see **Subheading 2.1.**). As a first step in CDR grafting, construct a structural model of the murine variable region using one of these programs (if a crystal structure is not available). The reader is encouraged to consult Chapter 4 of this volume for an in-depth discussion on antibody modeling, and Chapter 1 for a list of general molecular graphics and modeling software accessible on the worldwide web.

3.2. Human Acceptor Framework Selection

3.2.1. Structural and Sequence Analysis of Murine Variable Region

1. Assign Kabat numbering *(37)* to the murine Fv using the program SeqTest and determine the CDR canonical structures for L1, L2, L3, H1, and H2 using **Table 1.**
2. Inspect a 3D model of the murine Fv (crystal structure or molecular model from **Subheading 3.1.**) for potential framework-CDR and framework-antigen interactions. Focus initially on five groups of critical framework residues with special significance (**Table 2**). Take note of framework residues that are in the immediate vicinity of the six CDRs and those on the antigen-binding surface.
3. Determine the Kabat subgroup of the V_L and V_H sequences using the Subgroup tool from Kabat Database online. Proceed to the printed edition of the Kabat Database and manually align the Fv sequence with the subgroup. For each framework residue that differs from the consensus sequence, evaluate its degree of uniqueness within the subgroup and take note of the rare framework residues. These could have arisen from somatic hypermutation, and may contribute to antigen-binding affinity.
4. Load the Fv sequences into sequence alignment programs such as Genedoc, highlight the critical framework residues from **steps 2** and **3** on the Fv sequence and use as the blueprint for short-listing suitable human frameworks for CDR grafting.

3.2.2. Framework Selection

1. Search V-BASE or IMGT for the closest human immunoglobulin germline sequences to the murine V_L and V_H (*see* **Note 3**).
2. Align the translated germline sequences to the sequence blueprint compiled under **Subheading 3.2.1.**
3. Compare the short-listed sequences to the murine Fv in terms of framework sequence identity and the presence of critical framework residues highlighted in the sequence blueprint.
4. Choose, as the human acceptor frameworks, the V_L and V_H germline sequences that share the highest sequence identity with the murine frameworks, especially at the critical framework positions.
5. **Optional:** Construct a molecular model of the reshaped Fv using the murine CDRs and the chosen germline framework sequence (*see* **Subheading 3.1.**). Access the quality of the chosen frameworks by comparing the CDR conformations on the murine and reshaped Fv (*see* **Note 4**). Select alternative acceptor frameworks if necessary.

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Table 1
CDR Canonical Structures and Sequence Requirements

A number of CDR conformations have been observed with five of the six antibody hypervariable regions. Residue positions (Kabat numbering; **ref. *37***) required to maintain the different canonical structures are shown together with the most commonly observed amino acid(s). Residues from the framework regions are shown in bold (reproduced with permission from **ref. *73***).

CDR	Canonical class (loop length)	Residues important for canonical conformation: Kabat position [most common amino acid(s)]
L1	1 (10)[a]	**2**(I), 25(A), 30(V), 33(M, L), **71**(Y)
	2 (11)	**2**(I), 25(A), 29(V, I), 33(L), **71**(F,Y)
	2 (12)[b]	**2**(I, N), 25(A), 28(V, I), 33(L), **71**(F,Y)
	3 (17)	**2**(I), 25(S), 27B(V, L), 33(L), **71**(F)
	4 (15)[b]	**2**(I), 25(A), 27B(V), 33(M), **71**(F)
	4 (16)	**2**(V, I), 25(S), 27B(I, L), 33(L), **71**(F)
L2	1 (7)	**48**(I), 51(A, T), 52(S, T), **64**(G)
L3	1 (9)	90(Q, N, H), 95(P)
	2 (9)[a]	90(Q), 94(P)
	3 (8)	90(Q), 95(P)
H1	1 (5)	**24**(A, V, G), **26**[c](G), **27**[c](F, Y), **29**[c](F), 34(M, W, I), **94**(R, K)
	2 (6)	**24**(V, F), **26**[c](G), **27**[c](F, Y, G), **29**[c](I, L), 35(W, C), **94**(R, H)
	3 (7)	**24**(G, F), **26**[c](G), **27**[c](G, F, D), **29**[c](L, I, V), 35A(W, V), **94**(R, H)
H2	1 (16)	55 (G, D), **71**(V, K, R)
	2 (17)	52 (P, T, A), 55(G, S), **71**(A, T, L)
	3 (17)	54(G, S, N), **71**(R)
	4 (19)	54(S), 55(Y), **71**(R)
	5 (18)	52(Y), 54(K), 55(W), **71**(P)

[a] These canonical structures have only been observed in murine antibodies, but not in human antibodies.

[b] Approximately 20% of murine and 25% of human antibodies have 13 residues in canonical structure 2 or 14 residues in canonical structure 4. These minor variations in loop size result in changes at the tip of the L1 loop, but do not significantly alter loop conformation *(24)*.

[c] Residues at H26–H30 are defined as framework residues in the Kabat system, but structurally they are part of the H1 loop as defined by Chothia et al. *(24)*.

3.3. CDR Grafting and Expression of Reshaped Antibodies

Having finalized the acceptor framework sequences for the murine CDRs, the encoding DNA for the reshaped Fv is assembled by PCR. V_L and V_H sequences can be constructed in the form of "expression cassettes," which include additional upstream and downstream sequences to facilitate antibody

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Table 2
Important Framework Positions for Antigen Binding

In addition to CDR residues, five classes of framework residues frequently influence antigen binding directly or indirectly. The actual framework positions (Kabat numbering; **ref. *37***) or sequence motifs for these residues are shown.

Description	Significance	Framework positions	Reference
Vernier zone	A platform of residues directly underneath the CDRs	L2, L4, L35, L36, L46–L49, L64, L66, L68, L69, L71, L98; H2, H27–H30, H47–H49, H67, H69, H71, H73, H78, H93, H94, H103	*(74)*
Canonical residues	Maintenance of CDR canonical structures	See **Table 1** (bold residues)	See **Table 1** (bold residues)
V_L N-terminal residues	Form a contiguous surface with the antigen-binding pocket	L1–L6	*(75)*
Chain-packing residues	Residues buried at the V_L/V_H packing interface; may affect the relative disposition of CDRs	L34, L36, L38, L44, L46, L87, L89, L91, L96, L98; H35, H37, H39, H45, H47, H91, H93, H95, H100[a], H103	*(76)*
Putative N-/O-glycosylation sites	Carbohydrates may affect positioning of antigen in the binding pocket	See footnote[b,c]	

[a] The residue immediately N-terminal to residue H101 is a packing residue. The actual numbering of this residue varies with the number of loop insertions in CDR-H3.

[b] N-glycosylation consensus sequence: N-X-(S/T), where X is any amino acid *(77)*.

[c] No consensus exists for O-glycosylation, but four sequence motifs were found to predict O-glysolation *(78,79)*:

- X-P-X-X: at least one X is T.
- T-X-X-X: at least one X is T.
- X-X-T-X: at least one X is R or K.
- S-X-X-X: at least one X is S.

expression. The exact content of the cassettes will depend on the chosen vector and expression host, and whether the Fv is expressed as a full IgG or a single-chain Fv (scFv) fragment (*see* **Note 1**). Here, a general method (modified from **ref. *73***) is described for the PCR assembly of the reshaped Fv expression cassettes using overlapping oligonucleotides. Further details on antibody expression in *E. coli* and mammalian systems can be found in Chapters 14 and 15,

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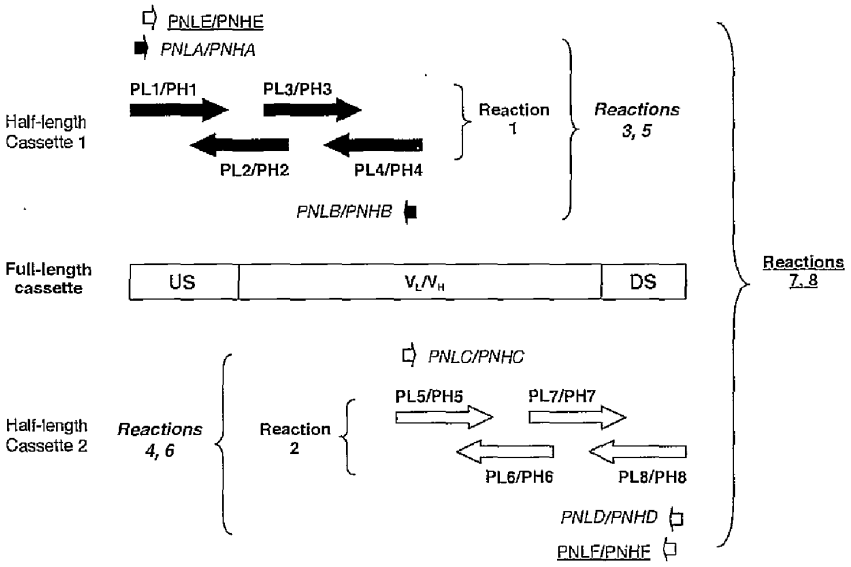


Fig. 3. Construction of reshaped Fv expression cassettes. The reshaped V_L and V_H expression cassettes are each produced by eight overlapping primers (block arrows) in eight PCRs. Overlaps between the megaprimers (PL1–8, PH1–8) are approx 24 bp. US = upstream accessory sequences, DS = downstream accessory sequences (tailored for individual expression vector systems).

respectively. A discussion on the design of monovalent and multivalent antibody fragments is also given in Chapter 12.

3.3.1. Design and Construction of the Reshaped Fv Expression Cassettes

1. Combine the nucleotide sequences of the chosen acceptor frameworks for V_L and V_H (from **Subheading 3.2.2.**) and the corresponding murine CDRs to form the reshaped Fv sequence. Append appropriate restriction sites, up- and downstream sequences as necessary for the chosen expression vector system.
2. Check the expression cassettes for the presence of internal restriction sites and stop codons. Remove these by alternative codon usage where necessary.
3. To construct the reshaped Fv cassettes, design eight partially complementary DNA megaprimers (approx 80 nucleotides each) and six nested primers (24 nucleotides each) covering the entire length of the V_L and V_H cassettes as illustrated in **Fig. 3.**
4. Using the Stemloop program in the GCG Wisconsin Package, check each megaprimer for secondary structure formation. Avoid stemloops with melting temperatures of above 40°C by alternative codon usage.

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5. Set up two sequential sets of four PCRs for the assembly of half-length V_L and V_H expression cassettes (*see* **Note 5**):
   a. Assembly PCRs (V_L reactions 1, 2 and V_H reactions 1, 2):

5 µL	Geneamp 10X PCR buffer with 15 m*M* $MgCl_2$
1 µL	10 m*M* dNTP mix
2.5 pmol each	PL1, 2, 3, 4 (V_L reaction 1); PL5, 6, 7, 8 (V_L reaction 2); PH1, 2, 3, 4 (V_H reaction 1); PH5, 6, 7, 8 (V_H reaction 2) (*see* **Fig. 3**)
2.5 U	AmpliTaq DNA polymerase
+ H_2O to 50 µL	

   Thermocycle: 94°C for 5 min (hot start); 8 cycles of 94°C for 2 min and 72°C for 5 min; and final extension at 72°C for 10 min.
   b. Amplification PCRs (V_L reactions 3, 4, and V_H reactions 3, 4):

5 µL	Geneamp 10X PCR buffer with 15 m*M* $MgCl_2$
1 µL	10 m*M* dNTP mix
5 µL	PCR product from V_L reaction 1 (V_L reaction 3), V_L reaction 2 (V_L reaction 4), V_H reaction 1 (V_H reaction 3) or V_H reaction 2 (V_H reaction 4)
40 pmol each	PNLA and PNLB (V_L reaction 3) PNLC and PNLD (V_L reaction 4) PNHA and PNHB (V_H reaction 3) PNHC and PNHD (V_H reaction 4)
2.5 U	AmpliTaq DNA polymerase
+ H_2O to 50 µL	

   Thermocycle: 94°C for 5 min (hot start); 20 cycles of 94°C for 1.5 min, 64°C for 1.5 min, 72°C for 2.5 min; and final extension at 72°C for 10 min.
6. Visualize reaction products from amplification PCRs on a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide, in 1X TBE buffer.
7. Gel-purify bands of the correct size using the Qiaquick gel extraction kit (V_L: ~225 bp, V_H: ~240 bp; approximate guide only, the exact fragment lengths will depend on the individual antibody sequence and the positioning of primers).
8. Clone half-length expression cassettes into plasmid vector using the TOPO TA cloning kit. Check integrity of constructs by DNA sequencing.
9. Re-amplify the half-length expression cassettes from clones harboring the correct sequence with PCR (V_L reactions 5, 6, and V_H reactions 5, 6):

5 µL	Geneamp 10X PCR buffer with 15 m*M* $MgCl_2$
1 µL	10 mM dNTP mix
40 pmol each	PNLA and PNLB (V_L reaction 5) PNLC and PNLD (V_L reaction 6) PNHA and PNHB (V_H reaction 5) PNHC and PNHD (V_H reaction 6)
2.5 U	AmpliTaq DNA polymerase
+ *E. coli* colony stab of the corresponding cloned half-length expression cassettes	
+ H_2O to 50 µL	

   Thermocycle: as described in **step 5b.**

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10. Visualize reaction products on an agarose gel and gel-purify bands of the correct size (V_L: ~225 bp, V_H: ~240 bp) using the Qiaquick gel extraction kit.
11. Set up two sequential sets of two PCRs to construct the final reshaped expression cassettes:
    a. Assembly PCRs (V_L reaction 7 and V_H reaction 7):

5 µL	Geneamp 10X PCR buffer with 15 m*M* $MgCl_2$
1 µL	10 m*M* dNTP mix
5 µL each	Gel-purified products from V_L reactions 5, 6 (V_L reaction 7) or: V_H reactions 7, 8 (V_H reaction 7)
2.5 U	AmpliTaq DNA polymerase
+ H_2O to 50 µL	

Thermocycle: as described in **step 5a.**

    b. Amplification PCRs (V_L reaction 8 and V_H reaction 8):

5 µL	Geneamp 10x PCR buffer with 15 m*M* $MgCl_2$
1 µL	10 m*M* dNTP mix
5 µL	PCR product from V_L reaction 7 (V_L reaction 8) or V_H reaction 7 (V_H reaction 8)
40 pmol each	PNLE and PNLF (V_L reaction 8) PNHE and PNHF (V_H reaction 8)
2.5 U	AmpliTaq DNA polymerase
+ H_2O to 50 µL	

Thermocycle: as described in **step 5b.**
12. Visualize reaction products on an agarose gel. Gel-purify appropriate-sized bands (V_L: ~450 bp, V_H: ~480 bp) and clone the expression cassettes into plasmid vector using the TOPO TA cloning kit. Check the integrity of the constructs by DNA sequencing.

3.3.2. Construction of the Murine Fv Expression Cassettes

The murine Fv should be expressed in parallel with the reshaped Fv as a control for binding analysis **(Subheading 3.4.)**. If the IgG format is chosen, it can be expressed with human antibody constant domains as a mouse/human chimeric antibody. For the scFv format, it can be simply expressed as a murine scFv fragment (*see* **Note 1**).

1. Append appropriate restriction sites, up- and downstream accessory sequences to the murine Fv sequence with PCR (*see* **Note 6**).
2. Visualize reaction products on an agarose gel, gel-purify bands of the correct size (V_L: ~450 bp, V_H: ~480 bp) and clone the expression cassettes into plasmid vector using the TOPO TA cloning kit. Check the integrity of constructs by DNA sequencing.

3.3.3. Antibody Expression

1. Digest the cloned reshaped and murine Fv expression cassettes with appropriate restriction endonucleases and ligate into the chosen expression vector(s). Arrange

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the inserts in such a way so that four initial antibody constructs can be expressed (*see* **Note 7**):

a. Murine V_L + Murine V_H
b. Reshaped V_L + Reshaped V_H
c. Murine V_L + Reshaped V_H
d. Reshaped V_L + Murine V_H

2. Clone the ligated vectors into *E.coli* and confirm the ligated sequences by DNA sequencing.
3. Transform/transfect these vectors into the expression host of choice and express the four initial antibody constructs.
4. Pass the antibody-containing media through a 0.4-µm filter, add sodium azide to 0.02% (w/v), and store at 4°C.

3.4. Binding Analysis and Framework Back-mutations

The binding activity of the reshaped antibody is accessed using ELISA. A quantification ELISA is first performed to measure antibody concentration in the expression medium; this is followed by a binding assay for the cognate antigen. Sample protocols are described here for the analysis of IgG antibodies; appropriate reagents can be substituted for the analysis of scFv fragments (*see* **Note 8**).

3.4.1. Antibody Quantification ELISA

1. Coat a Nunc Maxisorp 96-well plate with 1 µg/mL affinity-purified goat anti-human IgG (Fc-specific) in PBS (100 µL/well). Cover with an adhesive plate sealer and incubate at 4°C overnight (*see* **Note 9**).
2. Wash wells 4× with 200 µL/well of PBST. Block nonspecific binding sites by adding 200 µL/well of blocking buffer and incubate at room temperature with shaking for 2 h.
3. Discard the blocking buffer. Add, in duplicates, 100 µL/well of standardized concentrations (1 mg/mL and nine additional 1:2 serial dilutions) of purified human serum IgG1/κ in ELISA buffer to the assay plate (Rows A–B). (*see* **Notes 10** and **11**)
4. Add 100 µL/well of antibody-containing medium and nine 1:2 serial dilutions of each in ELISA buffer to the assay plate (Rows C–H). (*see* **Note 11**).
5. Incubate plate at 37°C with shaking for 1 h.
6. Wash wells 4× with 200 µL/well of PBST.
7. Prepare a 1:5,000 dilution of affinity-purified goat anti-human κ-chain horseradish peroxidase (HRP) conjugate in ELISA buffer. Add to the assay plate at 100 µL/well and incubate at 37°C with shaking for 1 h.
8. Wash wells 4× with 200 µL/well of PBST.
9. Add 100 µL/well of TMB substrate solution to the assay plate and incubate at room temperature for 5–10 min, until sufficient color development is reached.
10. Stop color development by adding 50 µL/well of 1 *M* H_2SO_4 and read end-point absorbance values at 450 nm on an ELISA plate reader.

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11. Construct a curve with mean absorbance values from the calibration standards (OD at 450 nm vs log [concentration]). Estimate sample IgG concentrations using absorbance values corresponding to the linear portion of the calibration curve.

3.4.2. Antibody Immunoreactivity ELISA

1. Coat a Nunc Maxisorp 96-well plate with 10 µg/mL antigen solutions in PBS (100 µL/well; *see* **Note 12**). Include the specific antigen(s) bearing the murine antibody epitope and several unrelated antigens to probe for nonspecific binding. Cover with an adhesive plate sealer and incubate at 4°C overnight.
2. Wash wells 4× with 200 µL/well of PBST. Block nonspecific binding sites by adding 200 µL/well of blocking buffer and incubate at room temperature with shaking for 2 h.
3. Discard the blocking buffer. Add antibody-containing medium to the assay plate at 100 µL/well (*see* **Note 13**).
4. Incubate plate at 37°C with shaking for 1 h.
5. Wash wells 4× with 200 µL/well of PBST.
6. Prepare a 1:4,000 dilution of affinity-purified goat anti-human IgG γ-chain HRP conjugate in ELISA buffer. Add to the assay plate at 100 µL/well and incubate at 37°C with shaking for 1 h.
7. Wash wells 4× with 200 µL/well of PBST.
8. Read plate as described under **Subheading 3.4.1., steps 9–10.**

3.4.3. Introduction and Analysis of Framework Back-Mutations

The expression and binding analysis of the four initial antibody constructs (**Subheading 3.3.3.**) should reveal whether back-mutations are required for V_L, V_H, or both variable domains of the reshaped antibody. Using these results as the basis, the following procedure describes the optimization of framework back-mutations by guided selection, a strategy that enables the individual analysis of each of the V_L and V_H mutants before the best ones are selected to form the final reshaped antibody (*see* **Fig. 4**).

1. Select the reshaped variable domain that resulted in a larger drop in antigen-binding affinity for initial mutagenesis. Align this with the sequence blueprint containing the critical murine framework residues (**Subheading 3.2.1.**) to identify which of these are absent from the human acceptor frameworks.
2. Construct hybrid Fvs in which the reshaped mutants are paired with the opposite murine domain. This can be conveniently achieved by mutating vectors constructed in **Subheading 3.3.3., steps 1c** and **1d,** using standard directed mutagenesis techniques (*see* Chapter 18). For example, if back-mutations are introduced to the reshaped V_L domain, prepare the following hybrid Fvs (*see* **Note 14**):
   a. Murine V_H-Reshaped V_L containing all Vernier zone back-mutations;
   b. Murine V_H-Reshaped V_L containing all canonical residue back-mutations;
   c. Murine V_H-Reshaped V_L containing all chain packing back-mutations;

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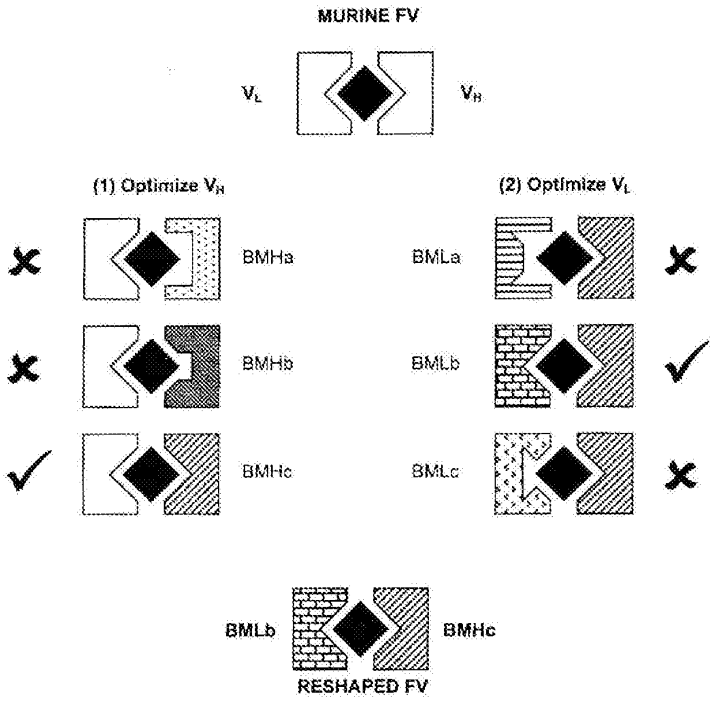


Fig. 4. Optimization of framework back-mutations by guided selection. The best combination of back-mutations can be identified by screening a series of hybrid antibodies for binding to antigen (◆). This figure illustrates the optimization of V_H back-mutations followed by V_L ones (the order can be reversed if appropriate, see under **Subheading 3.4.3.**). The murine V_L is initially expressed in combination with various reshaped V_H mutants (BMHx). The mutant V_L that gives the highest level of specific antigen binding is then used for the optimization of reshaped V_L mutants (BMLx), resulting in the final reshaped Fv.

d. Murine V_H-Reshaped V_L containing all V_L N-terminal back-mutations;
e. Murine V_H-Reshaped V_L containing all rare framework position back-mutations;
f. Murine V_H-Reshaped V_L containing all O- and N-glycosylation back-mutations;
g. Murine V_H-Reshaped V_L containing all of the above back-mutations.

In addition, prepare the following as controls:

a. Murine V_H Reshaped V_L containing no back-mutations; and
b. Murine V_H-Murine V_L.

3. Express these constructs in the expression system of choice (*see* **Note 1**). Perform quantification and immunoreactivity ELISAs as described under **Subheadings 3.4.1.** and **3.4.2.**

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4. Compare specific antigen-binding signals of mutants 2a to 2g with those of mutants 2h and 2i. Evaluate immunoreactivity data to narrow down beneficial back-mutations that restore binding to a level comparable to that of the murine antibody (mutant 2i). Perform further mutagenesis as appropriate to derive the optimum set of back-mutations that fully reconstitutes specific antigen binding (*see* **Note 15**).
5. Repeat **steps 1–4** for the opposite reshaped domain, using the first optimized reshaped domain as a guide. The reshaped mutant that gives maximum antigen binding is chosen as the final reshaped antibody.
6. Characterize the reshaped antibody in terms of its epitope specificity (Chapter 24) and binding affinity (Chapters 25–27), as required. A continuous source of the reshaped antibody (as IgG or scFv) can be established by the stable transfection of a mammalian cell line (e.g., Chinese hamster ovary cells or mouse myeloma NS0 cells) with suitable expression vectors (*see* Chapter 15).

4. Notes

1. For the expression of antibodies as full IgGs, eukaryotic vectors that provide the light- and heavy-chain constant domains are available from AERES Biomedical, London, UK (SuperVector Expression System) and Lonza Biologics, NH, USA (pCON vectors). For expression as single-chain Fv fragments (scFv), common prokaryotic vectors that allow periplasmic protein expression can be used (*see* Chapters 12 and 14 for further details). The supplied literature for these expression systems should indicate the sequence requirements for the expression cassettes (**Subheadings 3.3.1.** and **3.3.2.**).
2. In the homology approach, a structural template(s) is chosen based on sequence homology and length. Conflicts between amino acid side-chains are then resolved by energy minimization. This approach is especially useful for the β-sheet framework and CDR loops with known canonical structures (*see* **Subheading 1.1.**). In contrast, the conformational search approach seeks to build CDR conformations *ab initio* by generating a large number of theoretically possible conformations by varying the dihedral angles of the peptide backbone. The predicted loop conformation is obtained after energetic evaluation of the conformers. This is useful for CDRs that do not belong to any of the canonical classes and those that are poorly modeled by homology alone.
3. V-BASE and IMGT are online databases featuring human immunoglobulin germline sequences. A searchable interface is provided by both databases (V-BASE: DNAPLOT; IMGT: IMGT-VQUEST) for nucleotide sequences. Translation of the germline genes into amino acid sequences is also possible. *See* Chapter 3 for further information on the IMGT database.
4. Although this model provides an early indication on the ability of the chosen germline frameworks to support the CDRs in similar conformations for binding, it should only be taken as an approximate guide, as conformational correction mechanisms have been shown to adjust suboptimal CDR conformations upon binding to antigen (*see* **Subheading 1.1.**).
5. The Fv expression cassettes are initially synthesized as half-length inserts and sequenced before the two halves are stitched together. This allows the early detec-

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tion of PCR artifacts, which are likely at the numerous primer junctions, and avoids the subsequent need to sequence a large number of clones for the correct full-length cassette sequence.

6. Design overhanging primers that are partially complementary to the 5′- and 3′-ends of the murine V_L and V_H gene for the introduction of additional sequences.
7. The shuffling of the murine and reshaped V_L/V_H domains and subsequent analyses of the hybrid constructs will reveal whether the reshaped antibody retains the binding affinity and specificity of the murine antibody and, if not, the CDR grafting of which domain (V_L, V_H, or both) has resulted in the change in binding characteristics.
8. Possible capturing and detection agents include protein L (for kappa light chains) and anti-peptide tag antibodies (e.g., for hexahistidine, c-myc tags).
9. Coated plates can be sealed and stored at 4°C for up to 4 wk.
10. Purified immunoglobulins of a different isotype should be used if the reshaped/chimeric antibodies are not of the IgG1/κ isotype.
11. Serial dilutions can be made conveniently in the wells using a multichannel pipettor.
12. Antigen concentration (10–100 µg/mL) and coating buffer should be optimized if necessary.
13. Antibody supernatants should be diluted in ELISA buffer to equivalent concentrations for direct comparison.
14. Back-mutations of the same type are introduced initially as a group. The expression of these mutants with the opposite guide domain will provide an indication on the location of beneficial back-mutations. If a positive effect is seen within a particular group, further mutants containing different combinations of single back-mutations can be made to dissect out individual contributions. Priorities for individual mutations should be decided largely from results of structural analysis (**Subheading 3.2.1.**). After the analysis of the initial set of mutants, back-mutations of different types can then be combined to reveal any synergistic and/or antagonistic effects on antigen binding. If binding affinity cannot be restored to an acceptable level after back-mutating the critical framework positions, further back-mutations can be introduced at the remaining framework positions, again guided by the structural model of the murine Fv.
15. One should aim to achieve the maximum antigen-binding signal with the fewest number of back-mutations to keep the reshaped antibody as human as possible.

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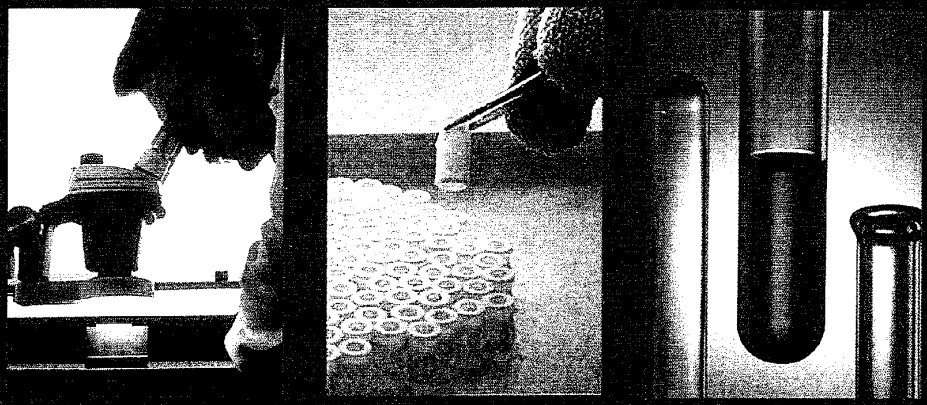
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Biologics Development:

A REGULATORY OVERVIEW

THIRD EDITION



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CHAPTER 5

Clinical Testing of Biologically Derived Therapeutics

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As it does for other classes of products, the clinical development of biologically derived therapeutics involves the investigation of a product's safety and efficacy through the conduct of clinical studies. The goal of these studies is to generate sufficient data to support market authorization. To obtain market authorization in the United States, biologically derived therapeutics discussed in this chapter require the submission and FDA approval of a biological license application (BLA).

This chapter outlines the clinical development of biological therapeutics with a focus on the key phases of the clinical development process and, in particular, on key development issues and study design elements relevant to biological products. It also discusses specific issues relevant to the clinical investigation of related therapeutic biological products, including cellular and gene therapies.

It is important to note that the clinical development of biologically derived therapeutics will continue to adapt to new scientific discoveries and changes in regulatory requirements. Consequently, the application of some of the concepts discussed here will almost certainly evolve in the future as technology moves forward and FDA organizational and regulatory requirements change.

Phases of Clinical Drug Development

Clinical development proceeds through a series of phases that are defined in the Code of Federal Regulations (21 CFR 312.21). Each phase of clinical investigation has different objectives and design issues (see exhibit below). Rather than distinct steps, these phases can be considered milestones along the continuum of clinical development that begins with the first-in-humans study, continues through market authorization, and extends into the postmarketing phase. The development of therapeutics often follows a prospectively designed clinical development plan consisting of several well-designed and well-executed studies. Taken together, these studies will characterize clinical aspects of the biological product and generate scientifically valid clinical data that will support the efficacy claim when that therapy is administered to the indicated patient population.

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As a roadmap to market authorization, the clinical development plan should be thoughtfully designed to generate clinical data that characterize specific aspects of the biological product that the sponsor hopes to include in the eventual product label. During labeling negotiations with the FDA, it is difficult for a sponsor to justify the inclusion of clinical claims that were studied in laboratory or preclinical testing but never investigated in the clinic. Thus, in addition to generating sufficient data to support the final indication, clinical study designs also should address clinically important questions of secondary interest and provide for the collection of appropriate data to describe the product as completely as possible in the label.

The clinical development plan should also reflect the lessons learned from prior clinical investigations or from regulatory experience gained during the development of products for the same or similar indications. Being the first to seek market authorization for a novel class of products or a product for an indication for which no therapies have been approved can be challenging. Inexperience in a particular field and the lack of regulatory precedents contribute to uncertainty regarding appropriate clinical trial design and regulatory standards.

Given the novelty of many biologically derived products, developers of these products should communicate with the FDA review team on a timely and effective basis throughout clinical development. Sponsor/regulator interaction allows the FDA to apprise developers of regulatory concerns, and allows sponsors to keep FDA informed of progress in clinical development.

FDA guidance documents are among the tools available to assist sponsors in planning and coordinating clinical investigations.[1,2] In addition to several guidance documents on the development of products for cancer, guidance documents for rheumatoid arthritis,[3] imaging agents,[4] and chronic cutaneous ulcers[5] have proven useful in the development of recently approved biologically derived products.

The amount of clinical data necessary to assess a biological product's safety very much depends upon the intended patient population and the availability of effective therapies for the targeted indication. Patients with severe or life-threatening illnesses or who have inadequate treatment options are generally more willing to accept greater levels of risk in order to experience clinical benefit. For these patient populations, the amount of safety data may be considerably less than that needed for patient populations with non-life-threatening diseases that require chronic treatment. In their respective pivotal studies, for example, rituximab (Rituxan™) was evaluated in 296 patients with relapsed or refractory non-Hodgkin's lymphoma, and trastuzumab (Herceptin™) was evaluated in 222 chemotherapy-experienced patients with metastatic breast cancer over-expressing HER2. In contrast to these patient populations with life-threatening malignancy, patients with psoriasis have non-life-threatening diseases and available treatment options. The safety databases for two recently approved biological products for patients with chronic plaque psoriasis were much larger: 1,350 patients for alefacept (Amevive™) and 2,762 for efalizumab (Raptiva™). For each product, safety and efficacy were evaluated in two Phase 3 studies in which the experimental therapy was administered to roughly 700 to 800 patients.

Because biologically derived therapeutics are often intended to treat patients whose disease significantly increases the risk of death or severe morbidity, many products have been approved on the basis of a single adequate and well-controlled clinical study, usually a Phase 3 study. The CBER/CDER 1998 guidance entitled, *Providing Clinical Evidence of Effectiveness for Human Drugs and Biological Products*[6] describes situations in which a single adequate and well-controlled clinical study may be acceptable to the FDA in supporting a product's efficacy. It is important to note, however, that the FDA's willingness to rely on a single study for initial market authorization will generally be limited to situations in which a study

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demonstrates a clinically meaningful effect on mortality, irreversible morbidity, or prevention of a disease with a potentially serious outcome, and in which confirmation of the result in a second trial would be impossible for practical or ethical reasons. As illustrated in the preceding example, for biological products intended to treat patients with less severe or chronic diseases (e.g., patients with psoriasis or rheumatoid arthritis), a larger clinical database, usually derived from two or more pivotal studies, will be required to substantiate the clinical results and to assess adequately the risk/benefit ratio in the intended patient population.

Increasingly, clinical development plans for biologically derived therapeutics should also include an evaluation of safety and efficacy in pediatric patient populations. Under the FDA's 1998 Pediatric Rule, BLAs for new biological products were required to include investigations in pediatric populations unless the FDA granted a waiver or deferral of pediatric studies.[7] Waivers were granted to products intended to treat diseases that rarely, if ever, occur in children (e.g., breast cancer, Alzheimer's disease) or when there was strong evidence that the product would be ineffective or unsafe in children. Deferral of pediatric studies allowed such studies to be completed following market authorization.

In an October 2002 ruling, however, the U.S. District Court held that the FDA lacked the statutory authority to require that industry conduct pediatric studies, thereby preventing the agency from enforcing the 1998 Pediatric Rule. Following this court ruling, the FDA has remained active in encouraging firms to propose and undertake clinical studies in pediatric populations. In November 2003, Congress passed The Pediatric Research Equity Act of 2003 (S650) to provide the agency with clear statutory authority to mandate pediatric clinical studies when necessary to ensure the safe and effective use of drug and biological products in children. The legislation was signed into law in December 2003.

Phase 1 Phase 1 studies are designed to assess the safety of a biologically derived therapeutic when administered for the first time in humans or to evaluate the product's safety in a new patient population. Because the primary objective of Phase 1 studies is to evaluate the safety of different doses, these studies tend to enroll a limited number of study participants and are closely monitored for safety events. In addition to safety, Phase 1 studies are designed to evaluate the pharmacokinetics, pharmacodynamics, and immunogenicity of the different doses being investigated.

Design Issues

Phase 1 studies are usually designed as open-label, dose-escalation studies that enroll sequential cohorts of patients. Although each cohort may include patients who receive a placebo, a placebo control, while useful, is not a critical element in Phase 1 studies because safety and effectiveness can be compared across cohorts.

Often, Phase 1 studies will first evaluate the safety of a single dose in a dose escalating fashion to determine the maximum tolerated dose (MTD). An evaluation of repeat dosing will be conducted either as a separate Phase 1 study or will be incorporated into the original Phase 1 study design as a second component whose conduct depends upon the safety data for the initial single doses evaluated (i.e., acceptability of the single dose's safety data to allow administration of repeat doses).

Obviously, sufficient preclinical toxicity data are necessary to support both the initial single and repeat dose investigations.[8, 9] In some cases, however, the preclinical evaluation of biologically derived therapeutics is not feasible (e.g., cellular therapies evaluating human cells such as $CD34^+$ hematopoietic cells and autologous tumor cells). For these types of products, the sponsor and FDA must discuss the degree to

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which an investigational product's safety can be extrapolated from clinical experience with similar products or other types of available data.

The Phase 1 study design should incorporate a measurement of the investigational product's biological activity along with its pharmacokinetics to determine the optimum biologic dose (OBD). Biological prod-

General Characteristics of Studies during Different Phases

Phase 1

- First-in-human studies for novel, biologically derived therapeutic products, or similar evaluation in a new patient population
- Primary objective is safety
- Explores safety of different product doses or dosing regimens
- Assesses pharmacokinetic and pharmacodynamic measurements
- May provide early evidence of effectiveness
- Generally open-label, single dose, dose escalation design
- For biological products, commonly conducted in patient population

Phase 2

- Hypothesis-generating studies in larger number of patients than Phase 1
- Expands safety and efficacy information regarding specific product dose or dosing regimen initially evaluated in Phase 1
- Explores potential endpoints, study population, and disease characteristics for the design of Phase 3 studies
- Generates clinical data intended to support Phase 3 studies
- Generally randomized, blinded, with or without a control

Phase 3

- Pivotal studies intended to support BLA for market authorization
- Generates information for product labeling
- Assesses risk-benefit relationship in intended patient population
- Generally randomized, double-blind, controlled design; often placebo-controlled
- Endpoints selected to support clinical benefit

Postmarketing Studies

- Collects additional clinical data in approved or closely related patient populations
- Studies to which the sponsor committed at market authorization (see Chapter 12)
- Studies required for accelerated approval
- Registries and studies as part of risk management program

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ucts may have a non-linear dose-response curve in which the MTD can be higher than the OBD. The lack of a direct correlation between dose and response may result from the naturally dynamic activity of a biological product. The pharmacodynamic effect of many biological products, for example, involves product-protein or product-receptor interactions that cause cellular or protein activity and subsequent clinical response.

Most gene therapy products are designed to demonstrate effect with gene expression following cell uptake, which makes correlating pharmacokinetics with biological activity difficult. Thus, clinical response often depends on a series of inter-related biological steps or cellular functions whose activities may vary among different disease states or in the presence or absence of disease.

A critical component of the safety evaluation for biologically derived therapeutics involves the assessment of the product's immunogenicity (see below). In Phase 1 studies, serum should be collected and analyzed to assess antibody development to the biological product following both single and repeat administration. The development of antibodies should be correlated to the occurrence of safety events. In addition to monitoring for acute events, researchers should monitor study participants for delayed safety events for at least 28 days following the administration of study agent. Because antibody development may alter the biological product's serum concentration or biological activity, the immunogenicity data should also be analyzed to determine effects on the biological product's pharmacokinetics or pharmacodynamics.

Since the risks associated with the investigational product are either unknown or were not characterized in the new patient population, the Phase 1 study design generally incorporates the use of predefined toxicity limits that will restrict dose escalation or trigger the discontinuation of the clinical study. Although the types and severity of all adverse events must be closely monitored, toxicities seen in the preclinical studies and any known product-class effects will help identify dose-limiting toxicities (DLT) of particular interest. In addition, the definition of DLTs will depend upon the nature of the study participants—that is, significantly lower grades or levels of DLTs may be acceptable in healthy volunteers or relatively healthy patient populations, while slightly higher grades of DLTs may be acceptable in patient populations with more severe or life-threatening diseases.

Frequently, Phase 1 studies for biologically derived therapeutics are conducted in the intended patient population rather than healthy volunteers. Because healthy populations cannot derive any therapeutic benefit by being exposed to biologically derived therapeutics, there are natural sensitivities to exposing such populations to theoretical or unnecessary risks. One particular concern is that the exposure to biologically derived therapeutics may trigger antibody development. The risk of antibody development to a native protein, which could theoretically result in a secondary deficiency state or the risk of antibody formation to a portion of a monoclonal antibody, may be considered unacceptable in healthy volunteers.

Other products whose bioactivity is disease-specific are evaluated in the intended patient population (e.g., a tumor-specific therapeutic vaccine or a monoclonal antibody directed against a tumor-specific receptor vaccine will be evaluated in patients with the targeted cancer). Since gene therapies, in which an inserted gene is intended to alter the disease process, pose unnecessary risks to healthy volunteers, these products are evaluated only in the intended patient population.

Despite the risks, Phase 1 studies for some biological products are conducted in healthy volunteers. In all cases, sponsors must provide sufficient preclinical data and a rationale to support proposals that Phase 1 studies be undertaken in healthy volunteers. When Phase 1 studies are conducted in healthy volunteers,

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safety events can be correlated with the study drug without the confounding influence of an ongoing disease process. In such cases, study participants can be enrolled and the study can be conducted in a more controlled setting at a single center than studies conducted in the intended patient population. Consequently, data collection is more uniform, and the studies can be conducted over a shorter period.

Phase 2 Due to limited resources and industry's desire for the rapid clinical development of biologically derived therapeutics, Phase 2 is a phase whose borders often overlap with those of Phase 1 and Phase 3 studies—that is, firms frequently conduct them as part of combined Phase 1/2, Phase 1b/2, or Phase 2/3 study designs. Because Phase 2 studies play an important role in the characterization of the therapeutic product, the abbreviation—or even omission—of these studies is often regrettable. Often, the design of and clinical data derived from Phase 1 studies are insufficient to permit the sponsor to identify the optimal dose or dose regimen, or to help the sponsor design a successful Phase 3 study that will support market authorization. Additionally, Phase 1 studies are generally too small to permit the sponsor to estimate the biological product's effects, which is important in calculating an adequate sample size for the Phase 3 studies. Such estimates are needed to provide some assurance that the critical Phase 3 studies have sufficient statistical power to allow an adequate comparison of the safety and efficacy of the biological product and the control.

Therefore, one of the goals of Phase 2 studies is exploring the effect and degree of effect that selected doses or dose regimens have on the investigational product's safety and efficacy, and to provide the information necessary for the selection of the appropriate dose or dose regimen that will be evaluated in Phase 3 studies. Phase 2 studies are also used to better define or clarify the appropriate patient population, clinical endpoints, and safety issues that could affect the design of Phase 3 clinical studies. When a sponsor plans to use a single large, multicenter Phase 3 clinical study to support approval, results from a well-designed Phase 2 study will provide supportive evidence for the Phase 3 study results. Lastly, from a practical perspective, Phase 2 studies permit sponsors to assess feasibility issues and fine-tune their Phase 3 study plan (e.g., feasibility of measuring certain endpoints and improving methods for data collection to avoid missing data).

Design Issues

Phase 2 studies are generally randomized, blinded studies that evaluate a larger number of patients than Phase 1 trials. These studies often include a control group that is administered either a placebo or an active treatment for comparison. For some indications, such as oncology, the Phase 2 study may have a single-arm design. In contrast to those in many Phase 1 trials, Phase 2 study participants are patients with the disease for which treatment is intended.

The dose or dose regimens evaluated in Phase 2 studies are chosen following an assessment of the Phase 1 study results. The Phase 2 study should be designed to collect and analyze additional pharmacokinetic, pharmacodynamic, and immunogenicity data. These data will provide a better understanding of the product's safety and exposure-response and exposure-toxicity relationships. Two guidance documents—the FDA's industry guidance entitled, *Exposure-Response Relationships—Study Design, Data Analysis, and Regulatory Application* (2003) and ICH's *E4: Dose Response Information to Support Drug Registration*—that discuss study designs and data analysis may be useful in designing these aspects of studies for biological products.[10, 11] When feasible, population pharmacokinetic analyses may be useful as well, particularly for analyzing exposure-response in subpopulations.[12]

The assessment of the biological product in a larger number of study participants provides a better estimate of the product's clinical effectiveness compared to placebo or other therapies. As noted, such esti-

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mates will be useful measures in determining a reasonable size for the Phase 3 studies (i.e., the so-called "power calculations"). The Phase 2 study design can incorporate an analysis of clinical response in subsets defined by covariates that may help the sponsor determine the appropriate study population for Phase 3. These covariates could include baseline patient characteristics (e.g., age, gender, concomitant medications or illness) or disease characteristics (e.g., duration or severity of disease, prior treatments).

For some indications, there will be a pharmacodynamic endpoint that correlates, with some degree of assurance, the product's bioactivity with clinical benefit. In these cases, the Phase 2 study could assess efficacy using the pharmacodynamic endpoint at an earlier timepoint than the timepoint used to assess clinical benefit in Phase 3. For example, the primary endpoint in a Phase 2 study evaluating an investigational therapy for multiple sclerosis might be the number of new enhancing brain lesions detectable on MRI at 6 months, while the primary assessment of clinical benefit in Phase 3 will be based on the change in Kurtzke Expanded Disability Status Scale at 12 or 24 months. For diseases in which appropriate clinical endpoints are not well defined, Phase 2 studies can evaluate potential clinical endpoints, provide insights on measuring the endpoints, and establish their reliability in demonstrating clinically meaningful effect.

Phase 3 The goal of Phase 3 studies is to generate definitive evidence of the product's safety and efficacy to support market authorization. During the Phase 3 studies, the sponsor must also collect sufficient clinical data to support statements within the package insert.

Design Issues

Phase 3 clinical studies are generally larger, lengthier, and more complex in design than studies conducted in the earlier phases of development. The studies are generally randomized, double blind, and placebo controlled whenever possible. When placebo controls are not feasible or are deemed unethical, an active control with known clinical benefit can be used as the comparator. The ICH's *E10: Choice of Control Group and Related Issues in Clinical Trials* (2001)[13] outlines general principles involved in choosing a control group in clinical efficacy studies, as well as other related study design issues, such as the utility of superiority and noninferiority designs.

Study participants in Phase 3 studies are patients representative of the intended patient population and are defined by the study's eligibility criteria. Although the study population should be sufficiently homogeneous to demonstrate the biological product's effect, it must also be sufficiently representative to allow generalization of the results to the larger patient population.

The efficacy endpoint studied in Phase 3 trials must be one that demonstrates clinical benefit in the intended patient population. Generally, the endpoint is a clinical outcome measure that has been accepted by regulators and physicians as having clinical meaning to patients. The ideal efficacy endpoint is an objective and reliable measure of clinical outcome that is clinically interpretable and sensitive to changes in disease states. Whenever possible, the method of measuring the clinical endpoint should be well established and validated. If a product's clinical endpoints are poorly defined, the Phase 3 study design may have to provide for the formal assessment of a combination of efficacy outcomes to provide a convincing demonstration of efficacy.

In Phase 3 studies, surrogate endpoints are often used as secondary endpoints to confirm the findings related to a primary efficacy endpoint and to further strengthen the evidence of a product's efficacy. Although surrogate endpoints do not measure clinical benefit directly, they are considered predictive of

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efficacy. For investigational products developed under the FDA's accelerated approval regulations, approval may be based upon a surrogate endpoint considered reasonably likely, based on epidemiologic, therapeutic, pathophysiologic, or other evidence, to predict clinical benefit. For other products, a surrogate endpoint can be used as a primary endpoint in a Phase 3 study only if that endpoint is validated. Validation requires the testing of both the surrogate and the clinical endpoint in the same trial as well as a prospectively defined analysis that establishes a link between the two.

The statistical analytical plan is an important component of the Phase 3 clinical study design. A prospective plan defines the study's hypothesis, identifies the type of data to be collected, and describes how the data are to be analyzed. The analytical plan should include a definition of the endpoint(s), calculations for statistical power, statistical methods used in the analyses, procedures to optimally address and minimize the frequency of missing data, and a definition of the study participants to be included in different analyses. The study design and statistical plan should include analyses of populations based on demographic differences (e.g., race/ethnicity,[14] sex, age[15]), which are required for market authorization.

The statistical analytical plan must be written prior to unblinding of the Phase 3 study and preferably prior to study initiation. *Post hoc* analyses of clinical data conducted after unblinding may provide information, but generally will lack the validity to support market authorization by themselves. The ICH's guidance document entitled, *E9: Statistical Principles in Clinical Trials*[16] (1998) addresses the statistical monitoring of trials, describes available statistical approaches, and presents the principles involved in their implementation.

Phase 3 studies are generally large, multicenter studies that include multinational sites. Given the complexity of Phase 3 trials, sponsors will often establish a data monitoring committee (DMC) to assist in monitoring a Phase 3 study. In addition to providing an objective assessment of safety events, DMCs can be chartered to monitor the conduct of the clinical study (e.g., rates of recruitment, accrual of important subsets), assess the impact of new data on the study (e.g., results of a related study, introduction of a new therapy), or conduct an interim analysis of unblinded data in an effort to address specific efficacy-related questions. Based upon its review, the DMC can make recommendations regarding the study conduct (e.g., early termination, increasing the sample size, modifications to the informed consent, discontinuation of a treatment arm). In a 2001 draft guidance entitled, *The Establishment and Operation of Clinical Trial Data Monitoring Committees,*[17] the FDA recommends situations in which DMCs should be used, and discusses related issues (e.g., independence) regarding their use in clinical trials.

Postmarketing Studies Postmarketing, or Phase 4, clinical studies often evaluate an approved biological therapeutic product in the approved or a closely related patient population to address certain safety or efficacy questions that arose during the BLA review, that were not completely answered during clinical development, or that remain of clinical interest to the sponsor and/or the FDA. A Phase 4 study, for example, could be conducted to further characterize a product's safety profile, evaluate a new indication, or assess a different delivery system.

Some Phase 4 clinical studies are required by the FDA (see Chapter 11). For products approved under the accelerated approval provisions, the FDA requires Phase 4 studies designed to confirm a product's clinical benefit. These Phase 4 studies may be designed to evaluate a similar or different patient population, to assess a different stage of the disease, or to utilize a clinical endpoint to demonstrate benefit. As noted below, a company's failure to demonstrate clinical benefit in such cases could lead to the FDA's withdrawal of market authorization. Sponsors discuss the design of these postmarketing studies with the FDA and, under the accelerated approval provisions, initiate such studies prior to market authorization. Phase 4 clin-

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ical studies are also required when the FDA grants a deferral of pediatric studies/data at the time of market authorization.

Other Phase 4 studies are conducted when, following discussion with the FDA at the time of approval, the sponsor commits to further evaluate remaining issues or questions regarding a drug's safety, efficacy or clinical use. Phase 4 studies may be a component of a risk management program that a sponsor implements in an effort to assess the effectiveness of programs designed to reduce risks to patients. Frequently, Phase 4 studies for biological products include a further evaluation of immunogenicity, the evaluation of long-term safety using a patient registry, or the establishment and evaluation of clinical data from a pregnancy registry.[18] Such postmarketing studies, including those involving registries, are based on a written protocol that describes the study design, patient population, and data collection and analysis.[19]

Special Topics in the Clinical Development of Biologically Derived Therapeutics

While a single chapter cannot address all aspects of clinical trial programs for therapeutic biological products, the following section discusses cell and gene therapies, which comprise a special category of biological therapeutics. This section also addresses a number of scientific and regulatory issues that can affect the clinical testing of biologically derived therapeutics.

Cell Therapy and Gene Therapy Relative to other biologically derived therapeutics, industry and the FDA have significantly less experience in the clinical development of cell therapies and gene therapies. At the same time, clinical programs assessing the value of cellular and gene therapies for numerous indications, ranging from chronic, non-life-threatening diseases to acute, life-threatening diseases remain extremely active.

Cell Therapy Over the past decade, the FDA has issued several notices or rules regarding the regulation of human cells, tissues, and cellular and tissue-based products (HCT/Ps). HCT/Ps include cellular therapies, human tissues and cells intended for transplantation, and reproductive tissue. The exhibit below identifies several types of HCT/Ps and specifies how they are regulated.

In most cases, the amount of *ex vivo* manipulation to which a product is subjected determines how the specific HCT/P is regulated. Human cells or tissues that are minimally manipulated (e.g., skin, cadaveric cornea) are regulated under section 361 of the PHS Act and 21 CFR 1271. The FDA is in the process of formulating regulations regarding the type of data that will be necessary to support the marketing of minimally manipulated human cell and tissues. The "tissue rules"—21 CFR 1271 and a pair of proposed rules (see discussion below)—are intended to minimize the risk of transmitting disease to recipients, to prevent contamination, and to preserve the integrity of the cells and tissues. Currently, clinical data from adequate and well-controlled studies are not required for such cells and tissues. FDA regulations (21 CFR 207, 807, and 1271) require establishments that process, test, or store human tissue for transplantation to register with the FDA. The FDA's proposed donor eligibility rule—*Suitability Determination for Donors of Human Cellular & Tissue-Based Products* (64 FR 52696)—would require that donors be properly screened and tested for communicable diseases. In its January 2001 Good Tissue Practices proposed rule, the FDA describes the proper handling and processing of cells and tissues as well as appropriate facility controls (Current Good Tissue Practice (cGTP) for Manufacture of Human Cellular and Tissue-Based Products; Inspection and Enforcement, proposed rule, 66 FR 1508).

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In contrast, products comprising more-than-minimally manipulated cells and tissues are regulated under 351 of the PHS Act and 21 CFR 312 and 600. When finalized, elements of the proposed regulations cited above revising CFR 1271 will apply to these products as well. Sponsors for these investigational therapies must demonstrate safety and efficacy in adequate and well-controlled clinical trials to support market authorization. As described in 21 CFR 312, the FDA reviews proposed clinical investigations through investigational new drug applications (see Chapter 3) and/or through investigational device exemption applications (IDE).

By definition, most somatic cell therapies are considered "more than minimally manipulated," and therefore clinical safety and efficacy data from adequate and well-controlled investigations are required to market these products. Somatic cell therapies involve the *ex vivo* propagation, expansion, selection, and/or pharmacological treatment of cells (or other modification of the cell's biological characteristics) intended to treat or cure disease. Given that the cell therapy field is evolving rapidly and that the clinical data requirements differ dramatically among various HCT/Ps, developers should seek FDA advice regarding the regulation of a particular cellular product early in the product development process.

In June 2002, the FDA requested that holders of cell therapy INDs and IDEs submit information regarding product manufacturing quality control procedures and clinical oversight (see Chapter 7). Issued in

Human Cells, Tissues, and Cellular and Tissue-based Products (HCT/P)

Regulated under Section 351 of the PHS Act and 21 CFR 312 and 600

Cell therapies (e.g., isolation and expansion of peripheral $CD34^+$ hematopoietic cells, activation and *ex vivo* expansion of lymphocytes, *ex vivo* expansion of bone marrow cells)

Genetically modified cells (e.g., autologous tumor cells genetically modified to express a cytokine, introduction of multi-drug resistance gene into stem cells, infusion of cells modified *ex vivo* with the Factor VIII gene for the treatment of patients with hemophilia A)

Non-homologous use of cells or tissues. Non-homologous cells are cells isolated from one tissue and used for a cellular function other than their normal *in vivo* function (e.g., the implantation of stem cells directed to differentiate into specific cell types, such as dopaminergic neuronal cells, for the treatment of Parkinson's disease, or insulin-secreting cells for the treatment of diabetes)

Cells whose clinical effect depends upon their systemic effect or metabolic activity (e.g., implantation of human pancreatic islet cells for the treatment of diabetes, neural cells for treatment of Parkinson's or spinal cord injury)

Any cells or tissues that are combined with a drug, biological product, or device (Note: Depending upon the cell product, materials such as matrices, other biological products, or drugs may be considered excipients, active components, or medical devices and be made subject to additional regulation.)

Regulated under Section 361 of the PHS Act and 21 CFR 1271

Human cells or tissues for transplantation (e.g., musculoskeletal tissue, dura mater, cadaveric cornea, human stem cells that are "minimally manipulated" and for homologous use)

Reproductive tissue

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response to safety-related concerns, this agency request harmonized the type of data that sponsors of cell and gene therapies must submit to the FDA. The FDA will continue to request that new INDs and IDEs for cell therapies include a comprehensive description of the clinical monitoring plan as well as the quality control procedures for the cellular product manufacture.

Gene Therapy Gene therapy involves the modification of a living cell's genetic material in an effort to treat or cure a disease. As discussed above, gene therapy can involve the administration of *ex vivo* genetically modified cells through infusion, implantation, or other means. Alternatively, a gene therapy can be administered directly *in vivo* (e.g., inhalation, injection into a tumor, intramuscular injection).

Currently, gene therapies are being evaluated for their ability to replace missing or defective genes, and to deliver genes that destroy cancer cells, promote growth or new tissue, or stimulate regeneration of damaged tissue. Numerous genes have been evaluated, including CFTR for cystic fibrosis, VEGF for coronary artery disease and peripheral vascular disease, and p53, E1A, and IL-2 for cancer.

Gene transfer vectors are designed to transfer the gene of interest into the target cell and regulate gene expression. To this point, a number of vectors derived from viral and non-viral components have been employed. Viral vectors take advantage of a virus' ability to infect different cell types and deliver the gene to the cell's nucleus. Due to the concern about the potential risks of cancer or viral infection, most vectors under active clinical investigation are replication incompetent or require a helper virus to replicate. Based on the target cell or therapeutic goal, components from poxviruses, adenovirus, herpes virus, and retroviruses have been used. Non-viral vectors consisting of plasmid DNA with the inserted gene are often packaged in liposomes to facilitate cell entry.

To regulate gene expression, the vector design will incorporate a promoter to control gene expression. Numerous promoters are being investigated, including those with cell-specific, inducible, and constitutive expression.

Needless to say, because of limited experience with different vector types and genetic insertions, both regulators and investigators recognize the need to exercise significant caution when investigating gene therapies. Therefore, proposals for clinical investigations involving new gene therapy products must be submitted to the National Institute of Health's (NIH) Recombinant DNA Advisory Committee (RAC). This additional level of review (i.e., in addition to the FDA's review of the IND) is unique to gene therapy. The RAC is responsible for conducting a public scientific and ethical review, and for discussing the novel application of the gene therapy and related public policy issues.

Given emerging safety-related concerns regarding gene therapy trials, the FDA used a November 1999 letter to outline the processes through which gene therapy investigators must submit documentation to the RAC and the FDA.[20] Following its initial review of a gene therapy proposal, the RAC may decide to publicly discuss the reviewed protocol. Factors that may influence the RAC's decision to hold a public hearing include the use of new vectors or new gene delivery systems, the use of gene therapy in a new disease, or the unique application of gene transfer. It is important to note that any adverse event reported to the FDA during the protocol study period should also be reported to the NIH's Office of Recombinant DNA Activities (ORDA), as required by the NIH Guidelines for Research Involving Recombinant DNA Molecules.[21]

Following the report of a patient death after the administration of an experimental gene therapy product in the United States, the FDA issued a March 2000 letter requesting that all sponsors/investigators involved

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in gene therapy trials submit a detailed plan describing the monitoring and oversight for the clinical study as well as information on the quality control procedures for the product's manufacture. In addition, sponsors were also required to submit an organizational chart identifying the units responsible for clinical study oversight as well as the individuals responsible for clinical study oversight (including their training and qualifications). Under this March 2000 Gene Therapy Monitoring Plan, CBER continues to require that gene therapy study sponsors routinely submit their monitoring plans, certain manufacturing-related information, and other information to the agency.

As part of the March 2000 initiative, the FDA discussed appropriate monitoring procedures for gene therapy trials with its Biological Response Modifiers Advisory Committee (BRMAC) in October 2001. Much of the discussion focused on the need for long-term monitoring of study participants exposed to gene therapies. Based upon the committee's recommendations, the FDA is requesting that sponsors of all new gene therapies, regardless of vector type, continue to monitor study participants for at least 15 years post-study. The advisory committee recommended that the principal investigator physically examine study participants for the first five years following study participation. Subsequent monitoring need not include physical examination, but may involve telephone calls, questionnaires, postcards, or other vehicles. Throughout the 15 year follow-up period, information on the occurrence of new malignancies or autoimmune, neurological, or hematological diseases should be collected annually. Study participants should also be asked about any changes in medication or the need for hospitalization. These recommendations may be modified in the future as clinicians, researchers, industry, and regulators meet to discuss this topic.

Studies involving gene therapies that employ retroviral vectors to transduce $CD34^+$ hematopoietic stem cells require special consideration. These types of studies were placed on clinical hold in January 2003 following a second report of leukemia in a France-based clinical trial involving children with X-linked severe combined immunodeficiency syndrome (X-SCID). Following a discussion at a February 2003 BRMAC meeting, the FDA allowed these studies to continue on a case-by-case basis after sponsors notified IRBs, investigators, and study participants of the potential risk of hematopoietic malignancy associated with the vector use.

In addition, the FDA requested additional analyses that will most likely apply to future investigations of gene therapy using retroviral vectors, particularly for the transduction of hematopoietic stem cells. Specifically, the FDA requested that sponsors analyze patients' peripheral blood mononuclear cells (PBMCs) for the clonality of vector integration sites at least every six months for the first five years after administration, and then annually for the next ten years. If a predominant clone is detected, then the researchers should assess the presence of a predominant integration site, something that would necessitate repeat analysis within three months to determine if the clone is persistent. If clonality is persistent, the integration site should be sequenced to determine any association with known human cancers, and the study participant should be closely monitored for malignancy. Needless to say, the design of all future investigations involving retroviral vectors, particularly those involving transduced hematopoietic stem cells, must take these known risks into account.

Immunogenicity Immunogenicity, or the stimulation of antibody formation to an investigational biological product (or some portion of the biological product), has become a major clinical and regulatory safety concern for biologically derived therapeutics in general. The exhibit below identifies possible causes of immunogenicity for biologically derived therapeutics.

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The impact of an antibody response to a biological product on safety and efficacy is variable and unpredictable. Antibody development can result in no apparent clinical effect, an altered pharmacokinetic profile, neutralization of the product's activity, or local or life-threatening systemic allergic reactions.

Due to species specificity, the utility of preclinical models to predict immunogenicity or its effects may be limited. Some therapeutic proteins are "human" or "humanized" and, therefore, naturally trigger an immune response in animals. Also, the biological product may not demonstrate the same activity in animals (e.g., differences in clearance or target protein, cell, or receptor). This variability in effect and the difficulty of assessing potential clinical effects of antibody formation from preclinical toxicology studies for some products make careful study monitoring even more important, particularly for adverse events in early human studies. Thus, an assessment of antibody formation and the effect of immunogenicity on safety and efficacy should be incorporated into the design of early clinical studies and should continue throughout clinical development. Obviously, the development and validation of sensitive assays are necessary for evaluating immunogenicity.

The degree of immunogenicity can vary depending upon the patient population, the route of administration, and the frequency of administration. The risk of antibody formation may be lower in immunocompromised study participants than study participants with an intact immune response. The risk may be also higher when biological products are administered intradermally as opposed to intravenously or intramuscularly, and when administered repeatedly or intermittently on a chronic basis. Although the proportion of

Possible Causes of Immunogenicity

- 'Non-self' immunogenic epitopes
  - Presence of non-human proteins (e.g., bacterial, bovine, porcine products)
  - Alteration in protein sequences (e.g., fusion proteins, chimeras)
  - Impurities
- Exposure of "hidden" epitopes
  - Glycosylation
  - Dissociation of multimeric proteins
  - Denaturation
- Breaking tolerance
  - Exposure to high levels of "natural" sequences
  - Exposure to altered "self" proteins
  - Stimulation of "clonally ignorant" B-cells
- Aggregation
  - Caused by non-glycosylation, oxidation, deamidation, shearing, heating, etc.
- Unknown factors

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patients who develop antibodies may be higher with some types of biological products, there may be no correlation between the frequency or degree of antibody response and its effect on safety and efficacy. All of these factors must be taken into account when designing clinical studies, and will ultimately determine the quantity of immunogenicity data that should be collected.

Safety with Chronic Administration Most, if not all, of the early clinical investigations of biologically derived therapeutics were conducted in study populations with severe or life-threatening diseases (e.g., patients with solid tumors or hematological malignancies). Over the last 10 to 15 years, however, increasing numbers of biological products have been developed to treat patients with moderate to severe stages of chronic diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, and asthma. The underlying pathology for these and other chronic diseases under investigation is a dysfunction in the body's immune response.

Currently, approved and investigational biologically derived therapeutics are intended to treat these diseases by modifying the body's response to either an auto- or exogenous antigen. The biological products differ in their primary target for the immune response, and in the degree of inhibition of the immune response. It is unknown what effect chronic administration of such immunomodulatory agents will have on long-term safety. Potential concerns for patients include an increased risk for infectious diseases, new onset autoimmune diseases, and cancer, particularly lymphoma.

Therefore, the long-term effects of chronically administered biologically derived products must be monitored carefully following approval. Given these concerns, the FDA has frequently requested that sponsors agree to monitor the long-term safety of these products in postmarketing studies or patient registries. Often, these registries are large (5,000 to 10,000 patients) and are conducted over five to ten years.

Regulatory Processes Because biologically derived therapeutics are commonly developed to treat patients with serious or life-threatening diseases, sponsors increasingly employ special regulatory processes designed to expedite the development and approval of these products. The so-called fast-track program and accelerated approval process are among the most commonly used for such products.

Fast Track Under the FDA Modernization Act of 1997 (FDAMA), Congress established the fast track process to facilitate the development, and expedite the review, of products that demonstrate the potential to address unmet medical needs in the treatment of serious or life-threatening conditions (Section 506 of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. 356). To become eligible for the benefits offered under the fast track program, a drug sponsor must first apply for, and obtain, a fast track designation for its drug product. The sponsor can seek an FDA fast track designation for a product at any point in the product's development, although most fast track designations have been granted during the clinical development process. CBER and CDER outline the fast track application and designation process in a 1998 guidance entitled, *Fast Track Drug Development Programs—Designation, Development, and Application Review.*[22]

The fast track program offers several benefits, although many are available under other FDA regulations and programs. Under the program, the FDA can approve a designated biological product after the sponsor demonstrates either the product's clinical benefit or its effect on a clinical or surrogate endpoint that is reasonably likely to predict clinical benefit (see discussion on accelerated approval below). Although most sponsors have found CBER reviewers to be open to meetings, the FDA's designation of a product as a fast track product can facilitate the agency/applicant meetings process. Further, fast track products generally are granted priority review status (i.e., six month review target).

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In addition, sponsors of designated fast track products can request, by submitting clinical data indicating that the product may be effective, that the agency accept for formal review completed portions of a BLA before the other sections of the application are submitted. If the agency permits this submission—sometimes called a rolling submission—the sponsor is required to provide a schedule for submitting the information necessary to make the BLA submission complete. The rolling NDA/BLA was the model for the "reviewable unit" concept that will be studied formally under PDUFA III's continuous marketing application (CMA) pilot program (see Chapter 8).[23,24] Sponsors intending to employ the rolling submission approach for their BLA must discuss the logistics and requirements with the FDA prior to its implementation.

Biologics sponsors have been active in pursuing fast track designations. Between March 1, 1998, and September 30, 2003, industry had submitted 114 designation requests to CBER, of which 66 have been granted. CBER had denied 44 of the requests (four additional requests were pending).

Accelerated Approval The FDA's accelerated approval program was implemented under a December 1992 regulation (21 CFR 601, Subpart E-biologics and 21 CFR 314, Subpart H-drugs) in an effort to expedite the review and approval process for significant new products that are intended to treat serious or life-threatening diseases and that provide meaningful therapeutic benefit over currently available therapies. A unique feature of the program is that it permits the FDA to approve products based upon either: (1) an effect on a surrogate endpoint considered reasonably likely to predict a clinical benefit (survival or the prevention of irreversible morbidity); or (2) an effect on a clinical endpoint other than survival or irreversible morbidity.

Generally, market authorization under the accelerated approval program is contingent upon an applicant's commitment to conduct new evaluations or complete ongoing studies to verify and describe the clinical benefit. In most cases, the Phase 4 studies are initiated prior to market authorization. If these trials fail to demonstrate clinical benefit (i.e., the surrogate did not correlate with clinical response), the FDA could withdraw its approval for the product. Thus far, no product approved under the accelerated approval program has had its market authorization withdrawn.

Special Protocol Assessment Under PDUFA II, the FDA introduced the "special protocol question assessment and agreement" process. As part of this process, the FDA agreed to evaluate Phase 3 clinical trial protocols (as well as carcinogenicity and stability protocols) to determine whether a trial's design and size are adequate to meet scientific and regulatory requirements identified by the sponsor. A review division may also consider other clinical protocols under the special protocol assessment process, provided that the division agrees and is aware of the clinical study's role within the overall clinical development plan. Within 45 days of receiving such a protocol and specific sponsor questions, the agency provides a written response that includes "a succinct assessment of the protocol and answers to the sponsor's questions."

In part, this process is designed to produce formal FDA commitments on study design issues. According to text that accompanied the FDA's PDUFA II commitments, "the fundamental agreement here is that having agreed to the design, execution, and analyses proposed in protocols reviewed under this process, the Agency will not later alter its perspective on the issues...unless public health concerns unrecognized at the time of protocol assessment under this process are evident." Language in the FDA Modernization Act of 1997 adds that, "any agreement regarding the parameters of the design and size of clinical trials of a new drug...that is reached between the [FDA] and a sponsor or applicant shall be reduced to writing and made part of the administrative records...[and] shall not be changed after the testing begins, except (i) with the written agreement of the sponsor or applicant; or (ii) pursuant to a decision...by the director of the review-

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ing division, that a substantial scientific issue essential to determining the safety or effectiveness of the drug has been identified...."

The special protocol assessment process is particularly valuable when a study involves an indication or disease for which there is no previously approved therapy or when the study's design or endpoints are unique. CDER and CBER released a 2002 industry guidance entitled, *Special Protocol Assessment* to outline the process under which such assessments will be conducted and agreements will be reached.[26]

Manufacturing Changes During Clinical Development During the clinical development program, manufacturing processes for the biologically derived therapeutic will undergo changes designed to reduce impurities, increase yield, or improve efficiency. Manufacturing process changes for biologically derived therapeutics have been known to affect a product's clinical safety or efficacy. Following the selection of a different cell line, for example, some biological products have shown a decline in efficacy in clinical studies. Changes in immunogenicity have been attributed to variation in the amount or types of aggregates, the structural presentation of the product, or the degree of denaturation that occurred following a manufacturing change.

Among the complexities associated with implementing biological product manufacturing changes is our inability to predict precisely what effect, if any, a given manufacturing change will have on a biological product's safety or efficacy. Many times, problems encountered in the clinic cannot be fully or logically explained, and may reflect our inexperience with certain classes of biological products or the inability of available assays to characterize the biological product fully.

Given these realities, it is critical that manufacturers analyze the product on a pre- and post-change basis to demonstrate comparability (i.e., a side-by-side analysis). The demonstration of comparability provides the FDA with assurance that the manufacturing change did not result in a significant change in the product's characteristics (i.e., identity, strength, quality, purity, and potency (efficacy)). It is important to note that the FDA decides whether the data are sufficient to demonstrate comparability. The agency has extensive scientific and regulatory experience with manufacturing changes across a number of different types of biological products and can often provide invaluable guidance. Therefore, manufacturers should consult with the FDA before implementing manufacturing changes during clinical development.

The demonstration of a product's comparability is based upon extensive chemical, physical, and biological assays and, when necessary, preclinical or clinical evaluation. Biological assays should be valid and, whenever feasible, reflect *in vivo* biological activity. Rather than detecting the presence of an expressed gene product or binding to the target, the *in vitro* and/or *in vivo* biological assay(s) should demonstrate the cellular or enzymatic response.

The need for additional preclinical (e.g., pharmacokinetics, immunogenicity, specific toxicology) or clinical (e.g., pharmacokinetic, immunogenicity, safety/efficacy) data will depend on the adequacy of the analytical and biological assays as well as the results of these tests. While a well-designed comparability program should circumvent the need for clinical evaluation, clinical data regarding pharmacokinetics or immunogenicity may sometimes be needed during clinical development (e.g., when animal specificity necessitates evaluation of pharmacokinetics in humans).

The FDA's 1999 guidance entitled, *Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products* outlines the types of data necessary to demonstrate comparability.[27] The ICH guidance entitled, *Q6B: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*[28] is

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another useful document in this area. The ICH parties have selected the demonstration of comparability of biologically derived therapeutics as a future topic for harmonization.

The timing of major manufacturing changes implemented during the later stages of clinical development could affect the acceptability of Phase 3 clinical data designed to support marketing approval. Ideally, the product used throughout Phase 3 studies should be manufactured using a process as close as possible to the process to be used following a product's commercialization . There are, however, cases in which significant manufacturing changes (e.g., scale-up of process, change in working cell bank, changes in fermentation processes, moving to a commercial manufacturing plant, etc.) are introduced during or after Phase 3. When these occur, the sponsor must discuss the type of manufacturing change and the anticipated impact on the Phase 3 clinical with the FDA.

When significant manufacturing changes are introduced during Phase 3, the FDA can analyze the clinical data for differences in safety and efficacy between pre- and post-change material. The absence of significant differences or trends in differences will support the comparability testing, and the results of the Phase 3 study will not be compromised. When differences are detected or the commercialization of the manufacturing process occurs following the clinical development program's completion, the FDA may require additional preclinical or clinical data (e.g., pharmacokinetic data, immunogenicity, or even safety/efficacy) to support comparability.

In summary, although process changes are routinely implemented during the clinical development program, the comparability of the biological product must be demonstrated. The quantity and type of data necessary to demonstrate the comparability of biologically derived therapeutics both during clinical development and after market authorization continue to evolve as the analytical tests used to characterize the product improve and as experience with post-change clinical effects for different biological products grows.

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Human constant regions influence the antibody binding characteristics of mouse–human chimeric IgG subclasses

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SUMMARY

Although antibody affinity is primarily determined by immunoglobulin variable region structure human IgG antibodies of the four subclasses specific for the same antigen have been shown to differ in their affinity. To explore the influence of the immunoglobulin constant region on functional antibody affinity, a set of V region identical mouse–human chimeric IgG subclasses specific for TAG72 (tumour-associated glycoprotein) were studied. Biomolecular interaction analysis (BIA) was used to determine the binding kinetics of whole IgG subclasses and $F(ab')_2$ fragments. Despite identical V regions, binding kinetics differed for the four subclasses. The apparent dissociation rate constants of the intact immunoglobulins ranked IgG4 < IgG3 < IgG2 < IgG1. In contrast, analysis of the binding characteriztics of the $F(ab')_2$ fragments derived from IgG1, IgG2 and IgG4 revealed identical binding kinetics. The structure of the constant regions of the humanized IgG subclass antibodies clearly influenced functional antibody affinity, as has been described for the murine IgG subclasses. The exact mechanism for this phenomenon remains obscure but such differences should be taken into account when designing or choosing antibodies for therapeutic use.

INTRODUCTION

Antibody binding affinity is defined as the strength of the interaction between a monovalent antibody and monovalent antigen whereas the bivalent binding of an antibody to a complex antigen is referred to as functional affinity or avidity.[1] The affinity of an antibody is determined by the 'fit' of the antigen in the binding groove of the Fab portion of the antibody formed by the heavy- and light-chain variable regions. Following encounter with an antigen *in vivo* an increase in antibody affinity over time is observed and this is known as affinity maturation.[2] Affinity maturation has been shown to be due to a combination of clonal selection and somatic hypermutation,[3] the latter resulting in a high rate of mutation in the complementarity determining regions (CDR) of the variable region genes. The functional affinity of an antibody plays a crucial role in determining biological activity such as the ability to clear antigen, with high affinity antibody being superior.[4] In the assessment of the immune response to vaccines and natural infection it is important therefore to characterize an antibody in terms of functional affinity as well as titre.

Whilst antibody affinity is predominantly determined by variable region structure, human antibodies of different IgG subclasses which bind the same antigen have been shown to differ in functional affinity. This was first described for tetanus toxoid[5,6] but has also been shown for a variety of other antigens including hepatitis B surface antigen,[7] *Moraxella catarrhalis*[8] and the neo-antigen keyhole limpet haemocyanin.[9]

The underlying mechanisms for differences in the functional affinity of IgG subclasses specific for the same antigen are at present not understood, but may be due to differences in epitope specificity and consequently different variable (V) region gene usage or differences secondary to the constant region structure. The latter hypothesis is supported by recent evidence that murine antibody structure remote from the V region may influence affinity and function.[10–15]

To explore the role of the human constant region in influencing functional antibody affinity we have utilized a set chimeric antibodies having different human IgG subclass constant regions linked to identical murine V regions directed against a mucin-like glycoprotein (TAG72, tumour-associated glycoprotein) purified from the human colonic cancer xenograft, LS174T.[16] This molecule is found in large amounts on bovine submaxillary mucin which has been utilized as the target antigen in these studies. Avidity and binding kinetics of the mouse–human chimeric antibodies were investigated by biomolecular interaction analysis (BIA) using a BIAcore™ instrument (Biosensor AB, Uppsala, Sweden). This technology utilizes surface plasmon resonance to detect binding events at

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the surface of a sensor chip composed of a dextran matrix to which binding partners of interest may be immobilized, allowing binding to be observed in real time and rate constants to be calculated.[17,18]

MATERIALS AND METHODS

B72.3 monoclonal antibodies

B72.3 chimeric cell lines (IgG1, Mouse Myeloma Cell; IgG2, IgG3, IgG4, Chinese Hamster Ovary) were obtained from Celltech Therapeutics Ltd (Slough, UK). For antibody purification cells were allowed to grow to saturation in 0·5 l Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Globepharm, Esher, UK), 50 U/ml of penicillin, 50 μg/ml streptomycin (Gibco) and 1% non-essential amino acids (Gibco). Culture medium was then centrifuged, the supernatant filtered through a 0·45 μm filter and 1 l concentrated to approximately 10 ml using stirred concentrator cells (Amicon, Stonehouse, UK). The antibodies were purified by affinity chromatography on a Sephadex–Protein G column using fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden). A buffer of 0·1 M glycine–HCl pH 2·7 was used to elute the bound antibody and the pH of this eluate was immediately neutralized with solid Tris. Antibodies were then concentrated and dialysed against phosphate-buffered saline (PBS) pH 7·2 containing 0·01% sodium azide using Centriprep-10 concentrators (Amicon). The presence of both heavy and light chains was confirmed using reducing 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and the absence of aggregates confirmed by non-reducing SDS–PAGE. The antibodies had the expected molecular weight (including the expected earlier elution position of the IgG3 product) following size exclusion gel filtration employing a Superdex-200 column (Pharmacia) linked to an FPLC system. The subclass composition of each chimeric antibody preparation was then analysed by antigen-specific enzyme-linked immunosorbent assay (ELISA), employing bovine submaxillary mucin (Sigma) as a capture antigen and mouse anti-human IgG subclass monoclonal antibodies (mAb) (Zymed, San Francisco, CA) to detect specific subclasses, in order to exclude contamination. Finally, protein concentrations were determined by measuring absorbance at 280 nm and assuming a value of $A^{1\%}_{1cm,280}$ of 13·6.

Preparation of F(ab')$_2$ fragments

F(ab')$_2$ fragments were obtained from the IgG1, IgG2, IgG3 and IgG4 subclasses by pepsin digestion. Antibodies were dialysed against 0·2 M sodium acetate buffer pH 4·5 overnight and pepsin (Sigma) was added at the following previously determined optimal enzyme : substrate ratios; IgG1, 1 : 100 for 10 hr; IgG2, 1 : 500–1000 for 1 hr; IgG3, 1 : 100 for 2 hr; and IgG4, 1 : 500–1000 for 1 hr. All digestions were carried out at 37° and the reaction was stopped by neutralization with 2 M Tris. F(ab')$_2$ fragments were then separated using a Superdex-200 (Pharmacia) column linked to an FPLC system and appropriate fractions were used without further concentration. The protein concentration of each preparation was determined by absorbance at 280 nm assuming a value of $A^{1\%}_{1cm,280}$ of 15·0.

Measurement of binding kinetics by biospecific interaction analysis

Antibody binding kinetics were measured using the BIAcore™ system (Biosensor AB) which permits real-time biospecific interaction analysis (BIA) by allowing analyte to pass over a sensor chip, to which antigen is covalently bound. Binding is detected by a change in refractive index expressed as resonance units (RU) depicted as a sensorgram. Analysis of the sensorgram permits the determination of rate constants.

Immobilization and kinetic measurements

Bovine submaxillary mucin (Sigma) was immobilized onto the dextran matrix of a CM5 sensor chip (Biosensor AB) in 10 mM citrate buffer pH 3·8. The dextran layer was activated for 7 min at a flow rate of 5 μl/min using an amine coupling kit (Biosensor AB) containing *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinamide (NHS). Approximately 900 resonance units (RU) of mucin were immobilized to the sensor chip by passing through the flow cell 35 μl of 200 μg/ml mucin in citrate buffer at a flow rate of 5 μl/min. Remaining active sites were then blocked with 1 M ethanolamine–HCl pH 8·5. Various concentrations of mAb were then passed over the immobilized mucin for kinetic analysis. After each measurement, bound antibody was washed out with 10 M NH_4SCN with no significant loss of immobilized mucin.

Kinetic calculations

Apparent rate constants k_a and k_d were calculated using BIAevaluation software from Biosensor AB. The interaction was treated as monovalent of the type antibody (Ab) + antigen (Ag) ↔ AbAg to simplify the mathematical treatment of the data, even though a portion of the AbAg complex is likely to have become an Ab2Ag complex. Detailed descriptions of the mathematical analysis have been reviewed extensively elsewhere.[19,20]

RESULTS

Kinetic measurements using real-time biospecific interaction analysis

The purity of the chimeric B72.3 mAb was checked by SDS–PAGE, specific ELISA and size exclusion gel filtration and found to be free of contaminating proteins, free of aggregates and to consist entirely of the relevant subclass (data not shown). Mucin-binding sensorgrams were obtained for all four B72.3 IgG subclasses and F(ab')$_2$ fragments of IgG1, IgG2 and IgG4 over a range of concentrations. The low yield of F(ab')$_2$ fragments from IgG3 precluded kinetic analysis. Each IgG subclass had different binding characteristics (Fig. 1). Maximal resonance units achieved for each subclass during the time–course of the injection differed with IgG4 having the highest maximal response. While antibodies at the same concentration might be expected to have similar maximal resonance units, the interaction curves observed here displayed a degree of heterogeneity. Furthermore, dissociation curves for the four antibodies showed subtle differences. IgG1 and IgG2 both demonstrated rapid initial dissociation phases while the dissociation curves for IgG3 and IgG4 appeared to be slower. These differences were observed at each concentration of antibody investigated.

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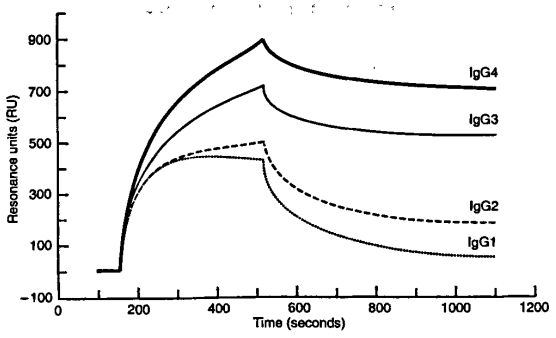


Figure 1. Sensorgrams for all four human IgG subclasses binding to mucin covalently attached to a sensor chip. Each preparation was analysed at a concentration of 170 nM.

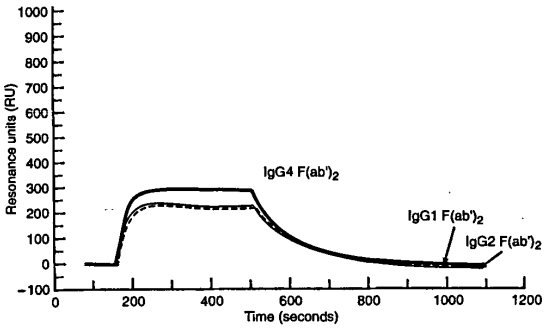


Figure 2. Sensorgrams obtained for $F(ab')_2$ fragments of human IgG1, IgG2 and IgG4 binding to mucin. All preparations were examined at a cocentration of 250 nM. The sensorgrams for the IgG1 and IgG2 fragments are virtually superimposable.

In contrast to the intact parent antibodies, binding curves for the $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 were strikingly similar to each other, showing rapid association followed by rapid dissociation (Fig. 2). For IgG2 and IgG4 there was a significant difference in the pattern obtained using the intact antibodies compared with the patterns obtained with the $F(ab')_2$ fragments.

Apparent rate constants

Association and dissociation rate constants were calculated for each IgG subclass and $F(ab')_2$ fragment studied. A range of five different concentrations of each antibody were analysed. Apparent association rate constants were found to be similar for both intact antibodies and $F(ab')_2$ fragments (Table 1). However, apparent dissociation rate constants were found to differ between the IgG subclasses with IgG1 having the fastest dissociation rate and IgG4 the slowest dissociation rate. The $F(ab')_2$ fragments were found to have dissociation rates similar to each other and all were faster than the respective intact antibodies.

DISCUSSION

We have utilized V region identical humanized antibodies of the four IgG subclasses to show that despite possessing identical V regions such antibodies exhibit differences in apparent dissociation rate constants. Whilst standard techniques of affinity measurement permit the derivation of equilibrium constants, BIA provides detailed analysis of the association and dissociation phases of the antibody–antigen interaction. The antibodies studied here showed a degree of heterogeneity in their association phase, although the major differences in the antibody kinetics was in their respective dissociation rates. Such differences appear to be due to the influence of the constant region on antibody binding, as shown by their abolition following the removal of the constant region by pepsin digestion.

Morelock *et al.*, using the less sensitive technique of competitive binding ELISA, were able to show functional affinity differences between mouse–human chimeric, V region-identical IgG1, IgG2 and IgG4 antibodies specific for intracellular

Table 1. Apparent rate constants of intact chimeric IgG subclass proteins and peptic $F(ab')_2$ fragments studied*

IgG subclass	Antibody range (nM)	k_a M^{-1} $second^{-1}$ ($\times 10^5$)	k_d $second^{-1}$ ($\times 10^{-3}$)
IgG1	170–10	1·92 (±0·08)	3·21 (±0·13)
IgG2	170–10	1·98 (±0·13)	2·56 (±0·03)
IgG3	150–20	1·41 (±0·10)	1·16 (±0·06)
IgG4	170–10	1·68 (±0·18)	0·69 (±0·06)
IgG1 $F(ab')_2$	250–15	1·62 (±0·03)	4·01 (±0·15)
IgG2 $F(ab')_2$	250–15	2·09 (±0·08)	3·93 (±0·12)
IgG4 $F(ab')_2$	250–15	1·93 (±0·13)	3·71 (±0·10)

* Rate constants were calculated as the mean ± standard error of a range of concentrations of antibodies. The same portion of the binding curve was chosen for analysis for each antibody concentration. Results are expressed as the mean value obtained from five different antibody concentrations, molar values were determined assuming a molecular weight of 146 000 MW for IgG1, IgG2 and IgG4, 170 000 MW for IgG3 and 100 000 MW for $F(ab')_2$ fragments

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adhesion molecule-1.[14] In their study the functional affinity of the antibodies was ranked IgG1 > IgG4 > IgG2 suggesting that the functional affinity differences could be explained by the respective subclass hinge flexibilities. The greater flexibility of the IgG1 hinge region may permit bivalent binding, in contrast to that of IgG2, the isotype thought to have the least flexible hinge region.[21–24] In our study, however, IgG1 and IgG2 had similar binding kinetics suggesting that hinge flexibility is unlikely to be the sole explanation for the differences noted above.

Differences in the functional affinity of IgG subclasses have been investigated more extensively in the murine system. Fulpius *et al.* showed that an IgG1 switch variant of an IgG3 parent lacked the expected rheumatoid factor activity despite having identical V regions.[12] Similarly, Schreiber *et al.* demonstrated functional affinity differences between an IgG1 switch variant of a V region-identical IgG3 parent specific for a *Pseudomonas* species, with IgG1 being of lowest avidity.[13] Cooper *et al.* have demonstrated differences in binding kinetics determined by BIA between mouse IgG1, IgG2b and IgG3 directed against *N*-acetyl-glucosamine (GlcNAc) of streptococcus group A carbohydrate with IgG3 being of the highest affinity.[15] The authors have suggested that the higher functional affinity of mouse IgG3 specific for GlcNAc is due to molecular co-operativity of IgG3, whereby IgG3 antibodies bound in close proximity to antigen undergo non-covalent Fc–Fc interactions stabilizing the complex. This might explain the consistent finding of higher IgG3 functional affinity in the murine literature although it is unlikely to explain differences in human IgG subclasses as they do not appear to display co-operative binding.[25] Furthermore, the human functional equivalent of murine IgG3 is IgG2 which is thought to have the least hinge flexibility[21–24] while human IgG3, in contrast to all the murine IgG subclasses, has a long flexible hinge. Such differences highlight the restricted structural and functional homology between the mouse and human IgG subclasses and underlines the danger in extrapolating data from the murine to the human IgG system.[26]

The functional significance of the differences in the apparent dissociation rate constants demonstrated here are as yet, unclear. However, the ability to analyse separately association and dissociation kinetics by BIA may prove crucial in the understanding of certain biological phenomena. Foote and Milstein have shown that the association constant of antibodies specific for the hapten 2-phenyl-5-oxazalone may be critical for B-cell selection and that concurrent with affinity maturation there is also kinetic maturation.[27] With regard to antibody function, the neutralizing capacity of a panel of antibodies reactive with the V3 loop of human immunodeficiency virus has recently been shown to correlate directly with dissociation rate.[28]

It appears likely that the differences observed here in the binding kinetics of chimeric mouse–human IgG subclasses may be due to structural differences in the human constant regions although the exact mechanism underlying such differences is at present unclear. In addition it remains to be established whether such differences exist between the different subclasses in a naturally occurring antibody response. Engineered antibodies may differ in their functional affinity and hence in their function, depending upon their subclass. Such consideration may be of importance when considering the choice of antibodies for therapeutic use.

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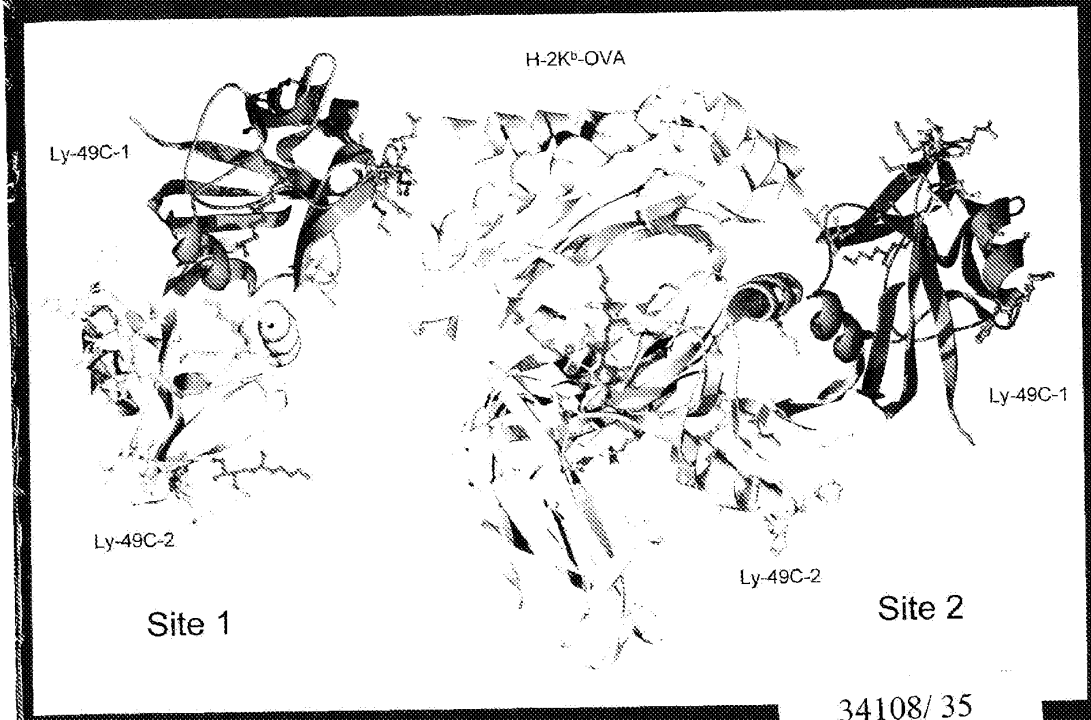
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On the cover: Model of Ly-49C and $H\text{-}2K^b$-OVA based on the crystal structure of Ly-49A-$H\text{-}2D^d$. Site 1 and site 2 interactions are shown. The $H\text{-}2K^b$ H chain is yellow, β_2m is gray, the peptide is orange, the Ly-49C-1 subunit is blue, and Ly-49C-2 is light blue. Ly-49C mutated residues that affected MHC class I binding: N160D/T162K are shown in orange, I195/P196 are shown in green, and K225/I226 are shown in red. Mutated residues that did not affect the Ly-49C-$H\text{-}2K^b$ interaction are shown as ball-and-stick molecules in backbone color. Ly-49C was threaded on the crystal structure of Ly-49A and was overlaid in the same orientation as Ly-49A in the Ly-49A-$H\text{-}2D^d$ structure (pdb entry 1Q03). The crystal structure of $H\text{-}2K^b$-OVA (pdb entry 1VAC) was aligned with the $\alpha_1\alpha_2$ domains of $H\text{-}2D^d$ in the crystal. The modeling and image were prepared using the Swiss-PDB Viewer, and the image was refined with the Persistence of Vision Ray Tracer. (Figure 5 from Sundbäck, J., A. Achour, J. Michaëlsson, H. Lindström, and K. Kärre. NK cell inhibitory receptor Ly-49C residues involved in MHC class I binding. *J. Immunol. 168:793*)

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Isotype Can Affect the Fine Specificity of an Antibody for a Polysaccharide Antigen[1]

Gary R. McLean,[2,3]* Marcela Torres,[2]* Natalia Elguezabal,* Antonio Nakouzi,* and Arturo Casadevall[4]*†

Ab specificity is determined by V region sequence. The murine Mab 18B7 (IgG1) binds to the *Cryptococcus neoformans* capsular polysaccharide glucuronoxylomannan and produces annular immunofluorescence (IF) on yeast cells. The heavy and light V regions of 18B7 were expressed with the human C regions μ, γ1, γ2, γ3, γ4, and α1, and the specificity and binding properties of these mouse-human chimeric (ch) Abs was determined. The chIgG1, chIgG2, chIgG4, and the chIgA produced annular IF, whereas the IgM and IgG3 produced punctate IF, despite identical V region sequences. Competition experiments with murine Abs that competed with mAb 18B7 and binding assays to peptide mimetics of glucuronoxylomannan provided additional evidence for altered specificity in some of the ch Abs. Expression of 18B7 heavy V region with murine μ C region produced IgM with a punctate IF, indicating that a change in fine specificity also accompanied the change from murine IgG1 to IgM. Our results show that Ab fine specificity can be a function of isotype. This phenomenon may be most apparent for Abs that bind to Ag with repeating epitopes, such as polysaccharides, where the quarternary structure of the Ag-Ab complex may be influenced by such constraints as Fab-Fab angles, Fc-Fc interactions, Ab size, and solvent accessibility to exposed surfaces. Alterations in Ab fine specificity following isotype change could have important implications for current concepts on the generation of secondary Ab responses to certain Ags and for the isotype preference observed in Abs to polysaccharides. ***The Journal of Immunology*, 2002, 169: 1379–1386.**

A central concept in our understanding of Ab structure and function is that the V region determines Ag specificity, whereas the C region determines the isotype, effector functions, and pharmacological characteristics of Ig molecules. Ab specificity and affinity are believed to result from the type of interactions made between V region amino acid residues and Ag surfaces. This concept is supported by overwhelming evidence from biochemical, immunological, and crystallographic studies showing that V regions contact Ag and that this interaction is responsible for the specificity of the Ab molecule. Isotype switching from IgM to IgG and IgA is considered to result in Abs that retain Ag specificity with the acquisition of new biological effector functions conferred by the different C region. The C region is not generally believed to contribute to specificity, but can have a major effect on the apparent affinity through its contribution to valency, and hence, avidity. Nevertheless, there is some evidence that C region interactions among Abs bound to Ags with repeating structural motifs can affect the types of complexes formed (1).

The construction of mouse-human chimeric (ch)[5] Abs is predicated on the premise that murine V regions confer and retain Ag specificity in the context of expression with human C regions. (2). Mouse-human ch Abs have been used for comparative studies of human C region function (3, 4) and for the calibration of serological assays as human Ab standards (5). Mouse-human ch Abs are more attractive for use in human therapy than murine Abs because they are less immunogenic, have longer serum half-life, and possess human C region effector function (6). Currently, ch Abs are under clinical evaluation for a variety of conditions. The use of mouse-human ch Ab in clinical and laboratory applications is based on the implicit assumption that they retain the specificity of the parent murine Ab.

The murine mAb 18B7 (IgG1-κ) is currently in clinical trial for evaluation as an adjunct to antifungal therapy in the treatment of *Cryptococcus neoformans* infections (7). This mAb binds to glucuronoxylomannan (GXM), which is the major constituent of the *C. neoformans* polysaccharide capsule. To improve effector cell function for human phagocytic cells and to reduce its immunogenicity in humans, we synthesized ch human-murine mAbs containing the heavy (V_H) and light (V_L) V regions from the murine mAb 18B7 and human C regions for the various Ig isotypes (μ, γ1, γ2, γ3, γ4, and α1; Ref. 3). To our surprise, the 18B7 mouse-human IgM, IgG3, and IgG4 ch Abs revealed differences in fine specificity from the other ch Abs and the parent murine mAb 18B7 despite identical V region sequences. Similarly, expression of 18B7 V_H with murine IgM resulted in altered specificity. The results imply that in certain conditions, the C region can affect the conformation of the V region that may in turn lead to changes in idiotype and fine specificity.

Materials and Methods

Murine and ch Abs

The IgG1 murine mAb 18B7 has been described (7). This mAb binds to the *C. neoformans* polysaccharide and is currently in clinical evaluation. Cloning of the 18B7 V_L regions into pLC and the 18B7 V_H region into pHC μ, γ1, γ2, γ3, γ4, and α1 expression vectors was done as described previously (8). With the exception of IgG3 ch, Abs were expressed in the murine

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[5] Abbreviations used in this paper: ch, chimeric; V_H, heavy V region; V_L, light V region; GXM, glucuronoxylomannan; IF, immunofluorescence.

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myeloma cell line NSO as described (8) and harvested from culture supernatants. Briefly, NSO cells were routinely grown in DMEM supplemented with 10% FCS and clones stably transfected with the pLC vector were selected in the presence of 6 μg/ml puromycin. L chain clones stably transfected with the pHC vectors were selected in the presence of 1.5 mg/ml G418 as described (8). Cells expressing ch Abs were grown in DME with 10% FCS, 10% NCTC 109, 1% nonessential amino acids and 1 mg/ml G418 at 37°C and 10% CO_2. Subsequently, the cell lines were adapted to conditions with 5% FCS. For the chIgG3, protein was obtained by lysis of the transfected cells. The IgM ch Ab was determined to be pentameric from migration of a nonreducing SDS-PAGE relative to native human IgM.

Generation of IgG1 switch variant from IgM hybridoma 13F1

An IgG1 isotype variant of the murine mAb 13F1 was recovered by selecting for a spontaneous IgG-producing hybridoma using the ELISA spot method as described (9).

Expression of 18B7 V_H with murine IgM

RNA was isolated from hybridoma cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). cDNA was generated using reverse transcriptase and the oligonucleotide primer p(dt)15 (Boehringer Mannheim, Indianapolis, IN). DNA containing the V_H was amplified using the primers 5′-ATAAAGCTTGCGGGGATATCCACCATGGAC and 3′-ATAGCTA GCTGAGGAGACTGTGA. The amplified DNA was cloned into the PCR 2.1 vector (Invitrogen, San Diego, CA) for sequencing and subcloned into a murine IgM expression vector, as described (10). The plasmid was then transfected into H chain nonproducing 18B7, 12A1, and 13F1 hybridoma cells by electroporation. Cells were selected with 1.5 mg/ml G418 (Geneticin; Life Technologies) in DME media with 20% of FCS (Harlan, Indianapolis, IN), 10% NCTC-109 (Mediatech, Herndon, VA), and 1% of nonessential amino acid solution (Mediatech).

Immunofluorescence

Glass slides were coated with poly L-lysine (0.1 mg/ml; Sigma-Aldrich, St. Louis, MO), a suspension containing 10^6 yeast cells was placed on the slide surface and the slide was air dried. The slides were then blocked by adding a solution 2% BSA and 0.5% goat serum and incubating for 30 min at 37°C. Abs were then added at a concentration of 5 μg/ml in blocking solution, and incubated overnight at 4°C. FITC-labeled goat anti-human IgG, IgM, IgA, or FITC-labeled goat anti-mouse IgM or IgG1 were added at 10 μg/ml after the incubation with primary Ab, and incubated at 37°C for 30 min. In other experiments, tetramethylrhodamine B isothiocyanate-labeled goat anti-human κ was used. Prior studies have established that the immunofluorescence (IF) pattern is the same regardless of whether one uses FITC-labeled primary or secondary Ab (11). The slides were washed three times with PBS between application of reagents. Finally, 30 ml of mounting medium (0.1 M *n*-propyl gallate–50% glycerol in PBS; Sigma-Aldrich) was added, and coverslips were placed. All labeled reagents were from Southern Biotechnology Associates (Birmingham, AL) except anti-human IgG, which was obtained from ICN Pharmaceuticals (Costa Mesa, CA). The slides were viewed with an Olympus AX70 microscope (Olympus, Melville, NY) equipped with FITC (fluorescein) and tetramethylrhodamine B isothiocyanate (rhodamine) filters. Fluorescent images were recorded with narrow band filter sets to ensure no cross-talk or spillover from one filter set to the others. Gray scale images were merged. This method is equivalent to multiple exposure photography, but has the advantages of wider linear response and dynamic range, optical separation of filters, and digital storage. Images were recorded in black and white. Color corresponding to the filter wavelength was subsequently added back during image reconstruction to reflect the actual color observed.

ELISA

mAb concentration was determined by ELISA relative to isotype-matched standard controls (Sigma-Aldrich). Polystyrene plates were coated with goat anti-human IgM, IgG, or IgA (Accurate Chemical and Scientific, Westbury, NY) blocked with 1% BSA in PBS and incubated with the ch Ab-containing solution. Bound Ab was detected using alkaline-phosphatase or biotin-conjugated goat anti-human IgM, IgG, or IgA (Accurate Chemical and Scientific). Ab binding to GXM was measured as described (6), except that bound Ab was detected using phosphatase-conjugated goat anti-human κ (Caltag Laboratories, San Francisco, CA). Competition ELISAs were done to determine whether murine mAb 12A1 could inhibit the binding of the ch Abs to GXM using the same protocols as described previously (7, 8). For this ELISA, polystyrene plates were coated with 2 μg/ml of GXM and incubated for 1.5 h at 37°C. The plates were then blocked with 1% BSA-PBS and incubated overnight at 4°C. Ch Abs and mAb18B7 were added and serially diluted, followed immediately by the addition of IgM mAb 12A1 (final concentration 1 μg/ml). The plates were incubated overnight at 4°C. Binding of murine IgM was detected by specific alkaline-phosphatase-conjugated goat anti-mouse reagents. Reactivity of ch Abs with the murine anti-idiotypic mAb 7B8 was also measured by ELISA. The anti-idiotypic mAb 7B8 binds to the Ag-combining site of 18B7 (9). Plates were coated with 1 μg/ml of goat anti-mouse κ, blocked with 1% BSA in PBS, and incubated with the ch mAb preparation. The plates were then incubated with 2 μg/ml solution of mAb 7B8 (IgG1) and binding was detected with phosphatase-conjugated goat anti-mouse IgG1. For peptide ELISA, polystyrene plates were coated with 1 μg/ml solution of streptavidin in PBS followed by blocking with 1% BSA in PBS. Biotinylated peptide was then added at a concentration of 2 μg/ml before testing mAb binding by titration. Specific binding of ch18B7 mAb was detected using phosphatase-conjugated goat anti-human κ Ab.

To prepare monomers of IgM, the ch mAb (4 μg/ml) was mixed with an equal volume of a 0.15-M solution of 2-ME in TBS and incubated for 1 h at 37°C immediately before addition to the ELISA plate for GXM binding or anti-idiotype ELISAs. The reduced product of IgM and IgG ch Ab contained both H and L chains by ELISA, indicating that the reduction had not disrupted the monomer.

Phagocytosis assay

J774.16 cells were cultured in DMEM with 10% heat-inactivated FCS, 10% NCTC-109 medium (Life Technologies), and 1% nonessential amino acids (Cellgro, Washington, DC). The protocol for in vitro phagocytosis was as described in earlier studies (11) with minor modifications. Cells were plated in 96-well tissue culture plates (Falcon; BD Biosciences, Franklin Lake, NJ) at a density of 10^5 cells/well in 96-well culture plates and stimulated with 50 U/ml recombinant murine IFN-γ (Genzyme, Cambridge, MA) and LPS (Sigma-Aldrich). Briefly, macrophages were stimulated with 50 U/ml IFN-γ/ml and incubated at 37°C overnight. The medium in each well was then replaced with fresh medium containing 50 U/ml IFN-γ and 1 μg/ml LPS, and *C. neoformans* cells were added at a ratio of 5:1 macrophages to fungi. Phagocytosis was measured in the presence or absence of ch Abs (2 μg/ml). After the addition of *C. neoformans*, the cells were incubated at 37°C for 3 h, washed three times with sterile PBS, fixed with cold absolute methanol, and stained with a 1/20 solution of Giemsa. PBS was used as a negative control and mAb 18B7 (IgG1) was used as a positive control, and phagocytic indices were determined. The phagocytic index is defined by: $PI = P \times F$, where P is the percentage of phagocytic macrophage and F is the average of yeast cells per macrophage. Experiments were done in duplicate and four different fields were counted per well.

Results

V region sequences

The 18B7 V_H and V_L nucleotide sequences have been previously reported based on direct mRNA sequencing (12). The 18B7 V_H and V_L region cDNA was cloned from 18B7 hybridoma cells by RT-PCR and inserted into human Ig expression vectors as described (8). V_H and V_L region sequence analysis revealed several differences from the previously published sequences (7, 12). For 18B7 V_H, there were three amino acid differences resulting from single-base changes. In the CDR1 region, positions 28 and 29 were Ser and Phe rather than Thr and Cys, respectively, and position 65 of the CDR2 was Arg instead of Lys. For the 18B7 V_L, there were two amino acid differences resulting from single-base changes in the cDNA sequence. Amino acid 15 of the first framework region was Leu rather than the original Ala, and CDR1 position 27C was Ala rather than Cys. These updated 18B7 V region sequences are deposited in GenBank under accession nos. AJ309276 and AJ309277. For each cell line producing the ch Abs, the V_H and V_L regions were sequenced and shown to be identical with each other and with those of the parent mAb 18B7. Multiple clones of each V region were analyzed.

Reactivity of ch Abs with GXM

The IgM, IgG1, IgG2, IgG3, IgG4, and IgA1 mouse-human ch Abs bound to GXM immobilized in polystyrene plates by ELISA (Fig. 1). Given that each ch Ab type had identical V region sequence, we

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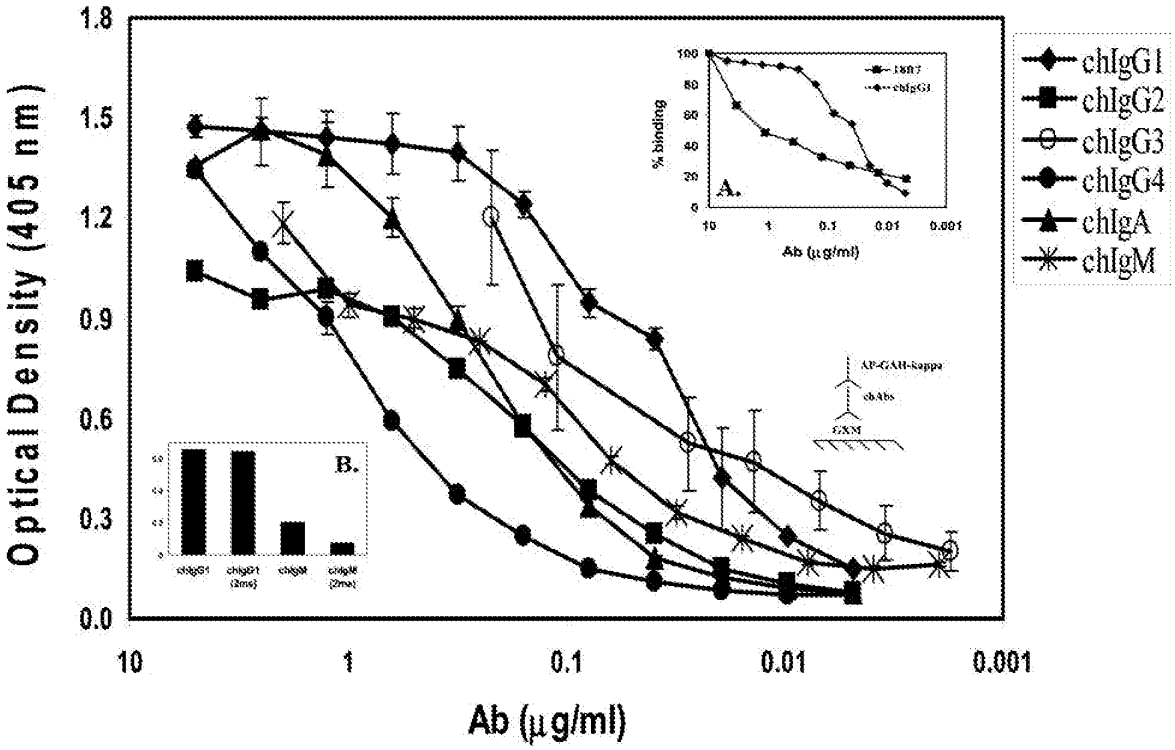


FIGURE 1. GXM binding of ch Abs by ELISA. The chIgG1, chIgG2, chIgG4, chIgA, and chIgM ch Abs bound to GXM immobilized in polystyrene plates by ELISA. The diagram represents the ELISA configuration. Data points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because there was insufficient Ab available. The chIgM and chIgG3 are from a different assay than the others, but are plotted here together because very similar results were observed in multiple assays in different days done by different investigators. *A*, A plot of chIgG1 relative to mAb 18B7 to compare their relative binding to GXM. *B*, Reactivity of chIgG1 and chIgM before and after 2-ME reduction.

were surprised to find large differences in binding to GXM (Fig. 1) and to mAb 18B7 (Fig. 1*A*). The relatively weaker binding observed for the chIgM was unexpected, since this molecule was pentameric as indicated by nonreducing SDS-PAGE (data not shown), and the higher valency should have conferred a higher avidity. To explore the potential contribution of valence to the binding of the chIgM, 2-ME was used to dissociate the IgM polymeric structures into monomers (13). Treatment of IgM with 2-ME resulted in complete loss of Ab reactivity, whereas identical 2-ME treatment of chIgG1 had no effect on GXM binding (Fig. 1*B*). Because IgM monomers have the same V region sequence as the IgG subtypes, the 2-ME reduction experiment indicated that the 18B7 V_H-human cμ chimera had significantly reduced affinity for Ag.

Immunofluorescence patterns of C. neoformans *with murine and ch Abs*

Prior studies have shown that the IF pattern resulting from Ab binding to encapsulated *C. neoformans* cells is a sensitive indicator of Ab specificity that correlates with biological activity (11). The IF pattern of mAb 18B7 with *C. neoformans* is annular (Fig. 2) and the expectation was that all the ch derivatives would also produce annular patterns upon binding yeast cells. The IF pattern observed with chIgG1, chIgG2, chIgG4, and chIgA1 Abs was annular (Fig. 2). Nevertheless, we noted differences in the IF pattern for the IgG chs as manifested by differences in fluorescence intensity throughout the capsule (Fig. 2). In particular, the chIgG4 gave a thick annular pattern that was different from the other IgG subtypes. Surprisingly, the IgM and IgG3 ch Ab produced a punctate pattern that was qualitatively different from that observed with the parent murine mAb or the other ch Abs (Fig. 2). The same patterns were obtained regardless of whether the secondary Ab was specific for the human H or L chain. The punctate patterns observed with the IgM and IgG3 chs were similar to that observed with the murine mAb 13F1, which is not protective and not opsonic (14, 15).

Competition experiments and reactivity with peptide mimetics

Because the altered IF patterns for chIgM and chIgG3 suggested an alteration in V region specificity as a result of expression in association with human μ and γ3, we proceeded to explore this possibility by Ab competition assays and reactivity with peptide mimetics. In previous studies, we have used the absence of Ag competition between two Abs as a criterion for different specificity (16). Competition experiments were performed with the murine mAb 12A1 (IgM) that produce annular patterns on binding *C. neoformans* and has similar specificity to 18B7 (17). As is evident in Fig. 3, murine mAb 12A1 inhibited the binding of ch Abs as follows; chIgA1 > chIgG1 > murine 18B7 $\gg$ chIgG2 > IgG4 $\sim$ IgM > IgG3. Absence of competition with an mAb that inhibits the parent mAb 18B7 provides strong evidence for altered specificity in the chIgM, chIgG2, chIgG3, and chIgG4 Abs. Competition experiments between mAb 18b7 and ch Abs revealed that it competed with chIgG1, chIgG2, and chIgG4, but not chIgM or chIgG3 (data not shown). As a second confirmatory method, we measured the binding of ch Abs to two GXM peptide mimetics that

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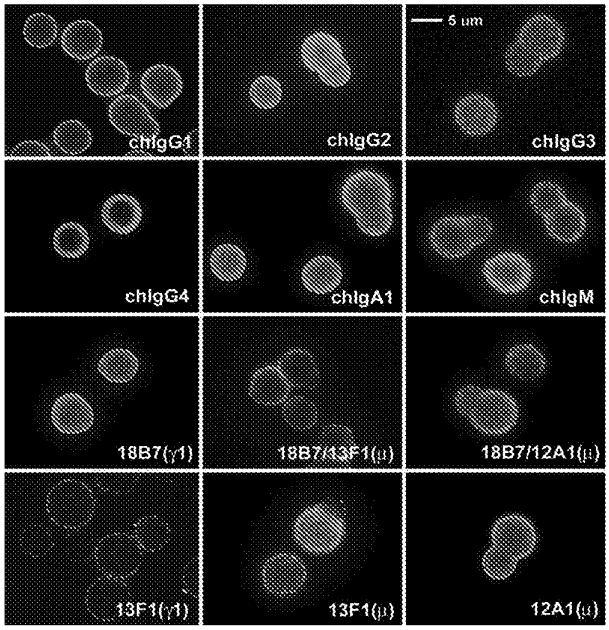


FIGURE 2. Immunofluorescence of *C. neoformans* stained with ch and murine Abs. Each photograph was taken under identical conditions. Cells shown are representative of patterns observed.

have proven useful for identifying small differences in fine specificity among mAbs with identical V region usage (18). Significant differences were noted for the reactivity of the various ch Abs and the parent mAb 18B7 for two peptides, providing additional evidence for alterations in specificity (Fig. 4).

Reactivity of parent and ch Abs with anti-idiotypic mAb

The idiotype of mAb 18B7 is recognized by the murine anti-idiotypic mAb 7B8 (19). The mAb 7B8 interacts with both the V_H and V_L at the Ag combining site (9). The 18B7 Id structure is presumably conferred by the primary sequence folded into the Ag binding site. mAb 7B8 bound strongly to chIgG1, chIgG2, and chIgA1 Abs, but weakly to chIgM, chIgG3, and chIgG4 (Fig. 5). Treating the IgM ch with 2-ME to create monomers did not restore

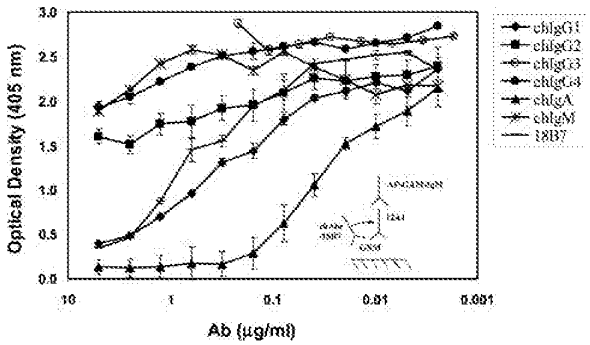


FIGURE 3. Binding of ch Abs and parental mAb 18B7 in the presence of mAb 12A1 by ELISA. The diagram represents the ELISA configuration. Points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. mAb 12A1 strongly inhibits binding of chIgA and chIgG1 and mAb 18B7, but is less effective at interfering with the binding of IgM, IgG2, IgG3, and IgG4 ch Abs.

the 18B7 Id. Hence, there were significant differences in the reactivity of ch Abs with an anti-Id mAb to mAb 18B7 despite identical V region sequences.

Opsonic efficacy of ch Abs

The chIgG1, chIgG2, and chIgG4 were strongly opsonic, whereas chIgA1 had weaker opsonic activity (Fig. 6). The opsonic efficacy of chIgG3 could not be evaluated because we lacked sufficient protein for this experiment. The opsonic activity of chIgG1, chIgG2, and chIgG4 was comparable to that of mAb 18B7. For murine IgM mAbs, the opsonic activity for *C. neoformans* correlates with the type of IF pattern such that Abs with annular IF efficiently promote phagocytosis, whereas those with punctate IF do not (14, 15). For chIgM, the opsonic efficacy was significantly lower than the opsonic murine IgM mAb 12A1, but higher than that observed for the nonopsonic murine IgM mAb 13F1.

Murine isotype and IF pattern

Because punctate IF patterns had previously been observed only with murine IgM mAbs (14, 15), we investigated whether punctate IF was an inherent property of the IgM isotype with certain V regions by carrying out two experiments. First, we selected a spontaneous switch variant from the hybridoma 13F1 that synthesized IgG1. The 13F1 IgG1 variant produced a punctate IF pattern like its parent IgM (Fig. 2). Second, we expressed the 18B7V_H with a murine μ C region to produce IgM. We attempted to express the 18B7 V_H-μ with 18B7V_L, but were unable to obtain an IgM-producing clone after screening >300 clones in three independent transfection experiments. Our inability to express 18B7 V_H-μ with 18B7V_L despite considerable effort suggests that the isotype switch that produced the B cell parent for the 18B7 hybridoma resulted in changes that precluded IgM expression. Instead, we resorted to expressing 18B7 V_H-μ with the V_L of IgM mAbs 12A1 and 13F1. Prior studies had established that the IF pattern is a property of the V_H (10). The 18B7 V_H-μ was expressed with the V_L of mAbs 12A1 and 13F1, which produce annular and punctate IF patterns, respectively, as a function of their V_H sequences (10). Binding of 18B7 murine IgM to *C. neoformans* produced a different IF pattern than that observed with the parent IgG1 mAb in combination with both the 12A1 and 13F1 V_L (Fig. 2). The 18B7/12A1 and 18B7/13F1 Abs each reacted with GXM, but only 18B7/13F1 was recognized by the anti-Id mAb 7B8 (Fig. 7). Competition assays with mAb 18B7 revealed competition with 12A1/13, but not 18B7/12A1 (Fig. 7). These results imply a change in the fine specificity of the 18B7 V_H when expressed with murine μ relative to that observed when expressed as γ1.

Discussion

Each of the mouse-human ch Abs derived from mAb 18B7 bound to the *C. neoformans* capsule and to soluble GXM. Hence, each ch Ab retained the Ag specificity of the parent murine mAb. However, to our surprise, there were differences in the serological properties of the ch Abs with GXM relative to the parent mAb 18B7. Most strikingly, chIgM, chIgG3, and chIgG4 displayed different IF after binding *C. neoformans* cells compared with that of mAb18B7, the other ch18B7 IgG subclasses, and the ch18B7 IgA1. Specifically, the chIgM and chIgG3 produced punctate IF, whereas the other ch Abs and mAb 18B7 produced annular IF. The chIgG4 produced a thick annular IF different from the other Abs. When comparing their binding to GXM, IF patterns, and reactivity with peptide mimotopes, each ch Ab was found to be different from the others and from the parent mAb 18B7. This result was unexpected since sequence analysis revealed that each of the ch

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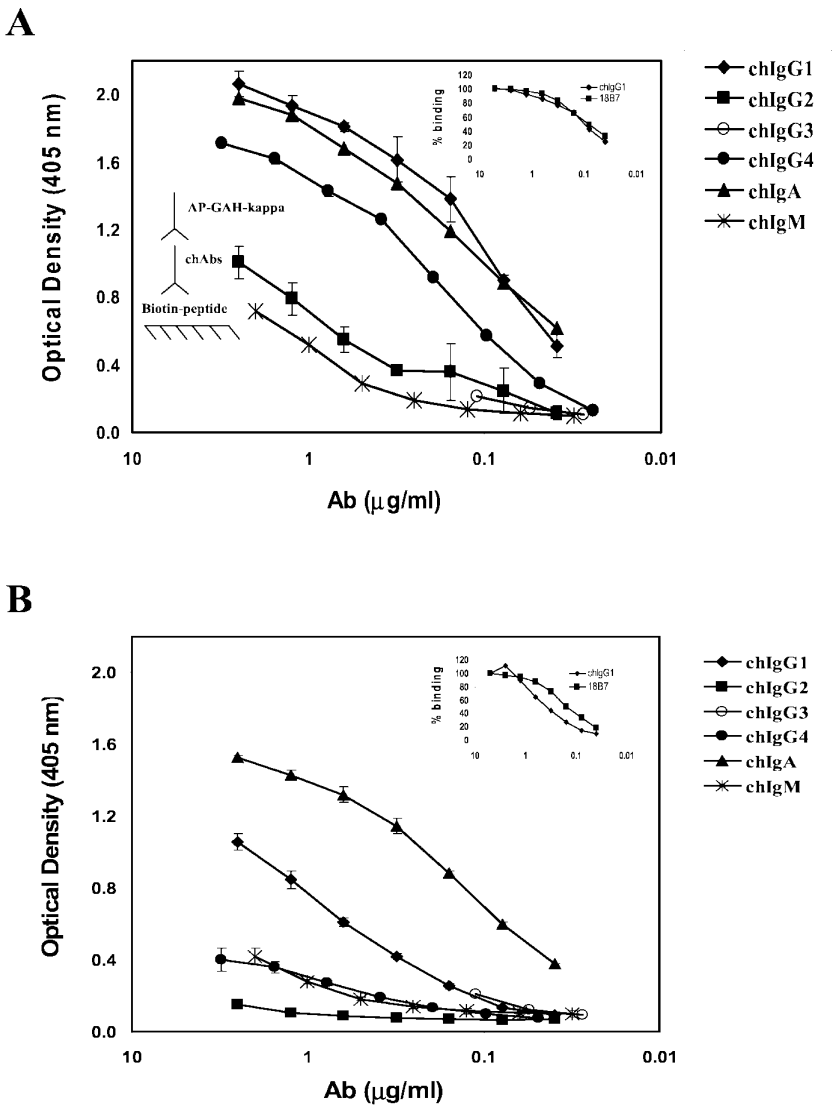


FIGURE 4. Reactivity of mAb 18B7 and ch Abs with GXM peptide mimetics P206.1 (*A*) and P601 (*B*) by ELISA. The diagram represents the ELISA configuration. Data points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because limited quantities of this Ab were available. Insets show plots of chIgG1 relative to mAb 18B7 to illustrate their relative binding to these peptides.

Abs had identical V_H and V_L sequence to the parent mAb 18B7. However, V region sequence analysis revealed several amino acid differences between the sequences amplified and cloned from the murine hybridoma 18B7 in this study and that reported previously based on direct mRNA sequencing (7). Because identical sequences were found repeatedly among independently amplified clones in this study, we attribute the differences between the current data and the earlier reports to errors associated with V region sequence determination by direct mRNA sequencing, which is known to be highly error-prone.

The observation that chIgM and chIgG3 gave a punctate IF pattern was intriguing because this type of IF pattern had previously been observed only with two nonprotective IgM murine mAbs (15, 16), which are not opsonic (14). We do not currently know the molecular structure of the carbohydrate domain recognized by the Abs binding in either punctate or annular IF. However, the differences in protective efficacy (15, 16), complement activation (20), and opsonic capacity (14) noted for mAbs that bind in annular in punctate IF patterns provide strong evidence that these represent different epitopes. For this discussion, we use the term "specificity" to denote the binding of Ab to the polysaccharide Ag GXM as manifested by reactivity with GXM, and the term "fine specificity" to denote the nature of Ab binding to GXM as manifested by the interaction of complexes formed by different Abs with GXM that is demonstrated by differences in IF and other serological assays. We are cognizant of the theoretical and practical difficulties involved in defining the terms specificity, fine specificity, and epitope in the absence of structural information (21, 22). Furthermore, we caution that Abs binding in annular or punctate IF are not necessarily binding at exactly the same site on the GXM or forming the same types of Ag-Ab complexes, a notion that finds support in this study.

We investigated whether expression of the 18B7 V_H with the human C regions altered Fab structure by evaluating the integrity of the 18B7 Id structure on the ch Abs. The mAb 7B8 demonstrated strong reactivity with chIgG1, chIgG2, and chIgA1, and weaker reactivity with chIgM, chIgG3, and chIgG4. Because the V region sequences of all the chs are identical, this result indicates that expression of the murine 18B7 V_H in the context of the human IgM, IgG3, and IgG4 altered the idiotypic structure, possibly a

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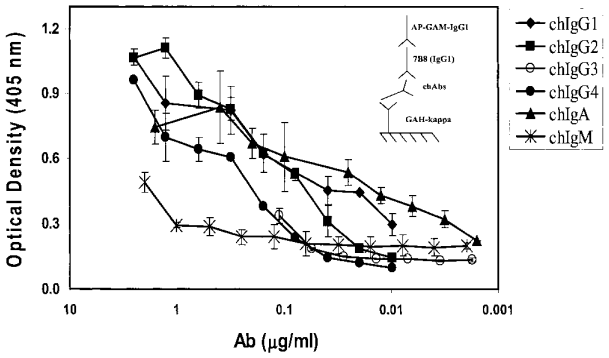


FIGURE 5. Reactivity of parent and ch Abs with the anti-idiotypic mAb 7B8 by ELISA. The diagram represents the ELISA configuration. Points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because there was insufficient Ab available. The chIgM and chIgG3 are from a different assay than the others but are plotted here together because very similar results were observed in multiple assays on different days done by different investigators.

result of differences in protein folding. This observation is consistent with the report that syngeneic V regions are more immunogenic, as IgM molecules when compared with IgG molecules, suggesting isotype-related differences in V region folding and exposure of antigenic epitopes (23).

Reduction of chIgM with 2-ME resulted in complete loss of Ag binding. This was unexpected because IgM monomers should bind to GXM with similar specificity and affinity as the IgG isotypes given identical V region sequences. Because the IgM C region sequence was that expected for this protein (8), mutations or deletions in the c_H domains can be excluded as an explanation for the altered fine specificity or the inability to bind as a monomer. Hence, for the chIgM, the change in fine specificity was accompanied by a reduction in apparent affinity and Id recognition consistent with an alteration in the protein structure at the Ag binding site.

Prior studies had shown that the punctate IF fine specificity was conferred by V_H (10), but we investigated whether expression of this pattern required the IgM valency structure. The approach was to generate an IgG1 isotype switch variant from a murine IgM that has punctate IF and to express 18B7 V_H as a murine IgM. An IgG1 isotype-switched variant was generated from the murine IgM mAb

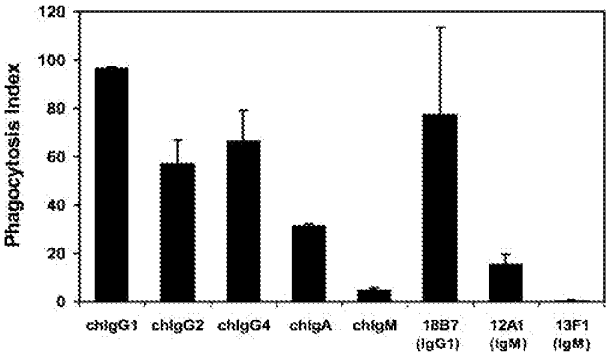


FIGURE 6. Opsonic efficacy of murine mAbs and ch Abs for *C. neoformans* with J774.16 cells. Values represent the average of eight measurements and brackets represent SD.

13F1 that displays punctate IF. Like the parent 13F1 IgM, the 13F1 IgG1 isotype-switched variant revealed punctate IF, a finding indicating that the IF pattern was not restricted to the IgM class. However, expression of the 18B7 V_H with the murine μ-chain to generate a murine IgM produced a different IF pattern than the parent IgG1 mAb 18B7. In fact, the pattern of the 18B7 IgM was very similar to the punctate IF observed with chIgM, chIgG3, and mAb 13F1. These results indicate that punctate IF patterns can be observed with both IgM and IgG isotypes. For the specific case of 18B7 IgM, the change from annular to punctate observed for IgG1 and IgM, respectively, may reflect a change in fine specificity resulting from greater avidity. This phenomenon may be similar to that observed to occur for murine IgG3 to streptococcal polysaccharide which can form different types of Ag-Ab complexes than other V region identical IgG isotypes because of ability to aggregate and increase its avidity (1).

chIgG3 also produced a punctate IF pattern, but retained reactivity with the anti-Id mAb, suggesting that steric and/or flexibility constraints may also contribute to the altered fine specificity observed for both IgM and IgG3. Among human IgG subclasses IgG3 is the most flexible, as evidenced by its ability to form ring dimers with anti-Id mAbs (24). IgG3 complexes also demonstrated the largest Fab-Fab angles among IgG subclasses bound to anti-Id mAbs, presumably due to their extended hinge region (24). However, human IgM monomers are even more effective than IgG3 at forming ring dimers, suggesting that it is the most flexible Ab class (25). Because GXM is a polysaccharide with repeating epitopes and both human IgM and IgG3 are multivalent ligands which display great flexibility for their Fab domains, it is possible that the punctate patterns reflect the formation of Ag-Ab complexes in the capsule with different geometry than those associated with the annular IF pattern. Consistent with this view is the observation that Abs which produce annular IF on wild-type *C. neoformans* strains produce punctate IF when binding to mutants that express *O*-acetylation-deficient GXM, which is more flexible polysaccharide structure and may form different types of Ag-Ab complexes in the capsule (26). Another factor that may contribute to the type of complexes formed is the nature of the exposed surfaces of the various isotypes. In this regard, liquid partition studies have shown that human IgM and IgG3 also appear to have different surface properties from each other and from other IgG subclasses which could conceivably affect the microenvironment in which the complexes are formed (27). For chIgG4, the alteration of the IF pattern to a thick annular type may reflect the ability of IgG4 molecules to self interact, a phenomenon proposed to account for both its apparent monovalence and its capacity to form bispecific molecules (28).

In summary, we observed a change in IF pattern from annular to punctate when 18B7 V_H was expressed with murine and human μ C region and with human γ3 c and γ4 c. For each Ab, the explanation for the change in fine specificity may be different. For chIgM, this phenomenon may reflect an altered Fab structure (as evidenced by loss of Id reactivity and reduced affinity), greater intrinsic Fab flexibility (25), or different Ig surface properties (27). For the murine 18B7 IgM, the change in specificity may reflect a difference in avidity as reported for Abs to streptococcal polysaccharide (1). For the chIgG3, the change in specificity may reflect the enhanced Fab flexibility of this isotype relative to other IgGs (24), or different Ig surface properties (27). Furthermore, we did note variations in IF after binding *C. neoformans* and in the reactivity with GXM and peptide mimetics for the IgG1, IgG2, and IgG4 chs, suggesting that more subtle changes in fine specificity may have also occurred in the other ch Abs.

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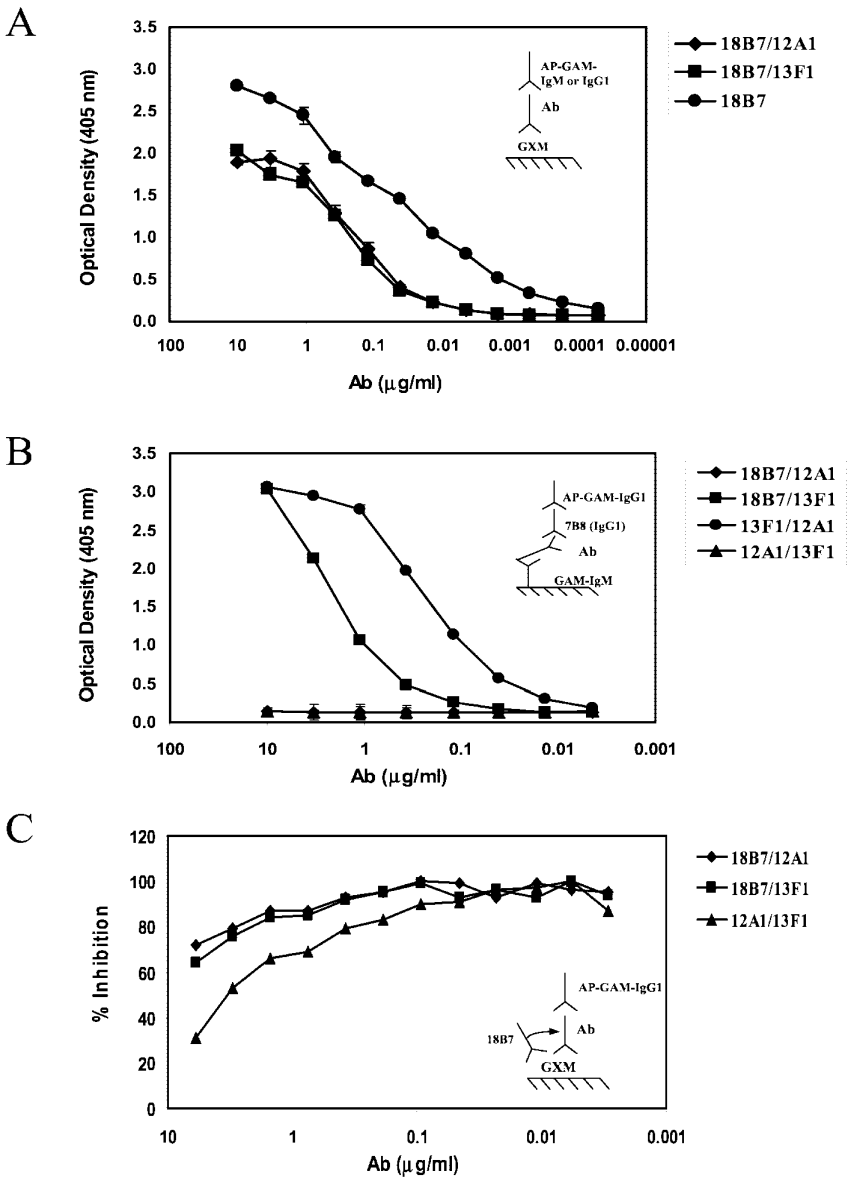


FIGURE 7. Reactivity of 18B7 V_H expressed as IgM with L chains of mAbs 12A1 and 13F1 with GXM (*A*), anti-idiotypic mAb 7B8 (*B*), and GXM in competition with mAb 18B7 (*C*).

The results of this study also shed light on the requirements for generation of the punctate IF pattern. Before this study, the punctate IF pattern had been observed only with two murine IgMs known as 13F1 and 21D2 (15). Recently, we used site-directed mutagenesis to establish that changing two amino acids in the in V region of an IgM mAb could change the IF pattern from annular to punctate (10). In this study, we demonstrate that the same V can produce annular or punctate patterns depending on which human or mouse C region it is associated with. Hence, the type of IF pattern is dependent on both the primary structure of the V region and the conformation of the Ag combining sites as determined by the associated C region.

Our observations add to the growing body of evidence that in certain circumstances, the Ig C region can affect the fine specificity of an Ab molecule. There are at least two potential mechanisms for this phenomenon. First, the C region could affect V region folding. This mechanism could explain isotype-related differences in Id immunogenicity (23) and the simultaneous loss of Id recognition for the chIgM and altered IF pattern on binding *C. neoformans* noted in this study. Second, the C region could influence the specificity of an Ab for an Ag with repeating epitopes through effects on segmental flexibility and the ability of certain Fc regions to associate (1). This phenomenon was demonstrated for a family of murine mAbs to group A streptococcal polysaccharide with identical V region which show isotype-related differences in their ability to bind bacterial strains expressing different epitope densities (1). Furthermore, there is evidence that C regions can affect the neutralizing activity of V-region identical Abs to human cytomegalovirus, possibly as a result of differences in the binding domain of the Ab to the target glycoprotein as a consequence of Fc-related effects (29). Another study reported that changing the C region of a viral enhancing murine mAb from IgG3 to a human IgG1 abrogated its deleterious activity in vitro, possibly as a result of changing the three-dimensional structure of the virus-Ab complex (30).

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The observation that the C region can affect the specificity of an Ab raises the possibility that isotype switching in the immune response can be accompanied by a change in specificity. If this is the case, then isotype switching may lead to loss of recognition of the original Ag as well as the recognition of new epitopes. In fact, this could suggest an explanation for the observation that the primary and secondary response to certain Ags can arise from different precursors (31). A variation of this theme was suggested by the observation that IgA1 bind tubulin with significantly greater affinity than IgG1 as a consequence of c_H segmental flexibility, raising the possibility that affinity maturation can occur by isotype switching alone (32). Furthermore, our findings imply that certain types of Ag-Ab interactions may be limited to certain C regions and suggest a potential explanation for the biased preference of certain isotypes in polysaccharide responses. From a practical consideration, our observations suggest that fine specificity changes may be a relatively common phenomenon when ch or humanized Abs are constructed. This phenomenon may be limited to certain murine V regions, and/or may apply primarily to V regions that bind to Ags with repeating epitopes such as polysaccharides. Furthermore, the results are consistent with the view that differences in hinge angle and flexibility, possibly in combination with other variables such as C region size and glycosylation can affect the type of Ag-Ab complexes made. Our findings provide additional validation for the theory proposed by N. S. Greenspan (33) that valency can influence the specificity of the Ag-Ab interaction. Given the increasing use of mouse-human ch Abs in human therapy, it is critical that each ch Ab be carefully tested for specificity to insure the fidelity of the Ab engineering process.

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Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

U. S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
February 28,1997

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IPR2019-00739
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Date: February 27, 1997

From: Kathryn C. Zoon , Ph.D., Director
Center for Biologics Evaluation and Research
Food and Drug Administration

Subject: Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

To: Manufacturers of Biological Products and Other Interested Persons

This Points to Consider (PTC) document has been developed for manufacturers of monoclonal antibody products for human use. These "Points" are not regulations nor are they guidelines, but represent the current thinking that the Center for Biologics Evaluation and Research (CBER) staff believe should be considered at this time. This 1997 PTC document supersedes the 1994 PTC document of the same title, announced in the Federal Register of August 3, 1994 (59 FR 39571).

It is our intention to continuously update and revise this document in order to improve its usefulness. We invite your review and comment on the "Points". Comments should be identified with the docket number 94D-0259. Two copies of any comments should be submitted except that individuals may submit one copy. All comments should be addressed to:

Dockets Management Branch (HFA-305)
Food and Drug Administration
12420 Parklawn Drive, Room 1-23
Rockville, MD 20857

_____-s-_____
Kathryn C. Zoon, Ph.D.

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Points to Consider in the Manufacture and Testing
of Monoclonal Antibody Products for Human Use

[Docket No. 94D-0259]

For further information regarding this document, contact:
Sharon Carayiannis
Center for Biologics Evaluation and Research (HFM-630)
Food and Drug Administration
1401 Rockville Pike, Suite 200N
Rockville, MD 20852-1448
301-594-3074

Submit written comments on this document to:

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Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use February 1997

I. INTRODUCTION

A. BACKGROUND

Points to Consider documents provide a flexible approach in which FDA provides and updates its guidance on regulatory issues in many areas of drug development. Such documents are particularly useful in the rapidly evolving field of biotechnology-derived drugs and other biologics. The Center for Biologics Evaluation and Research (CBER) set out to revise the "Points to Consider (PTC) in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" with several objectives. An important goal was to facilitate initial development of monoclonal antibodies for serious or life threatening indications. Additionally, it was felt that some of the information in the 1994 document required updating and streamlining. Finally, it was necessary to review this document for consistency with current CBER policy and with International Conference on Harmonisation (ICH) documents dealing with this category of products. This updated document supersedes the 1994 version, and is designed to assist sponsors and investigators regarding monoclonal antibody (mAb) product development, including information to submit when filing Investigational New Drug Applications ("INDs") and License Applications. Although this document does not create or confer any rights for or on any person and does not operate to bind FDA or the public, it does represent the agency's current thinking on monoclonal antibody products for human use.

For mAb, as for other biologics, certain regulations contained in 21 CFR Parts 200-299 and 600-680 apply and should be consulted. In common with the other PTC, the mAb PTC are not intended to be all-inclusive. They represent recommendations on how to conduct the clinical development of a product up to and after licensure, not checklists of items to be provided before or after phase 1 trials are initiated. Specific products which raise issues that are not considered in these "Points" will be evaluated on a case-by-case basis. The discussion on abbreviated product testing for feasibility trials in serious and immediately life-threatening conditions and on generic and modular virus clearance studies does not apply to human products made in human cell substrates. Consultation with CBER is strongly advised for sponsors considering the application of abbreviated testing policies to products that have the potential to be contaminated by human pathogens. For aspects of manufacturing and of the production facility that are not included in this discussion or in applicable regulations, sponsors should consult with the Office of Therapeutics Research and Review and the Office of Establishment Licensing and Product Surveillance respectively.

B. DEFINITIONS

For the purpose of this document, the terms "**antibody**" and **"monoclonal antibody"** (mAb) may be used interchangeably and refer to intact immunoglobulins produced by hybridomas, immunoconjugates and, as appropriate, immunoglobulin fragments and recombinant proteins derived from immunoglobulins, such as chimeric and humanized immunoglobulins, F(ab)′ and F(ab′)$_2$ fragments, single-chain antibodies, recombinant immunoglobulin variable regions (Fvs) etc. Recommendations on the manufacture of recombinant products are contained in other PTC documents from CBER (1,2). Some of these recommendations pertaining to recombinant mAb produced in cell substrates other than hybridomas are reiterated in this document for convenience of consultation. This document applies to mAb used as therapeutic or *in vivo* diagnostic agents, as well as to **ancillary products**, i.e. mAb used in the manufacture of other products for *in vivo* use. The latter include mAb that are used alone or in

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conjunction with devices, for example, for *ex vivo* purging of cells to remove immune or tumor cells, for *ex vivo* cell collection (e.g. hematopoietic stem cells), or for purification of other products intended for *in vivo* administration. Generally, these mAb should meet the same criteria for safety and freedom from adventitious agents as mAb intended for direct administration to patients. Likewise, reagents that are commonly used in conjunction with mAb for *ex vivo* manipulations of cellular products intended for *in vivo* administration (e.g. complement, DNAase) should meet the same safety standards as mAb intended for direct administration to patients. However, in such cases, some procedures for virus inactivation or removal may be performed on the downstream product rather than on the mAb or other reagent (see II.C.7). Complete information on products that will be used in conjunction with the mAb, such as rabbit complement or DNAase, should be submitted before clinical studies begin. This information can be submitted as a part of the original IND submission or in the form of a Master File.

As used in this document, "**cocktails**" are defined as two or more mAb administered at a fixed ratio. Relevant targets may include multiple antigens on infectious pathogens and multiple tumor-associated antigens. The rationale for combining the products should be clear and based on the clinical context or previous clinical experience with individual products. Lack of interference among the mAb in the combination should be shown and synergistic or additive effects should be characterized. Dose-ranging for each of the components is highly desirable. In some instances, dose-setting may be based on preclinical or clinical data that show the necessity or superiority of a particular dose and ratio of mAb in the combination.

As used in this document, "**panels**" are defined as sets of mAb directed against related antigens from which one or more members would be used for an individual patient based on target antigen characterization. Such panels could be submitted for approval in a single license application. Examples of panels might include anti-idiotype mAb for lymphoma and mAb directed against different bacterial or viral serotypes. Dose-ranging for each mAb would be necessary. During the phase 3 trials to establish efficacy of the entire panel, some clinical experience with each member of the panel should be obtained.

C. FILING INFORMATION

It is not necessary to have all of the information discussed in this document available in the initial IND submission. Rather, much of the information may be developed during clinical development, with guidance from CBER or other appropriate Centers by means of frequent dialogue. At pre-IND meetings, CBER staff may provide guidance in planning clinical development and establishing the format and content of initial IND submissions. Such meetings may be particularly useful when the product is a novel molecular entity or is produced by a novel process, and when drug development plans are unusually complex.

The manufacture of mAb that are produced and controlled by similar procedures in the same facility may in some cases be documented in a single Master File. This may be particularly helpful when data from generic or modular virus clearance studies are used for multiple antibodies that differ only in the variable (v) or complementarity-determining region (CDR) and when multiple antibodies are purified by identical procedures (see Section II.C.6).

See references 3 and 4 for information on filing biologics license applications. An Establishment License Application is no longer required for mAb intended for in vivo use (3).

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II. PRODUCT MANUFACTURE AND TESTING

A. GENERAL PRINCIPLES AND DEFINITIONS

Traditionally, most mAb are produced by hybridoma cell lines through immortalization of antibody-producing cells by chemically-induced fusion with myeloma cells. In some cases, additional fusions with other lines have created "triomas" and "quadromas". We anticipate an increase in recombinant mAb (e.g. chimeric or humanized mAb, single-chain or dimeric Fvs, mAbs derived from phage display libraries etc.) and human mAb in the future. These may be produced in animal cell lines (e.g. CHO, SP2/0) transfected with recombinant DNA constructs, in human cells (e.g. immortalized lymphoid cells), in bacteria, yeast, insect cells etc. Novel methods of production for mAb or mAb-derived recombinant proteins may include insects, plants or transgenic animals,.
The principles reviewed in Sections II.B. 1 through 4 may be applied, in general to all hybridoma and heterohybridoma generated products, regardless of the species of origin. All steps in manufacturing of mAb to be used in trials intended to support licensure and of licensed mAb should comply with current Good Manufacturing Practices (cGMPs), as appropriate for the stage of product development.

While manufacturing details and safety issues may be different for different expression systems, some general principles can be applied. The establishment of a reliable and continuous source from which the antibody can be consistently produced is highly recommended (e. g. master cell banks for cell cultures, seed banks for transgenic plants, founder strains for transgenic animals). If transient expression systems are used, master vector seed stocks should be generated, and the genetic stability of the expression constructs used should be tested. Appropriate in-process testing which takes into consideration the specific safety concerns of the expression system used should be instituted. Sponsors are encouraged to consult the most recent available versions of the Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, the Points to Consider in the Production and Testing of New Drugs and Biologicals produced by Recombinant DNA Technology or the Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived From Transgenic Animals (1, 2, 5), the 1996 CBER/CDER Guidance Document on the Submission of Chemistry, Manufacturing and Controls Information for a Therapeutic Recombinant DNA-derived Product or a Monoclonal Antibody Product for In Vivo Use (4), as well as relevant International Conference on Harmonization (ICH) documents (e.g. 6, 7), if applicable to their expression systems. Sponsors considering novel expression systems not specifically covered by guidance documents are encouraged to consult with CBER.

B. MANUFACTURE AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

1. Cell lines

The following information should be provided in the IND or biologics license application:

a. Source, name, and characterization of the parent cell line, including any immunoglobulin heavy or light chains that it synthesizes and/or secretes, the fusion partner in the case of hybridomas, or the host cell line in the case of transfected cells producing recombinant mAb.

b. Species, animal strain, characterization, and tissue origin of the immune cell.

c. Description of immortalization procedures, if any, used in generating the cell line.

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d. Identification and characterization of the immunogen. A complete biochemical characterization may not be possible or necessary in all cases. However, we recommend that as much information as possible be gathered on the nature and characteristics of the material used as an immunogen. Such data can be useful in choosing appropriate potency assays, as well as in evaluating potential for cross-reactivity and possible clinical usefulness. For example, a determinant which is not expressed on the surface of target cells bind necrotic cells better than intact cells.

e. Description of the immunization scheme. In the case of human mAb, any *in vitro* or *in vivo* immunization procedures should be described, as well as any relevant aspects of the subject's medical history.

f. Description of the screening procedure used. In the case of human mAb, steps performed in order to enrich antigen-specific human B cell populations should be described.

g. Description of the cell cloning procedures. If changes in cell culture process (e.g. cells adapted from serum-containing to serum-free medium) are shown not to affect product quality, it is not necessary to reclone the cells or rebank the MCB or WCB. In this context, product quality includes not just the identity, purity, potency and pharmacological characteristics of the purified product, but also its safety profile. For example, possible changes in types and/or titers of viruses detectable in the unpurified bulk material and the ability of the purification process to remove or inactivate them should be addressed).

h. For transfected animal or plant cell substrates, as well as for microbial cell substrates (bacteria, yeast), a detailed description of the vector(s) and final construct(s) generation, including whether or not extraneous amino acid sequences are introduced into the product as a result of subcloning, and description of transfection/transformation, screening and selection procedures (see refs. 1-6). Determination of cDNA sequence(s) of the predominant transcript(s) is acceptable as an indication of clonality of transfected cell lines.

i. For cell culture systems using autonomously replicating vectors (e.g. baculovirus or other transient expression systems) a detailed description of the vector system, construct generation, selection, vector banking procedures, and infection/transfection procedures should be provided (1-6).

j. For all cell substrates, description of the seed lot system for establishing and maintaining the master cell bank (MCB) and the working cell bank (WCB).

2. Production in cell culture

The following information should be provided in the IND or biologics license application:

a. A description of the culture procedures if production is entirely *in vitro* or if cells are passaged *in vitro* prior to mouse inoculation.

b. A description of the culture media used, including certification and testing. Serum additives used in hybridoma propagation should be free of contaminants and adventitious agents.

c. The steps taken to prevent or control contamination by viruses, bacteria, fungi, mycoplasma and transmissible spongiform encephalopaties (TSE) agents. These include, among other things, a description of the equipment, transfers, room classification, employee gowning procedures etc.

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d. The acceptance criteria for cells or tissue culture supernatants intended for further manufacture.

3. Production in animals or plants

The following information should be provided in the IND or biologics license application:

a. A description of the cell line used as the inoculum (if any) should be provided (see 2.a. above).

b. Animal care should be in accordance with the NIH Guide for Care and Use of Laboratory Animals. For ascites production, the use of specific pathogen free (SPF) mice is recommended. To ensure manufacture of consistent, high quality ascites for production of mAb, an animal health monitoring program should be in place that encompasses quarantine procedures, sentinel animals, and an in-house health surveillance program (including screening for mycoplasma). Frequency of serological testing of sentinel mice should be established and is usually based on the incidence of virus contamination. Screening programs for known infectious agents should be updated to reflect advances in the knowledge of infectious diseases. Sponsors should be responsible for the adequacy of screening programs.

c. All protocols for ascites production should also incorporate information on: *i*) species, sex and age of animals used; *ii*) animal supplier; *iii*) volume of pristane; *iv*) volume and concentration of cell inoculum; *v*) timing of priming, inoculation, and ascites harvesting; *vi*) frequency and procedure for ascites harvesting; *vii*) definition of a batch; *viii*) animal bedding, food and water; *ix*) number of animals housed together; *x*) environmental conditions under which each procedure takes place and *xi*) number of times cells are passaged from one animal to another, if applicable.

d. For production in transgenic animals, the vectors, constructs and procedures used for gene transfer should be described. The genetic background and characterization of founder animals, the generation and selection of production herds and animal maintenance procedures should be described as well (see ref. 5 for details). Health monitoring programs for animal herds or colonies should be in place, including screening for zoonoses known to exist in captive animals of the relevant species in North America. Programs for screening and detection of known infectious agents should be tailored for the animal species and periodically updated to reflect advances in the knowledge of infectious diseases. Sponsors should be responsible for the adequacy of screening programs (see paragraph b above). When initially establishing transgenic animal strains, the following considerations should be kept in mind: *i*) non-transgenic animals to be used for breeding or gene transfer procedures should be obtained from closed herds or colonies that are serologically well characterized and as free as possible of pathogens of concern for the animal species or for humans; *ii*) the use of imported animals or first generation offspring from imported animals is discouraged and *iii*) animals from species in which TSE have been documented should be obtained from closed herds with documented absence of dementing illnesses and controlled food sources.

e. For production using autonomously replicating vectors (e.g. baculovirus) in live insect larvae, larvae maintenance procedures should be described in detail, including procedures used to control and monitor bioburden.

f. For production in plants (e.g. transgenic plants or autonomously replicating vectors using plants as bioreactors), early consultation with CBER staff is recommended.

4. Purification

Purification schemes for mAb should be described in the IND or biologics license application. We

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recommend that mAb purification schemes incorporate the following characteristics:

a. Production techniques that will prevent the introduction of and eliminate contaminants, including animal proteins and materials, DNA, endotoxin, other pyrogens, culture media constituents, components that may leach from columns, and viruses.

b. Incorporation of one or more steps known to remove or inactivate retroviruses in excess of the endogenous particle load, whenever applicable (see Reference 8 and discussion of virus clearance studies in Section II.C.). As a general guidance, we recommend that each purification protocol include at least two orthogonal (i.e. based on different mechanisms) robust virus removal steps (see below). Including these steps would not obviate the need for virus clearance studies, except in the case of products intended for use in feasibility trials in serious or life-threatening conditions (see Section II.D.2.)

> *i.* **Robust virus removal/inactivation steps** are defined as those that have been shown to work well under a variety of conditions (e.g. pH or ionic strength of column buffers) with a variety of mAb. Robust steps include low pH, heat, solvent/detergent treatments, and filtration (see Table III). Sponsors have the option of providing adequate evidence indicating that a step different from these is robust, or is reliably effective for removal/inactivation under the conditions employed. An estimate of the efficiency of robust steps in removing virus may be demonstrated by: (a) cross-referencing master files or reliable scientific literature published in peer-reviewed journals or (b) generic or modular clearance studies (see Section II.C.6. for definition).

c. Demonstration of the ability of the purification scheme to remove adventitious agents and other contaminants, by means of a clearance study. For some contaminants, e.g. DNA, pristane or protein A, such a clearance study, if appropriately carried out, may be an acceptable alternative to routine testing for the contaminant. In the case of virus clearance studies, we recommend the use of several model viruses encompassing large and small particles, DNA and RNA genomes, as well as chemically sensitive and resistant lipid enveloped and non-enveloped strains. Human blood products should be avoided in production of other biologicals. When human blood products that may be contaminated with hepatitis C virus (HCV) or other infectious agents must be used in production (e.g. as media additives), such schemes should include viruses that are acceptable models for HCV, such as bovine viral diarrhea virus (BVDV) or Sindbis virus. Retrovirus clearance studies should be performed prior to phase 1 trials, except for products intended for use in the setting of serious or life-threatening conditions in feasibility trials (see Section II.D.). Clearance studies for other viruses and/or other contaminants should be carried out prior to production for phase 2/3 trials and may need to be repeated if the final manufacturing process has changed. ICH guidelines are currently being drafted to address in further detail the viral safety evaluation of biotechnology products derived from cell lines of human or animal origin.

d. Limits should be prospectively set on the number of times a purification component (e.g. a chromatography column) can be reused. Such limits should be based upon actual data obtained by monitoring the component s performance over time.

e. As a product is developed, retention samples from each production should be saved under appropriate conditions so that side-to-side comparisons may be made to determine product comparability (see Section II.E.).

f. A description of the purification room(s) design features, HVAC and other support systems, equipment, transfers and personnel should be provided. Emphasis should be placed on operational features that minimize the risk of contamination from the environment or cross-contamination from other products.

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5. Characterization of purified unmodified mAb

Before a mAb is studied in humans, a precise and thorough characterization of antibody structural integrity, specificity, and potency should be conducted and described in the IND. The mAb should be as free as possible of non-Ig contaminants. A properly qualified in-house reference standard with known characteristics, specificity, and potency, and that is stored under appropriate conditions and periodically tested to ensure its integrity, should be used for lot-to-lot comparisons. Reference standards should be updated as a product evolves but should be finalized by the start of phase 3 trials. Appropriate standard operating procedures (SOPs) should be developed for qualification of a new reference standard.

a. Structural Integrity

A combination of SDS-PAGE, IEF, HPLC, mass spectrometry, or other appropriate physicochemical methods should be used to show that the purified antibody is not fragmented, aggregated, or otherwise modified (e.g. loss of carbohydrate side chains). Side-by-side comparisons of production lots to the in-house reference standard should be performed.

b. Specificity

Assays should provide evidence that the binding of the mAb to the target antigen is specific. Once the specificity of the antibody is characterized, it should be screened for cross-reactivity with human tissues (see Section III.A.). The following are some suggestions on the design of specificity studies:

i. Direct binding assays should include both positive and negative antibody and antigen controls. At least one isotype-matched, irrelevant (negative) control antibody should be tested. Negative antigen controls should include a chemically similar, antigenically unrelated compound, if available (e.g. similar chemical nature, size, charge, and charge density).

ii. Whenever possible, the protein, glycoprotein, glycolipid, or other molecule bearing the reactive epitope, should be biochemically defined, and the antigenic epitope, itself, determined. If the antigenic determinant is a carbohydrate, the sugar composition, linkage, and anomeric configuration should be established.

iii. If possible, fine specificity studies using antigenic preparations of defined structure (e.g. oligosaccharides or peptides) should be conducted to characterize antibody specificity by means of inhibition or other techniques. For complex biological mixtures, the lots of test antigen and/or inhibitors used for direct binding tests should be standardized. Inhibition of antibody binding by soluble antigen or other antibodies should be measured quantitatively.

iv. Once the specificity of an antibody has been determined, it is important to quantitate antibody binding activity by affinity, avidity, immunoreactivity, or combinations of these assays, as appropriate. A number of published methods are suitable for measurement of antibody binding activity (9, 10).

c. Potency Assays and Potency Specifications

Potency assays are used to characterize the product, to monitor lot-to-lot consistency, and to assure stability of the product. Potency may be measured by a binding assay, a serologic assay, activity in an

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animal model, and/or a functional assay performed *in vitro* or *in vivo*. It is desirable that the assay(s) bear the closest possible relationship to the putative physiologic/pharmacologic activity of the product and be sufficiently sensitive to detect differences of potential clinical importance in the function of the product. In particular, when the performance of the antibody depends not only upon antigen binding but also on other critical functions, it is desirable that the potency assay(s) measure all such functions. Documentation of the potency assay's performance, including sensitivity, intra- and inter-assay variation and robustness, should be provided.

i. Antibody binding activity may be quantitated by ELISA, RIA, radioimmune precipitation, cytotoxicity, flow cytometry, or any other standard, appropriate method. Activity should be expressed as specific antigen-binding units per mg or : g of antibody. Product should be compared to an in-house reference standard. Appropriate measurements of antibody affinity, if established, may be a useful adjunct to other assays. Parallel line bioassay or a similar, valid statistical procedure should be used in calculating potency.

ii. The potency of a mAb may also be tested by measurement of *in vivo* function in animal models, although such assays are often cumbersome and difficult to standardize and should not be the sole measure of potency.

iii. The permissible range of values in potency assays that reflects adequate biological activity of a product should be based on experience with a particular antibody. Ideally, potency assays should be correlated with *in vivo* activity in order to develop control tests which will ensure an effective product. This implies that multiple production lots should be used during the clinical development program and potency assay results should be correlated with clinical performance. When clinical performance is measured by *in vitro* tests used as surrogates of efficacy, such tests should be validated in a phase 3 clinical trial of appropriate design.

6. Anti-idiotype vaccines

The following issues should be addressed for anti-idiotype vaccines:

a. In the case of an anti-idiotype vaccine (Ab2 vaccine), the Ab2 immunogen should be characterized as to the Ab2 type, e.g. classical type (Ab2") or antigen mimic (Ab2$) (11).

b. Ab2$ vaccines should be shown to be reactive with the appropriate population of human Ab1 (antibody to nominal antigen) if such antibodies are available.

c. The Ab2 preparation should be studied for the appropriateness of response (to target antigen) in xenogeneic as well as syngeneic animals (12).

7. Monoclonal antibodies conjugated with toxins, drugs, radionuclides or other agents (immunoconjugates)

Immunoconjugates are typically produced by chemical processes using specific reagents to link the unconjugated antibody with a non-antibody agent. Alternatively, immunoconjugates can be obtained as chimeric recombinant proteins containing non-immunoglobulin and immunoglobulin sequences in the same polypeptide chain. In addition to previously discussed recommendations for unconjugated (naked) mAb, manufacturers of immunoconjugates should address the following:

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a. Construction of the Immunoconjugate.

A full description of the reagents and the process used to construct an immunoconjugate should be submitted, including:

i. A description of components such as toxins, drugs, enzymes, and cytokines that are linked to the mAb, including: the source, structure, production, purity (including demonstration of freedom from adventitious agents), and characterization of all components (if components are purchased, a certificate of analysis should be supplied).

ii. A description of chemical components, such as linkers and chelating agents, that will be used in preparing the immunoconjugate. These should include documentation of the sources of reagents and method of preparation and determinations of residual impurities from synthesis or purification. Charts of the synthetic reaction pathways and any relevant published or in-house data concerning the toxicity of chemicals used in the production of an immunoconjugate should be provided.

iii. The average ratio of coupled material to antibody and the number of conjugated moieties per antibody should be determined as the first step in establishing lot release criteria for the final product and developing the relationship between immunoglobulin substitution number, potency, and stability.

iv. Products prepared using recombinant DNA technology (e.g., derived from transfected cell lines or microbial cell substrates, chimeric, reshaped, complementarity determining region [CDR] grafted, single chain Fv antibodies, and recombinant immunoconjugates) should follow recommendations discussed in references 1-7, as appropriate. The stability of recombinant immunoconjugates should be studied carefully, as such chimeric proteins may have altered conformational stability, solubility or tendency to aggregate compared to their component polypeptides in their native structures. Loss of specific immunoreactivity due to denaturation or formation of aggregates (e.g. diabodies formed by recombinant Fvs) may lead to altered pharmacokinetics and/or binding to non-target tissues.

b. Purity of the Immunoconjugate

i. Special care should be taken to ensure that the antibody preparations are as free as possible of extraneous immunoglobulin and non-immunoglobulin contaminants as such contaminants could react with nuclides, toxins or drugs during the construction of the immunoconjugate.

ii. The amount of free antibody and free components in the final product should be determined with limits set for each. Reactive intermediates should be inactivated or removed.

c. Immunoreactivity, Potency and Stability of the Immunoconjugate

Coupling of toxin or drug to an antibody may alter the activity of either component.

i. Immunoreactivity before and after coupling should be assessed using appropriate methodology (9, 10).

ii. Activity of the non-immunoglobulin component of immunoconjugates, should be assessed by a potency assay whenever appropriate (e.g., toxins, cytokines or enzymes, but not radio-

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immunoconjugates intended for use in imaging)

iii. Limits on the percent change in immunoreactivity resulting from construction of the immunoconjugate should be established as part of product specifications.

iv. The immunoconjugate should be tested for stability *in vitro* by incubation in pooled human serum at 37° C under sterile conditions. Plasma may be used instead of serum, provided that the anticoagulant used does not affect the stability of the immunoconjugate (e.g., chelating agents may react with some radioisotopes, heparin may interact with basic proteins, etc.). Aliquots should be analyzed at timed intervals for the concentration of intact immunoconjugate and degradation products. The conditions under which product stability is evaluated and the positive and negative controls used should be fully described. Stability in human serum or plasma is not relevant for topically administered immunoconjugates which are demonstrated not to be absorbed into the bloodstream. It should be established whether or not such immunoconjugates or their components are detectable in plasma after topical administration, and whether or not they elicit an immune response.

d. Specific Issues Related to mAb Coupled to Radionuclides

The preparation of the radioimmunoconjugate should be performed in a standardized, well-controlled, and validated manner. Methods should be developed to estimate the percent radioactivity in each of the three species of concern: free isotope, conjugated mAb, and labeled, non-mAb substances.

i. It is recommended that the initial IND submission for a radiolabeled mAb contain analytical results from two to three radiolabeling runs that demonstrate the preparation of an immunoreactive, sterile, and pyrogen-free product. These radiolabeling runs should be performed by the same personnel who will radiolabel the mAb for the study, using the reagents that will be used for the study.

ii. Radiopharmaceutical grade isotopes should be used when preparing immunoconjugates. The sterility and pyrogen-free nature of each isotope should be documented by submission of a certificate of analysis and letters of cross-reference for manufacturing information.

iii. The concentrations of covalently-bound and free isotope in the final product as well as residual levels of labeling reagents and their decomposition products should be determined during the trial labeling runs.

iv. Quality control tests that will be performed before and/or after each patient administration should be described.

v. When appropriate, colloid formation by the radio-immunoconjugate should be determined and limits set for it.

C. QUALITY CONTROL AND PRODUCT TESTING

1. Cell line qualification

Qualification of the cell line for production of a mAb to be used as a biologic therapeutic should include screening the master cell bank (MCB) and the working cell bank (WCB), at least on a one-time basis, for endogenous and adventitious agents utilizing the tests outlined in Table I and described in the Points to

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Consider in the Characterization of Cell Lines Used to Produce Biologicals (1). Because the WCB is derived from the MCB and propagated for only a few additional passages in tissue culture, abbreviated testing for detecting newly introduced contaminants is acceptable. Any virus contaminant should be quantified and, wherever possible, identified in order to establish the extent of virus clearance that the purification process should achieve (see also Section II.C.5.). The tropism of virus contaminants for human cells should be determined by appropriate infectivity assays. In the case of tissue culture or fermenter production, end-of-production cells (EPC) should be tested at least once to evaluate whether new contaminants are introduced or induced by the growth conditions. EPC should also be re-examined when there are changes in culture medium or in the scale of production. Cells at the limit of *in vitro* age used for production can be tested in lieu of EPC to allow for possible extensions in the length of time cells are kept in culture as manufacturing schemes are developed. The term EPC will be used throughout this text for ease of consultation. For cell lines which are known not to be susceptible to infection with mammalian viruses (e.g. plant cells, some insect cells), bacterial and fungal sterility testing, and in some cases testing for Mycoplasma or other mollicutes such as Spiroplasma, will be the most important concerns. Consultation with CBER is advised before using these cell substrates.

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Table I
Cell Line Qualification

Tests	MCB	WCB	EPC
Sterility	+	+	+
Mycoplasma	+	+	+
Virus			
Adventitious	+	-	+
Species-specific*	+	-	-
Retrovirus	+	-	+
Authenticity	+	+	+

* Tests for rodent, primate, or human viruses (other than retroviruses), as appropriate
Retrovirus testing is not required for murine hybridomas. All other cell substrates should be tested as described in Section II.C.1.d.

a. Cell lines should be free from bacterial and fungal contamination as demonstrated by sterility testing. Recommended testing procedures for mycoplasma (cultivable and non-cultivable) are described in Ref. 1.

b. Screening for adventitious viruses (other than retroviruses) should include routine *in vivo* and *in vitro* tests (1).

c. Screening for species-specific viruses (other than retroviruses):

i. The mouse antibody production (MAP) test for murine cell lines (see Appendix II), the HAP test for hamster lines, and the RAP test for rat lines should be used. *In vivo* testing for lymphocytic choriomeningitis (LCM) virus, including non-lethal strains, is recommended. Testing of hamster cell lines should include minute virus of mice (see III.B.1.c.).

ii. Material that is contaminated with LCM, reovirus, Sendai virus, or Hantaan virus should not be used for mAb production.

iii. Cell lines from non-human primates should be screened for the following: herpes viruses (simiae and SA-8), cytomegalovirus (sCMV), encephalomyocarditis virus, simian hemorrhagic fever virus (SHF), varicella virus of simians (sVZV), adenovirus, SV-40, monkeypox, rubeola, and Ebola virus. Any other zoonotic agents suggested by the cell line derivation history should be screened for.

iv. Human cell lines should be screened for Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis B (HBV) and HCV, human herpes virus 6 (HHV-6), and any other viruses that are suggested by the medical history of the donor and type of tissue used to establish the original line. Cells from patients who are known to have developed Creutzfeld-Jakob disease (CJD) or other TSE, or from persons with two or more genetically related family members with CJD, should not be used.

v. Heterohybridomas using cells from 2 different species should be tested as appropriate for both species of origin.

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vi. For cell lines of other species please consult with CBER.

d. Retrovirus testing of cell lines: Retrovirus contamination of cells from different species varies. The following should be considered when designing studies to detect retrovirus:

i. Murine cells used to produce monoclonal antibodies should be considered inherently capable of producing infectious murine retrovirus. The amount of retrovirus in the unprocessed bulk should be quantitated on a series of bulk harvests and shown to be consistent from lot to lot (1). Endogenous virus particle burden should be determined at the end of a typical fermentation, prior to purification, preferably by thin-section EM on material pelleted by ultracentrifugation. Particle burden determination is preferable to infectivity assays at this stage of production because it does not depend upon the susceptibility to infection of the cell lines used for virus amplification and it provides a "worst case scenario" of the level of viral contamination. Thin-section EM also allows morphological observation of viruses. Other, novel methods of equal or superior sensitivity and general applicability may be acceptable, if appropriately validated. Sufficient retrovirus removal by the purification scheme should be demonstrated (see also Section II.C.4.).

ii. Rat myeloma cell lines and hybridomas may not express retrovirus (13).The absence of detectable retrovirus, however, should be demonstrated by co-cultivation of the test article with a cell line(s) susceptible to a wide range of retroviruses combined with a sensitive detection assay, including examination of EPC and several production lots. If retrovirus is not detected by infectivity assays or electron microscopy, further clearance studies may not be needed. It is suggested that purification schemes for mAb produced by rat cell lines include one or more robust retrovirus inactivation or removal step.

iii. CHO cell lines express defective retrovirus particles (14). Whether hamster cell lines express infectious retroviruses has not been shown. Sponsors should demonstrate the lack of infectious hamster retroviruses by means of the most sensitive infectivity assays available. These include co-cultivation of the test article with a cell line(s) susceptible to a wide range of retroviruses combined with a sensitive detection assay. As a product moves into pivotal clinical trials, it may be necessary to make additional attempts to detect potential infectious virus by utilizing a wider range of indicator cells, including human cell lines (15, 16). Because of uncertainty about the validity of infectivity assays for hamster retroviruses, sufficient retrovirus particle removal by the purification scheme should be demonstrated (see also Section II.C.4-5). It is suggested that purification schemes for mAb produced by hamster cell lines include one or more robust retrovirus inactivation or removal step.

iv. Hybridomas or transfected clones produced from cells of non-human primate or human origin should be examined for the presence of retrovirus. Generic assays, such as transmission electron microscopy (TEM) or reverse transcriptase (RT) can be used to assess the presence of retrovirus. Other assays may be used, as long as they are appropriately validated. In addition, all primate cell lines should be screened for simian immunodeficiency virus (SIV), simian T lymphotropic virus (STLV), Foamy virus, human T lymphotropic virus (HTLV), and human immunodeficiency virus (HIV). Cell lines from non-human primates should be additionally tested for presence of simian retroviruses (SRV).

e. Each cell clone generated by stable transfection of widely used parental lines (e.g. CHO) should be considered as a new cell line from the standpoint of viral safety. Such clones may have significantly

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different genetic characteristics compared to the parental line as a result of the transfection procedure itself, the clonal selection process and positional effects due to random integration of vector DNA into the cellular genome. Each clone should be screened for retroviruses and for adventitious viruses as described above and in Table I. Screening for species-specific viruses can be done once, on the MCB of the parental line.

f. Authenticity testing should confirm the cell line's species of origin, identity, and lack of cell-line cross-contamination.

2. Lot-to-lot quality control monitoring of unprocessed bulk lots and purified bulk lots, and final product specifications

Quality control monitoring should be performed on each lot of product, as defined in 21 CFR 600.3(x). Table II summarizes lot-to-lot product safety testing.

Table II
Lot-to-lot product safety tests

Tests	Unprocessed Bulk	Purified Product	Final Product
Sterility	+*	+	+
Mycoplasma	+	-	-
Virus			
Adventitious	+	-	-
Species-specific	+	-	-
Retrovirus	+	-	-
Polynucleotide	-	+	-
Endotoxin	-	-	+

*Bioburden testing with acceptable limits is sufficient at this stage.

In vitro testing with three indicator cell lines should be performed routinely for non-ascites material. *In vivo* testing is generally done once for non-ascites material but should be repeated when production methods change.

MAP, RAP or HAP testing for ascites only..

Quantitation of retrovirus (preferably by TEM) in the unprocessed bulk is important for murine hybridomas. For other hybridomas, generic assays for detection and quantitation of retrovirus, such as TEM coupled with appropriate co-cultivation assays are important if MCB or EPC are positive.

a. Unprocessed Bulk Lots

i. There should be set limits for bacterial bioburden in unprocessed bulk material. If bioburden testing of pooled ascites harvests shows the presence of viable contaminants, they should be quantified, and allowable limits for bacterial contamination should be set based on manufacturing experience. The identity of the bacterial species should be determined on a periodic basis and whenever the allowable limits for contamination have been exceeded. Filtration of ascites

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harvests through a # 0.45 : m filter prior to storage is recommended (see also II.C.2.a.*ii*.).

ii. Tests for cultivable and non-cultivable mycoplasma should generally be performed on unprocessed bulk hybridoma supernatants, prior to any clarification by filtration (1). The filtration of unprocessed bulk ascites through a 0.45 : m filter followed by storage at #-60° C prior to testing for mycoplasma is acceptable if samples of unfiltered material are retained for testing. If mycoplasma contamination of animals or unpurified bulk ascites or hybridoma supernatants is detected, these should not be used or processed further.

iii. In vitro virus testing with three indicator cell lines (e.g. Vero, MRC5, 3T3) should be performed routinely. *In vivo* testing is generally done once (as part of cell line qualification, Section II.C.1) but should be repeated when production methods change (1). Bioreactors containing hamster cells can become contaminated with minute virus of mice that may escape detection in routine *in vitro* assays. MAP testing or PCR testing for this virus appear to be more sensitive. In all cases, the frequency of monitoring should be specified in SOPs and justified based on actual experience when using continuous production in contrast to batch production. When contamination with a particular virus is encountered in a facility, consideration should be given to modifying the routine testing program in order to detect that virus.

iv. Species-specific virus testing should be performed (see Table II and II.C.1.c.).

v. Murine retrovirus contamination should be quantitated routinely for bulk ascites harvests. This may not be necessary if the sponsor can provide data showing that little variation exists in the concentration of mAb and retroviral load of their bulk ascites over several consecutive manufacturing runs, and the purification scheme used can remove substantially more than the highest load observed. If ascites production uses different groups of mice, periodic serologic monitoring for species-specific viruses should be performed on each group prior to their use for producing ascites. For tissue culture harvests, retrovirus contamination should be quantitated on three clinical grade production lots in order to establish the level of virus contamination for the specific cell line and manufacturing process (1) (see II.C.4). This quantitation of retrovirus should be done preferably by generic assays such as TEM or alternatively by sensitive infectivity assays (see also Section II.C.1.d.). Quantitation should be repeated when changes in tissue culture media, duration or scale of culture are made.

vi. For hybridomas of non-murine origin or other cell substrates, see section II.C.1.d. for appropriate assays to determine whether retrovirus is present. In those cases where MCB or EPC are positive for retrovirus, each lot of unpurified bulk should be examined for detection and quantitation of retrovirus by generic assays, such as TEM, coupled with appropriate co-cultivation assays.

b. Purified Bulk Lots (Drug Substance)

In addition to lot-to-lot safety testing summarized in Table II, routine testing on purified bulk lots of unmodified and modified mAb product should include the following determinations (for discussion of immunoconjugates see Section II.B.7.):

i. If a cell bank containing a known infectious agent is used, CBER staff should be consulted before proceeding with development, and clearance studies should be conducted to demonstrate the removal/inactivation of this agent by the mAb purification scheme. Testing for murine

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retrovirus during clearance studies should employ infectivity assays which detect ecotropic recombinant murine retrovirus (ERV) and the polytropic or mink-cell focus-forming murine retrovirus (MCF). The infectivity assay should be comprised of an amplification period on a cell line sensitive to infection by these murine retroviruses (for example, Mus dunni cells, 17) coupled with an appropriate indicator assay (for example, PG4 S+L- assay for MCF virus, immunofluorescence assay with appropriate antibodies for detection of ERV). Assays which do not rely on infectivity, such as PCR-based assays, may be substituted, provided they have been validated for sensitivity and specificity, and that their results are correlated with those of infectivity assays. The first consecutive 3-5 lots of purified bulk should be tested to confirm that the contaminant was removed by the purification scheme. For cell lines containing viruses or virus-like particles, the absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However, for cell lines for which the endogenous particles have been extensively characterized, such as CHO cells, and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of non-infectious particles in purified bulk. Should a human infectious agent be identified in the cell bank, every lot of purified product should be tested and consultation with CBER staff is recommended before extensive product development.

ii. Chemical purity including the residual amounts of extraneous animal proteins, e.g., albumin, immunoglobulin or other contaminants in the final product. An SDS-PAGE analysis, under both reducing and non-reducing conditions, of increasing amounts of purified material should be provided. Generally, silver staining methods are more sensitive but less quantitative than Coomassie blue for SDS-PAGE.

iii. Molecular integrity, including the presence of aggregated, denatured or fragmented product.

iv. Immunoglobulin class or subclass, if used as a test of identity.

v. IEF pattern of the antibody (or its heavy and light chains) in each bulk lot with comparison to the in-house reference standard.

vi. Sterility.

vii. Lot-to-lot testing for DNA content, prior to any excipient addition, is recommended as a way to monitor purification efficiency and reproducibility. DNA content in the final product should be as low as possible, as determined by a highly sensitive method. Low cell viability at harvest may contribute to high DNA levels in unprocessed bulk. It is suggested that, whenever possible, the final product contain no more than 100 pg cellular DNA per dose (18). It is suggested that a method with a sensitivity of 10 pg be used to determine DNA levels (2). An appropriately conducted clearance study for DNA removal may be an acceptable substitute for lot-to-lot testing.

viii. Tests for detection and quantitation of potential contaminants or additives (e.g., antibiotics, other media components, host cell proteins, chromatography reagents, preservatives, or components that may be leached from affinity chromatography columns such as protein A). Whenever possible, contaminants introduced by the recovery and purification process should be below detectable levels using a highly sensitive analytical method. However, for many of these potential contaminants, depending upon their potential for toxicity or immunogenicity, an

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appropriately conducted clearance study may be an acceptable substitute for lot-to-lot testing. For products intended for marketing, at least 3 exhibit lots should be tested to confirm the removal of the contaminant(s) for which clearance studies have been conducted. Such clearance studies for product contaminants may have to be repeated when manufacturing schemes are changed. We recommend that antibiotics, particularly penicillin or other beta lactams, not be used. However, if they are used, their removal must be demonstrated by an adequate clearance study. The acceptability of trace contaminants that cannot be removed by standard methods should be discussed with CBER prior to the submission of an IND. Pristane, if used in the propagation of ascites fluid, should be shown to be undetectable by a sensitive test.

ix. A brief description of the formulation process and areas to be used for it should be provided. This description should incorporate information on the processing area, support systems, personnel and product transfers in sufficient detail to highlight the design or operational features utilized to minimize contamination or cross-contamination.

c. Final-Filled Product (Drug Product)

The following tests should be performed on the contents of final containers from each filling of product as defined in 21 CFR 600.3(y). In certain situations (e.g. user-radiolabeling), special approaches to final container testing may need to be developed on a case-by-case basis after discussion with CBER:

i. Protein quantity.

ii. Potency (21 CFR 610.10).

iii. Purity (21 CFR 610.13). Electrophoretic migration of the product in both the native and reduced states on polyacrylamide gels with comparison to the in-house reference standard can be used as a test for purity.

iv. Sterility (21 CFR 610.12).

v. A test for endotoxin. The Limulus Amebocyte Lysate (LAL) assay may be an acceptable equivalent method as allowed in 21 CFR 610.9 when validated by the rabbit pyrogen test as described in 21 CFR 610.13. A U.S. licensed test system should be used to perform the LAL assay. Comparative testing should be repeated when LAL lots made by a different manufacturer are used. Conditions necessary for comparative testing of the rabbit pyrogen and LAL assay procedures should be discussed with CBER on a case-by-case basis. See also "Guidelines on Validation of the LAL Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices", December , 1987 (19).

vi. An appropriate identity test (21 CFR 610.14).

vii. Moisture (21 CFR 610.13) testing, when appropriate.

viii. Preservative (21 CFR 610.15) testing, when appropriate.

ix. Excipients, when appropriate

x. pH, when appropriate.

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xi. The areas used for final fill of drug products should follow the recommendations provided in the Guidelines on Sterile Drug Products Produced by Aseptic Processing (20). For information on validation of equipment used in aseptic processing, refer to the Guidelines for Submitting Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products (21).

3. Stability of product

Product stability should meet the demands imposed by the clinical protocol. Accelerated stability testing data may be supportive but do not substitute for real-time data for product approval and labeling.

a. A stability testing program should be developed that includes tests for physico-chemical integrity (e.g., fragmentation or aggregation), potency, sterility, and, as appropriate, moisture, pH and preservative stability, at regular intervals throughout the dating period. See also "Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics" (22) and the relevant ICH document (7). For products that are in clinical trials, significant changes that occur during storage should be reported to CBER. For license applications, and preferably prior to each stage of development, stability tests that support the proposed dating period should be performed on the final-filled product, using the container and closure configuration intended for distribution. For biologics license applications, storage of production intermediates (e.g. unpurified bulk, purified bulk) also should be supported by stability testing.

b. Stability tests for assuring biological activity (e.g. quantitative *in vitro* potency assays) should include a manufacturer's in-house reference standard. Whenever possible, a single lot of test antigen (e.g. purified antigen, cells or tissue) should be used throughout the study. A quantitative potency assay(s) should be used to permit a meaningful comparison of activities.

c. Accelerated stability studies, i.e. stability testing after storage at temperatures exceeding routine storage temperatures, may help to identify and establish which tests are stability-indicating. Specific parameters that indicate stability should be monitored by trend analysis on every lot on the stability testing program.

4. General considerations on quantitation and removal of a retrovirus contaminant

The amount of any retrovirus contaminant in the unprocessed bulk product should be estimated. If a retrovirus is detected, any possible or suspected tropism for human cells should be explored (e.g. by co-cultivation assays). This should be followed by demonstration of removal of the contaminant, using the contaminant itself or a representative analogue of the known contaminant (e.g. a model retrovirus) in a model purification system (1).

Unprocessed bulk supernatant concentrates or ascites should be assayed prior to any manipulation other than clarification by low speed centrifugation, unless it can be shown that virus testing would be made more sensitive by initial partial processing. We recommend that retroviruses contaminating ascites or supernatants produced by rodent cell lines be quantitated by TEM of concentrated samples of supernatant. If TEM results are negative, it should be assumed that the titer of retrovirus is equivalent to the lowest limit of detection ($1x10^6$/ml). Ascites and supematants produced by non-rodent or hybrid cell substrates should be assayed as described (see Section II.C.2.b.*vi.*). Demonstration that retroviruses are removed or inactivated by the purification scheme should occur prior to phase 1 studies, except for mAb intended for use in feasibility trials in serious or life-threatening conditions (see Section II.D.). References 1, 2 and 8,

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and paragraphs 5 and 6 below, also discuss the design of studies to demonstrate retrovirus removal. The goal of such studies is to demonstrate that the purification process is able to remove substantially more virus than is estimated to be present in a single dose equivalent of starting material.

5. General considerations on the design and interpretation of virus clearance studies

These considerations are provided here solely for guidance purposes in the design of virus clearance (i.e., removal/inactivation) studies.

a. General Experimental Design

The objective of a virus clearance study is to provide a quantitative estimate of the level of virus reduction provided by the removal/inactivation procedures. Thus, the study should be designed, conducted and analyzed in a manner that will provide accurate information to reliably assess the ability of these procedures to remove/inactivate viruses. The study, including virus infectivity assays, should be designed according to good scientific practice to yield data with accuracy and precision that are amenable to statistical analysis. Each clearance study should include an appropriate control experiment performed in parallel to the experimental condition to assess virus inactivation caused by experimental manipulation (dilution, concentration, filtration, storage etc.). Any observed difference should be used to adjust the virus reduction/inactivation values for each removal/inactivation procedure. It is preferable that the study be conducted under current Good Laboratory Practices (21 CFR, Part 58).

b. Statistics

Virus clearance studies should be supported by appropriate statistical analysis demonstrating that the study is valid and reliable. Virus infectivity assays used to quantitate the virus titer should be sensitive, reproducible and conducted with sufficient replicates to demonstrate statistical accuracy. Sufficient sample volumes should be tested to ensure that there is a high probability of detecting virus in the sample if present. The experimental variability present in virus titration should be determined in virus infectivity assays and reported using confidence intervals. Within assay variability should be calculated using conventional means (standard deviation, standard error of the mean, etc.). Between assay variation can be monitored by the inclusion of an appropriate virus reference preparation run in parallel with unknown samples. The 95% confidence intervals for within assay variation should be established and reported for each virus infectivity assay, and should be in the order of " 0.5 $\log_{10}$ of the mean. The quantitative estimate of virus reduction for each procedure should be reported as the reduction factor. This is defined as the $\log_{10}$ of the ratio of the virus load in the starting material to the virus load in the post reduction material ($\log_{10}$ (volume x virus titer of starting material / volume x virus titer of post-reduction material)). In general, this virus reduction factor should be based upon the amount of virus detected in the spiked starting material. The 95% confidence interval for each reduction factor is calculated from the 95% confidence interval of the virus infectivity assays at the beginning (+s) and end (+a) of each procedure using the formula " $\sqrt{a^2+b^2}$. Such confidence intervals should be calculated whenever possible in studies of clearance using relevant or specific model viruses (with respect to viruses actually detected in the cell line as opposed to non-specific model viruses). The reduction factors for each procedure are summed to calculate an overall reduction factor for the entire process. Additivity of reduction factors assumes that different steps are independent, i.e. have different mechanisms of action so that virus forms (mutant, aggregates etc.) escaping one step do not have a higher likelihood of also escaping the other. Reduction factors from two steps with the same mechanism of action (e.g. two incubations at low pH) are not necessarily additive. Due to the high intrinsic variability of infectivity assays, reduction factors of one log or less are considered negligible and are not included in the overall reduction factor.

6. Generic or modular virus clearance studies

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a. A generic clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model antibody. These data may then be extrapolated to other antibodies following the same purification and virus removal/inactivation scheme as the model antibody.

b. A modular clearance study is one that demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, etc). Each module in the purification scheme may be studied independently of the other modules. Different model mAb may be used to demonstrate viral clearance in different modules, if necessary. If the purification process of a product mAb differs at any of the virus removal or inactivation modules from the model mAb, this module must be studied independently from the model. The other, identical modules in the procedure may be extrapolated to the product mAb.

c. Applicability: For monoclonal antibodies manufactured at one site, generic or modular clearance data may be extrapolated to other monoclonal antibodies of the same species and H and L chain class and, generally, subclass, derived from the same source (e.g. ascites or tissue culture) and cell substrate. Generic and/or modular clearance will apply only when the mAb have similar biochemical properties and are purified by identical methods. Particular attention should be paid to column elution buffer conditions, including pH and ionic strength, the sequence of columns, protein concentration, dwell times, flow rate, pressure, temperature, and potential problems associated with scale-up, at all steps of virus inactivation and removal. In some cases, sponsors may demonstrate virus removal/inactivation for a particular module at two different values of a given parameter (e.g. ionic strength, dwell time, temperature) and use any values of that parameter falling within this range. In order to apply generic or modular clearance algorithms to a specific product, it is necessary to determine the virus load in the unpurified bulk for each specific product using at least 3 lots (see Section II.C.2.). This information is then used to determine whether the total reduction/inactivation factor provided by the purification procedure ensures that substantially more virus is removed or inactivated than the estimated unpurified bulk titer. For example, generic clearance might apply to a series of murine mAb of different specificities but of the same H and L chain isotypes that are purified in an identical manner, or to a series of humanized mAb of the same H and L chain isotype but with different CDRs, that are purified by identical methods. The concepts of generic and modular virus clearance studies do not apply to products of entirely human origin or to products that have the potential to be contaminated by human pathogens. Consultation with CBER is advised before applying generic or modular virus clearance studies.

d. Virus Clearance Master Files: A sponsor purifying mAb for a variety of applications or a manufacturer producing mAb for a variety of sponsors may submit a Master File containing the data demonstrating virus removal or inactivation for different purification schemes. This Master File may then be cross-referenced in INDs/IDEs or license applications using mAb purified by these schemes if applicability criteria are met.

7. Product testing requirements for mAb used as ancillary products

Testing requirements for mAb used as ancillary products (see I.B. for definition) vary depending on the clinical indication and the stage of product development. From a safety standpoint, they should be characterized in the same way as products intended for *in vivo* administration. However, production steps which follow purification of the mAb can be used as part of the virus removal/inactivation scheme. These include, for example, conjugation of mAb to solid phases for affinity purification, sanitation of affinity columns etc. The concepts of generic and modular clearance studies apply to ancillary products as well. Purity of the ancillary product may not be as critical as for products intended for *in vivo* administrations, provided that the performance of the ancillary product in the production of the final product is acceptable

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and reproducible, limits are set for impurities and the nature of impurities is known. Leaching of mAb or impurities from the mAb preparations into the final product should be taken into consideration in testing the final product, as appropriate. Removal of mAb or impurities from the mAb preparation may be demonstrated by means of a clearance study. Labeling for the final product may need to carry precautionary statements about potentially toxic or immunogenic residual impurities.

D. PRODUCT SAFETY TESTING FOR FEASIBILITY CLINICAL TRIALS IN SERIOUS OR IMMEDIATELY LIFE-THREATENING CONDITIONS

1. General considerations

The extent of product testing necessary before a particular clinical trial is initiated depends on the source and nature of the product, the stage of product development and the clinical indication. Abbreviated testing needs described in this Section (II.D.) apply to feasibility clinical trials in serious or immediately life-threatening conditions for which no effective alternative treatment exists. Abbreviated testing does not apply to human products made in human cell substrates, but may apply to recombinant products made from transfected human genes, depending on the cell substrate. Consultation with CBER is strongly advised for sponsors considering the application of abbreviated testing in this setting to products that have the potential to be contaminated by human pathogens. For the purpose of this document, the following definitions should be considered:

> *i*) **Feasibility clinical trials.** These are pilot studies whose objectives include, among others, early characterization of safety and initial proof of concept in a specific patient population. These trials are limited in scope, and are generally conducted in a single center, with a small number of patients (e.g. 5-20). These trials cannot be used by themselves to support licensure of a product. Studies conducted in normal volunteers are not included in this definition.
>
> *ii)* **An immediately life-threatening condition** is "a stage of a disease in which there is a reasonable likelihood that death will occur within a matter of months or in which premature death is likely without early treatment" (21 CFR 312.34).

See 21 CFR 312.34 and the Federal Register vol. 52, No. 99 (May 1977) for a discussion of serious or life-threatening conditions. Application of abbreviated testing requirements to serious conditions which are not immediately life-threatening as defined in 21 CFR 312.34 will depend upon an assessment of the potential risks and benefits to the patient(s). Factors that should be considered in such a risk-benefit analysis include, among others: *i*) the nature and manufacture of the product; *ii*) the nature and severity or stage of the disease; *iii*) the anticipated effect(s) of product administration (e.g., diagnosis, palliation or cure); *iv*) the availability of comparable or satisfactory alternate treatments *v*) characteristics of the patient population (e.g. age, response to previous therapy); *vi*) the number of patients involved and *vii*) the design of the clinical trial (e.g. patient follow-up, safety monitoring etc.). Pre-IND consultation with CBER staff is strongly recommended for sponsors planning to use the abbreviated testing described below for serious but not immediately life-threatening diseases. The guiding principle for these trials is that sufficient information should be provided before testing in human subjects to assure that patients and their contacts will not be put at unacceptable risk. Informed consent issues for these trials are discussed in Section IV.A.1.e. The limited testing described below should not be used to support development beyond the stage of feasibility trials. Therefore, sponsors are encouraged to plan for additional testing and characterization as described in Section II.C. when they intend to pursue advanced clinical development and seek licensure. In designing the purification process, it is advisable to include at least two orthogonal robust virus inactivation/removal steps. This would further reduce the testing necessary to begin initial clinical trials.

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2. Product safety data needed before the initiation of feasibility trials in serious or immediately life-threatening conditions

a. Sterility (bacteria and fungi) testing should be performed on the final product. Three vials of final product should be tested. Routine methods in use in the sponsor's hospital accredited clinical diagnostic laboratory can be used for these tests. Mycoplasma and endotoxin testing are strongly encouraged.

b. *In vitro* and *in vivo* testing for adventitious viruses : If the unpurified bulk product is free of adventitious viruses by *in vitro* and *in vivo* tests or if the purification scheme does include at least two orthogonal robust steps (see Table III), these tests are not required.
Table III shows ranges of retrovirus removal (expressed in decimal logs) that might be expected with various robust inactivation/removal steps and is presented to aid manufacturers in the design of mAb purification schemes.

Table III

Inactivation Step	Reported log virus removal
pH # 3.9	**3-4**
heat	**4**
solvent/detergent	**5**
filtration (15-40 nm)	**4-8**

If the mAb is produced as an ascites fluid and the mice used have been MAP tested and found free of species-specific viruses, adventitious virus testing of the final product is not necessary. When testing is necessary, we recommend that a minimum amount of product equal to 3 maximum human doses be used for these tests. For the purpose of these trials, MAP testing can be limited to known human pathogens: Hantaan, LCM, Reovirus and Sendai virus. For mAb produced in primate cell lines or in non-murine cell lines whose potential for contamination by human pathogenic viruses in unknown, the cell lines or the EPC or the unpurified bulk product should be tested as described in Section II.C.1. c and d.

c. Murine retrovirus testing of the final product is needed on the final-filled product only when the antibody is produced in a murine cell substrate and the purification scheme does not include at least two robust orthogonal virus inactivation/removal steps (see Table III). We recommend that a minimum amount of product equal to 3 maximum human doses be used for these tests. Testing of the final product, when necessary, should be done by a highly sensitive infectivity assay, such as amplification in Mus dunni cells followed by detection in PG4 cells or by other sensitive means as outlined in II.C.2.b.*vi.*

d. MAb used as ancillary products (see I.B. for definition) in feasibility clinical trials in serious or life-threatening conditions. Two cases can be distinguished:

> *i.* When the final product can be tested and the results are available prior to administration (e.g. purified recombinant proteins or cells that can be stored frozen), safety testing can be carried out on the final product itself, as determined during review of the final product.

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ii. When the final product is administered prior to or without any safety testing and/or processing, testing of the mAb should be performed as described above in paragraphs D.2. a. through c. In this case, amounts of mAb comparable to those used in one run of final product purification should be used.

The same safety considerations apply to complement, DNAase and other biological reagents used as ancillary products for cell depletion in conjunction with mAb.

E. ISSUES RELATED TO MANUFACTURING CHANGES (DEMONSTRATION OF PRODUCT COMPARABILITY)

1. General

Changes in the product manufacturing scheme frequently occur during clinical development of mAb. Sponsors should develop a plan for demonstrating that the products made by the old and new schemes are comparable, particularly when preclinical or clinical data developed prior to the production changes will be used to support further clinical trials and/or marketing applications (23). Similar considerations apply in the case of significant scale up in the manufacturing process (with or without modification of the general manufacturing scheme) implemented during or after completion of phase 3 trials.

When changes in manufacturing occur during early clinical development, plans for evaluation of product comparability should be incorporated into product development strategies. Such plans should be discussed with CBER and, when appropriate, submitted to CBER for review (see ref. 23).

In-process specifications may be affected by manufacturing changes or process scale-up and appropriate revisions should be undertaken. Similarly, process validations (e.g., virus clearance studies, removal of contaminants or leachables) for all affected steps should be repeated after any significant manufacturing change. In the case of process scale-up, it is recommended that, whenever possible, a column geometry and a ratio between sample volume and bed volume as close as possible to that of the original process be maintained. This is particularly important for those steps (e.g., size-exclusion chromatography) where these parameters are critical to the chromatographic process. Ref. 23 should be consulted for more details on demonstrating product comparability.

2. In vitro evidence of product comparability

In general, when a product is obtained by a modified or scaled-up manufacturing scheme, the results of a rigorous physico-chemical characterization and *in vitro* functional comparison (see Product Manufacture and Testing, Section II.B.) will dictate whether additional data (e.g., pre-clinical and/or clinical data) will be needed. A protocol for demonstrating physico-chemical, immunochemical and biological comparability of two products should prospectively define acceptable variation in the results of individual assays and acceptance criteria for product comparability. For quantitative assays (specific biological assays) accurate estimates of inter and intra-assay variations should be provided. Assays with high intrinsic variability are poorly suited for the evaluation of product comparability. Comparisons should test a number of separate product lots in parallel in order to demonstrate the reproducibility of the new manufacturing scheme. An *in vitro* biochemical characterization of mAb comparability should include a side by side comparison of the two products by a number of different techniques. Properly stored retention samples from previous lots should be used for such side by side comparison. A list of techniques could include SDS-PAGE under reducing and non-reducing conditions, Western blot, size-exclusion analytical chromatography, reverse phase high performance liquid analytical chromatography, isoelectrofocusing, mass spectrometry, an

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analysis of glycosylation including carbohydrate content and composition, peptide mapping or other appropriate tests. *In vitro* functional comparison should include assays aimed at the characterization of the biological function of the antibody (e.g., binding, cytotoxicity, epitope modulation, etc.). Whenever possible, a comparison of the affinity constants of the two products is highly recommended.

3. Animal studies

Depending on the quality of the data and the type of *in vitro* assays, the nature of the manufacturing change and the types of product differences observed or anticipated, a program of comparative testing (pharmacokinetics, etc.) in appropriate animal models may be considered in lieu of human clinical data when biochemical testing shows differences or cannot exclude significant differences in two products. In some cases, pharmacokinetic studies are complementary to *in vitro* studies. Pharmacokinetic studies in animals may be informative, even in the absence of the target antigen, depending upon the question to be addressed and the expected contribution of antigen binding to the biodistribution of specific mAb in humans. The extent of animal toxicity testing that may be needed to assess comparability will depend upon the safety profile of each specific product, the magnitude of the changes in manufacturing, the presence or absence of detectable differences in purity, structure or *in vitro* activity. Sponsors are encouraged to discuss plans for comparative testing of the two products in animals with CBER or to submit proposal for such testing to CBER for review and comment. The proposed program should be appropriate in view of biochemical data and include statistical considerations.

4. Clinical studies to support manufacturing changes

Comparative clinical evaluation of the products produced by different or scaled-up manufacturing schemes may be needed in certain situations:

a. Product activity cannot be adequately characterized by analytical testing.

b. Biochemical or biological testing show differences in the products.

c. Animal testing reveals significant pharmacokinetic or other differences in the products.

d. The formulation of the product has been changed in a way that can affect its bioavailability. The latter changes generally dictate a need for clinical pharmacokinetic studies.

Pharmacokinetic, safety and/or efficacy data may be required depending upon the nature and magnitude of the observed changes in the biochemical and or biological properties of the product.

Additional information on product comparability testing can be found in ref. 23.

III. PRECLINICAL STUDIES

A. TESTING CROSS-REACTIVITY OF MAB

When the same or related antigenic determinant is expressed on human cells or tissues other than the intended target tissue, binding of the antibody to this tissue may be observed. Non-target tissue binding may have serious consequences, particularly when pharmacologically active antibodies or cytotoxic immunoconjugates are used. Accordingly, cross-reactivity studies with human tissues (or cells if

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applicable) should always be conducted prior to phase 1 to search for cross-reactions or non-target tissue binding. In the special case of bispecific antibodies, each parent antibody should be evaluated individually, in addition to testing of the bispecific product.

1. *In vitro* testing for cross-reactivity

Human cells or tissues are presently surveyed immunocytochemically or immunohistochemically. Appropriate newer technologies should be employed as they become available and validated.

a. Reactivity of the antibody or immunoconjugate should be determined with the quick-frozen adult tissues listed in Appendix I. Surgical samples are preferred. Post-mortem samples are acceptable with adequate tissue preservation. Tissues from at least three unrelated human donors should be evaluated in order to screen for polymorphism. The effect of fixatives on tissues that are known to be positive should be evaluated to ensure that the target antigen is preserved during tissue processing.

b. In special situations it may be appropriate to assay cross-reactivity on representative cultured cell lines, stem cells, and embryonic/fetal tissue.

c. Several concentrations of the product should be tested. The ability to detect cross-reactions may depend on antibody concentration. Antibody affinities as well as expected achievable peak plasma concentrations should be considered when choosing the proper concentrations for tissue binding studies. An "ideal" concentration for these studies may be the lowest mAb concentration that produces maximum (plateau) binding to the target antigen. An attempt should also be made to compare the ratio of specific binding to target tissue to specific binding to cross-reactive tissue. Because non-specific as well as Fc-mediated binding may be observed, it should be distinguished from specific cross-reactions using inhibition assays with purified antigen, when available.

d. Positive and negative controls are essential for interpreting study results. Controls confirm the acceptable condition of the tissues and adequacy of the assay. Anti-transferrin receptor mAb may be a useful positive control, since transferrin receptor is a common and abundant molecule on the surface of growing normal and tumor cells.

e. If a conjugated, chemically modified antibody or antibody fragment is to be used clinically, it should be tested in that form if at all feasible. The substitution of antibodies of similar specificity for cross-reactivity testing is discouraged.

f. When cross-reactions are encountered and there is a reason to suspect genetic polymorphism of the target antigen, studies should be expanded to a larger panel of tissues to better characterize this polymorphism.

g. A comparison of *in vitro* cross-reactivity in tissues from different species is important in determining the most relevant animal for subsequent toxicology studies.

2. *In vivo* testing for cross-reactivity

Cross-reactivity of a monoclonal antibody with non-target human tissues should dictate a comprehensive *in vivo* investigation in animals, when appropriate models are available. This finding, particularly with cytolytic immunoconjugates or antibodies with ADCC activity, generally indicates the desirability of more extensive preclinical testing, including studies in more than one animal species over a range of doses and

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repeat dose animal studies. Localization to non-target tissues should be kept in mind when designing clinical trials.

B. PRECLINICAL PHARMACOLOGY AND TOXICITY TESTING

1. General considerations

a. Preclinical safety testing of mAb is designed to identify possible toxicities in humans, to estimate the likelihood and severity of potential adverse events in humans, and to identify a safe starting dose and dose escalation, when possible. Preclinical testing concerns surrounding mAb products include their immunogenicity, stability, tissue cross-reactivity, and effector function(s). Species differences may complicate the design and interpretation of preclinical studies. CBER recognizes that animal models expressing the antigen of interest or a closely related, highly cross-reactive epitope are not always available. Pharmacokinetic and pharmacodynamic properties of mAb that are dependent upon specific antigen binding may not be evident in animal studies conducted in species which do not express the antigen of interest. In some cases, xenograft models can be developed by introducing cells expressing the antigen of interest into immunodeficient mice (e.g. SCID or nude mice). Such models can provide information on specific targeting of desired cells, especially with radiolabeled mAb or immunoconjugates. Transgenic models expressing the antigen of interest are another possibility, if available. Whenever they are available, parallel models which explore the effects of mAb against the animal homolog of the antigen of interest can be informative. *In vivo* activity models have proven valuable in providing data which support a rationale for the proposed product use and in defining safety and toxicity. Animal disease models are available to study the effects of mAb on many inflammatory and autoimmune diseases, and allograft rejection. The extent of preclinical safety testing and the results of such testing will influence safety considerations for initial clinical trials (e.g. starting dose, dose escalation scheme, etc.).

b. Preclinical testing schemes should parallel to the greatest extent feasible those anticipated for clinical use with respect to dose, concentration, schedule, route, and duration. The range of doses selected for study should include at least one dose that is equivalent to and one dose that is a multiple of the highest anticipated clinical dose, with appropriate adjustments for interspecies differences in body size. A broad dose range should be explored. The highest doses tested should elicit adverse effects, whenever possible. Dose ranges are best established with a minimum of three doses. The linearity and overall shape of the dose response curve should also be defined by investigation of several doses and dosing intervals. If changes in manufacturing and/or formulation are made subsequent to conduct of preclinical studies, the decision to repeat some or all preclinical studies should depend on an assessment of the impact or likely impact of these changes on the product (see Section II.E.).

2. Animal toxicology studies

When planning toxicity testing for mAb, the following should be considered:

a. If the test article is an unconjugated antibody and there is no animal model of disease activity or animal that carries the relevant antigen, and cross-reactivity studies with human tissues are clearly negative, toxicity testing may not be necessary.

b. When a relevant animal model is available, an attempt should be made to study the dose-dependence of pharmacodynamic effects. The use of a broad range of doses, including high doses may allow a better prediction of the therapeutic index.

c. The properties of a relevant antigen in the animal should be comparable to those in humans in

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biodistribution, function, and structure. For example, studies of $CD34^+$ progenitor cells in the baboon are useful because the same cell fractions in both species express the $CD34^+$ antigen and produce hematopoietic engraftment. Absolute equivalence of antigen density or affinity for the mAb, however, is not necessary for an animal model to be useful. Differences in binding, for example, may be compensated for by alterations in the dose or dosing frequency. Differences between the animal and human in antigen number, the affinity of a mAb for the antigen, or the cellular response to mAb binding, should be identified. This will allow more accurate extrapolation of safe human starting dose and estimation of the margin of safety.

d. Routine assessments of mutagenicity are not generally needed for mAb.

e. Reproductive and developmental studies including teratogenicity in an appropriate animal species should be carried out in instances in which the product is intended for repeat or chronic administration to women of childbearing potential. Results of such peri- and post-natal developmental studies should be submitted for marketing approval. Evaluation of male fertility, when appropriate, should be completed before phase 3 trials.

3. Pharmacokinetics and pharmacodynamics

A pharmacokinetic model may aid in the interpretation of preclinical activity and toxicity, and in the recommendation of an appropriate dosing regimen and thereby improve the design of clinical trials. Such studies should aim at determining pharmacokinetic and pharmacodynamic endpoints. Of particular importance to the selection of clinical dosage is determining the relationship activity to area under the curve (AUC) of tissue or blood concentration over time. In considering the relationship of activity to AUC, factors related to the pharmacodynamics of the monoclonal antibody should be used in evaluating potential clinical effects. These factors include pathophysiologic status, threshold for effects as well as molecular events like the rates of association and dissociation for the site of action. Studies of biodistribution may provide the initial evidence for inappropriate tissue targeting by a mAb or explain toxicities that are observed in animals. Interpretation of data should consider species of origin, isotype, whether the mAb is an intact immunoglobulin, a fragment or an immunoconjugate, method of labeling, stability of the immunoconjugate, level of antigen expression in the recipient, binding to serum proteins, and route of administration. Even if antigen is expressed in an animal model, the mAb may bind the human target antigen and its animal counterpart with different affinities. MAb half-life may also be affected by glycosylation, susceptibility to proteases, presence of circulating antigen, and host immune response. The presence of antibodies to the product may alter biodistribution and elimination. In some cases, informative pharmacokinetic studies may be obtained in animal models which do not express specific antigenic determinants, depending upon the role played by antigen binding in product biodistribution, biotransformation and excretion.

a. Selection of the animal species for pharmacokinetic and pharmacodynamic testing should be guided by the following considerations:

i. Preference should always be given to study of a mAb in an animal model in a species that shares a cross-reactive or identical target antigen with humans, whenever such a species is available. For unconjugated mAb directed at human antigens not expressed in animal models or foreign antigens (bacterial, viral, etc.), studies in animal species lacking the target antigen may not be necessary unless they are designed to address manufacturing issues (see Section II.E. on Product Comparability).

ii. Study of non-human primates is appropriate for unconjugated mAb when there are antigen

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binding data that indicate that primates are the most relevant species.

iii. Normal rodent and murine xenograft models should be critically evaluated for their likelihood of predicting accurately human pharmacokinetic behavior of mAb. Xenograft models may be more useful in evaluating the ability of mAb to bind to human tumors *in vivo*.

b. Changes in manufacturing or formulation may result in significant changes in biological activity. Therefore, it is recommended that the material used in the preclinical studies be manufactured using the same procedures as used or intended for use in manufacturing material for clinical trials. In some cases it may be appropriate to modify the components of the formulation for preclinical testing. For example, substitution of the homologous animal serum albumin for human serum albumin that is used as a carrier will prevent the formation of anti-albumin antibodies in animal studies and thereby increase the relevance of preclinical testing.

c. Pharmacokinetic parameters should be defined using one or more assay methods (e.g., a radiolabeled mAb should be assayed by ELISA and by measurement of radioactivity). In the case of immunoconjugates of any type, intact conjugate should be distinguished from free mAb and free ligand (e.g. toxin, drug, or radionuclide). Pharmacokinetic parameters that are most important for product characterization, as well as most useful for determining product comparability include T_{max}, C_{max}, $T_{1/2}$ and AUC.

d. The development of anti-immunoglobulin antibodies greatly complicates study and interpretation of the effects of repeat dosing in animals. Murine antibodies are non-immunogenic in mice but are immunogenic in humans, making it difficult to extrapolate the results of repeat dose studies in mice to planned repeat dose administration in humans. The reciprocal problem will occur with fully human, chimeric or "humanized" mAb. Repeat dose studies in rodents in this case may be of little value.

4. Preclinical *in vivo* studies with immunoconjugates

a. Immunoconjugates should be tested for stability *in vivo*.

i. Individual components of an immunoconjugate should be measured during pharmacokinetic and tissue distribution studies in animals and compared to the distribution of unconjugated antibody.

ii. The target tissues for the various components and the potential toxicities that they may cause should be established.

b. Immunoconjugates containing radionuclides, toxins, or drugs should undergo animal toxicity testing even when the target antigen is not present in an animal species, because of possible conjugate degradation or activity in sites that are not the result of mAb targeting. Depending upon the nature of the components of the immunoconjugate and the stability of the conjugate itself, separate studies of the components may be warranted. The toxicity profile of each component should adequately describe the incidence and severity of possible adverse effects. Results should be correlated closely with studies of conjugate stability. Studies of the immunoconjugate should be performed in a species with the relevant target antigen or disease model, whenever available and generally in rodents if a target antigen-positive species is not available. Toxicity testing of free toxin or nuclide may be performed in a different species.

c. For immunoconjugates containing radionuclides:

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i. Animal biodistribution data may be used for initial human dose estimation.

ii. Animal models that express the targeted antigen, whenever such models are available, are more likely to reveal the effects of antigen "sinks" or tissues with unexpected antigen expression on biodistribution and/or toxicity.

iii. Xenograft models may evaluate tissue targeting and antigen non-specific radioimmunoconjugate distribution problems, but are not helpful at identifying areas of normal tissue cross-reactivity.

iv. An adequate number of animals should be studied to achieve radiation dose estimates with an acceptable coefficient of variation (usually less than 20%).

v. There should be complete accounting of the metabolism of the total dose of administered radioactivity and an adequate number of time points to determine early and late elimination phases.

vi. Radioimmunoconjugates should be tested for stability *in vitro* by incubation in serum or plasma (see Section II.C.7.). Methods should be developed to estimate the percent radioactivity in each of the three species of concern: free isotope, conjugated mAb, and labeled, non-mAb substances.

IV. CLINICAL STUDIES

MAb administered to humans have usually been well-tolerated. Instances of serious or fatal adverse events have generally resulted from intended or unintended binding of mAb to specific antigens. These events emphasize the importance of screening tissues for mAb cross-reactivity, particularly when relevant-antigen animal models are not available. The results of preclinical tests may alert physicians to potential toxicities and may indicate that more conservative dosing schemes are justified during dose escalation.

A. CLINICAL CONSIDERATIONS FOR PHASE 1 AND 2 STUDIES

1. General

a. Different approaches to Phase 1 and 2 studies may be warranted depending on the nature of the mAb. Initial studies of therapeutic mAb in phase 1 are generally escalation studies of single-doses of the mAb. The goal should be to determine a presumed optimal biologic dose (OBD) that is usually defined by pharmacokinetics or pharmacodynamic measurements (e.g., degree of antigen binding or saturation or target blood levels, determined on the basis of preclinical studies) and, where appropriate, by the tolerability to the agent (e.g., the maximally tolerated dose [MTD]) (24-26). In the case of a therapeutic unconjugated mAb, studies that identify the MTD may not be necessary. Instead, determination of a presumed OBD may be a more appropriate goal. Immune activation, when relevant to the mechanisms of action or toxicity of the mAb, should be evaluated. In the case of radiolabeled therapeutic mAb or immunotoxins, undesired tissue targeting and release of conjugate due to degradation are major safety concems. Patients receiving immunotoxins should be monitored for capillary leak syndrome and for hepatic, renal, and muscle toxicities.

Some antibody-specific side effects are more likely to occur with certain subclasses of immunoglobulins.

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These antibodies (e.g., human IgG1 and IgG3, mouse IgG2a) are more likely to activate complement or activate antibody-dependent cell-mediated toxicity (ADCC) via their Fc regions, leading to lysis of bound cells. MAb may also cause desired or adverse effects by blocking or inducing functions of target cells (e.g., cytokine release syndrome following stimulation of T-cell receptors by anti-CD3).

b. In general, subjects in clinical trials of therapeutic and diagnostic products, including mAb, should be representative of the population targeted for eventual product use. Because of the potential immunogenicity of mAb, healthy volunteers may not be appropriate candidates for phase 1 trials. The nature of the mAb, the target antigen, and the proposed clinical application should be considered before deciding to enroll healthy volunteers in a trial. Situations in which healthy volunteers might be used in early trials include the following:

i. When the risks of studying a new agent initially in the index population are too high, such as when the index population expresses abnormally high levels of antigen (raising specific toxicity concems) or when the index population may be particularly vulnerable to toxicity because of serious illness or significant organ dysfunction.

ii. When the index population is so ill that safety data are confounded and difficult to interpret.

When healthy volunteers are considered for inclusion in initial studies of a mAb, the informed consent should reflect the absence of direct medical benefit. For healthy volunteers, as for patient volunteers, the informed consent process should also illustrate the potential immediate and long-term risks of receiving xenogeneic proteins. These include possible toxicity, allergic reactions, and, in the case of murine and other non-human mAb, potential future inability to receive or benefit from a diagnostic or therapeutic mAb because of the development of an immune response against the foreign protein.

c. Sponsors and investigators should carefully consider whether single doses of the mAb, multiple doses of the mAb in a single course, or multiple courses of therapy will be most likely to optimize benefit over risk. Concomitant therapies or repeat administration of the mAb may alter its safety and efficacy profiles. Changes in antigen mass (e.g., due to binding and clearance, or to antigen modulation by the mAb) and immune responses to the mAb, for example, may prevent extrapolation of single dose data to multiple-dose schedules. Furthermore, repeat administrations in the face of an antibody response against the therapeutic agent may lead to toxicity and/or loss of therapeutic benefit.

d. Subjects with prior parenteral exposure to xenogeneic proteins or with a history of xenogeneic protein allergies should be excluded from phase 1 studies of mAb products that have been derived from the same or a closely related species.

e. Informed consent issues in feasibility clinical trials in serious or immediately life-threatening conditions:
If applicable, informed consent forms for these trials should clearly state the following in language understandable to the patients:

i. only a limited characterization of the processes used to prepare the product for their ability to remove endogenous or exogenous infectious/toxic agent(s) was performed, and

ii. there may be potential health risks, including hitherto unknown risks, derived from exposure to such agents if they are present in the product.

2. Dose-setting

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a. Whenever possible, the selection of the phase 1 starting dose should be based on safety and toxicity information derived from testing in a relevant animal model. When extrapolating from animal doses to human doses, information about the relative affinity of the mAb for the human antigen as compared to its animal analogue may be of great value. The target *in vivo* dose or concentration range should be based both on *in vitro* studies of cells for which antibody-antigen affinity and functional activity (e.g., immune modulation, cytotoxicity) have been measured, as well as on study of a relevant-antigen animal model, if available, to assess *in vivo* activity. If animal studies are judged to be impossible or of no relevance and initial *in vivo* studies are to be performed in humans, testing should begin at a low dose that is based on extrapolation from tissue culture studies or from available information gathered in clinical trials of a similar mAb.

b. Initial studies of radioimmunotherapeutic mAb should also employ escalating single-doses of the mAb, with the lowest and highest doses based on animal dosimetry and on the projected tolerance of normal organs to radiation. Both the elimination half-life of the mAb and the elimination half-life of the radioactivity should be characterized.

c. If a multiple-dose regimen of a mAb is anticipated, multiple-dose schedules should be explored in late phase 1 or phase 2 trials, after basic data on toxicity, peak levels, clearance, distribution, and biologic effects are available from single-dose studies. The time required for recovery from the biologic effects of single doses (e.g., immune recovery after $CD4^+$ cell depletion or modulation, return of bone marrow function after radioimmunotherapy) should also be well understood prior to initiation of multiple-dose regimens. The rationale for dosing schedules should be provided. The rationale should be based on dose tolerance, available pharmacokinetic and pharmacodynamic data in humans, and on relevant animal models of safety and efficacy. Modified dosing regimens to compensate for a high antibody response against the agent or circulating antigen may need to be studied. Pharmacokinetic studies to determine the relationships of human anti-mAb antibody titers and circulating antigen levels with organ distribution, clearance, and toxicity may be necessary.

d. Before repeat administration of a radioimmunotherapeutic or immunotoxin, the investigator should characterize all organ toxicities and pathology resulting from single dose administration. The timing of recovery from all toxic effects should be determined. Intra-patient dose escalation may confound interpretation of safety data because it may be difficult to determine whether toxic effects (e.g., to bone marrow) are due to prolonged therapy or to increased dosing levels. Intra-patient dose escalation may be appropriate if no toxicity is seen at the initial dose levels or if it is possible to use initial safe "test" doses and if cumulative toxicity is deemed unlikely. If intra-patient dose escalation is performed, consideration should be given to threshold and carryover effects, as well as to the reversibility of clinical and laboratory adverse events.

e. Design of pharmacokinetic studies should include consideration of the species in which the immunoglobulin is produced, the immunoglobulin class and subclass, and the structure of the antibody (e.g., whole mAb, Fab fragment) or immunoconjugate. A relevant study population will have the appropriate antigen and antigen mass. If antigen mass is likely to alter the bioavailability of the mAb, this should be determined in pharmacokinetic studies so that its impact on dose setting and on stratitifcation and analysis of later trials can be considered. Aside from obtaining estimates of common pharmacokinetic parameters, pharmacokinetic studies may also be very useful in situations in which the comparability of different products or formulations is to be determined (see Sections II.E. and III.A.). Pharmacokinetic studies optimally include the following:

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i. Determination of plasma concentration profiles, distribution, and clearance of the mAb.

ii. Determination of doses for further study based on dose-concentration effect relationship and correlation with desired concentrations estimated from *in vitro* studies.

iii. Determination of peak and trough mAb levels and elimination rate constants.

iv. Determination of the organs and sites where the mAb is distributed, metabolized, and eliminated.

v. Determination of the fate of immunoconjugates by assaying the whole molecule and its components.

vi. Investigation of the relationships between the elimination rate and the method of administration, antigen load, and presence of a circulating antigen or of an antibody response against the therapeutic agent.

B. IMMUNOGENICITY: CLINICAL CONSIDERATIONS

Monitoring of antiglobulin titers and immune activity is of great importance in evaluating the safety and efficacy of mAb and in designing protocols involving repeat administration. Immune responses to mAb may have little or no effect, or may interfere significantly with the safety and/or efficacy of a mAb.

1. Monitoring the development of antibodies to mAb

Depending on the source of the mAb, assays for anti-immunoglobulins will need to be developed to detect human anti-mouse antibodies (HAMA), human anti-rat antibodies (HARA), human anti-human antibodies (HAHA), human anti-chimeric antibodies (HACA), and anti-idiotype antibodies. As appropriate for the mAb, assays should be developed to detect human immunoglobulins directed against humanized or primatized antibodies, immunonuclides and immunotoxins, their individual components (e.g., ricin), and neoantigens formed by the linked antibody/toxin/nuclide.

a. The timing of sample collection for evaluating the presence of an anti-mAb antibody should take into account whether the mAb is intended to be given as single or as multiple doses. Titers of the anti-mAb antibody should always be established at baseline to account for pre-existing antibodies (including anti-globulin or anti-conjugate antibodies, when appropriate), and also before readministration of the mAb. Post-administration samples may be drawn early (e.g., two weeks after administration), but should also be drawn at later times (e.g., at six-eight weeks).

b. The assay(s) used to detect the anti-mAb antibody should be standardized to the greatest extent possible. Aliquots of a "reference" preparation of antibody, e.g. anti-mouse antibody for a HAMA assay, with defined specificity from a human or primate source should be aliquoted and frozen to facilitate future intra- and inter-study comparisons. The reference preparation can also be used to establish a standard curve for routine testing. The assay(s) should be validated by establishing sensitivity, specificity, precision, and accuracy. Inhibition or competition studies with both negative and positive controls should be used to demonstrate that the assay detects antibodies to the mAb product. Studies should assess the range of reactivity of normal individuals and should evaluate potential interference by serum components such as bilirubin and lipids.

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c. The specificity of the immune responses to the mAb should be identified and characterized in a sample of patients. These studies should establish whether the responses are generated against a heavy-chain isotype determinant, a light-chain, constant (C) region, variable (V) region, idiotypic epitope(s), immunoconjugate, or neoantigen. These data will demonstrate whether it is possible to use an anti-mAb antibody test with broad specificity (e.g., detecting human antibodies reactive with antigens of both heavy and light chain constant regions of all foreign immunoglobulin classes), or whether a more restricted anti-mAb antibody test that is idiotype-specific is necessary. In certain instances the anti-mAb antibody assay should include the actual mAb product as the detection antigen.

d. The choice of the appropriate assay for anti-mAb antibody depends on the proposed use and labeling of the product. Development and validation of the assay should accompany the clinical development of the new mAb. The results of the anti-mAb antibody testing should be correlated with product efficacy and adverse events.

e. A license application submitted for a mAb to be administered in a repeated dosing regimen should include a clinically available, validated test that reliably measures human antibody responses to the mAb, if an anti-mAb response may affect the safety, efficacy or dosing of the product. If a commercially available HAMA (or other anti-mAb antibody) test kit, is available, it may be used provided it has been demonstrated to reliably detect antibody response against the new mAb product. In most cases, humanized mAb require an assay specific for the product itself. If no appropriate anti-mAb antibody test is available, a properly validated test system should be developed by the sponsor.

2. Clinical consequences of immunogenicity

a. When a patient is found to have developed an antibody response against the therapeutic or diagnostic mAb, adverse events should be anticipated and appropriate precautions taken. MAb are generally given in facilities where acute resuscitative care is immediately available. The use of non-hospital settings for mAb administration (e.g., clinic or home) should be justified by clinical safety data. Vital signs should be observed closely for at least one hour after completion of the mAb administration. The possibility of delayed adverse effects from immune responses to mAb should be considered and reflected in the trial design, including appropriate clinical and laboratory testing.

b. Anaphylaxis, anaphylactoid and other immune reactions

i. True IgE-mediated anaphylaxis to whole mouse immunoglobulins is infrequent. It is theoretically possible that anti-human allotype responses of an allergic nature could occur but they have not been reported to be of clinical importance to date. If the mAb is conjugated to chelating agents or toxins, the likelihood of allergic reactions may be greater. In all cases, repeated administration of a mAb increases the likelihood of a hypersensitivity response. Immediate hypersensitivity reactions may range from mild to severe. Skin testing is not advised because it is a poor predictor of sensitivity to mouse immunoglobulins and can cause sensitization.

ii. Infusional reactions such as chills, rigors, aches, and low grade fever, are common during or immediately following mAb administration (the incidence is approximately 5% with several antibodies). The mechanism of these reactions is not clear. The frequency and intensity of such reactions can often be controlled by using slower infusion rates or by pre-medication.

iii. Serum sickness is unusual following mAb administration but has been described. Unlike anaphylaxis and infusional reactions, which occur during or immediately after antibody treatment,

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serum sickness is delayed by several hours. The correlation between circulating levels of soluble antigen and immunocomplex-mediated adverse events such as serum sickness should be explored if such adverse events are observed.

c. Anti-mAb antibodies can interfere directly with some antibody-based clinical tests for antigens, such as CA-125 and CEA, by binding to the murine detection antibody. Indirect interference with diagnostic assays is theoretically possible if mAb administration induces anti-idiotypic responses that mimic the antigen. When appropriate, evidence for either type of interference should be systematically sought using well designed *in vitro* studies. Ideally, attempts should be made to circumvent such interference and alternative clinical assays should be validated.

d. When subjects are selected for testing a mAb, the risk that future therapy with a monoclonal antibody may be compromised by elicitation of an antibody response against the therapeutic agent or other mAb should be considered and reflected in the informed consent form.

C. PRODUCT-RELATED CONSIDERATIONS FOR PHASE 3 STUDIES

When planning manufacturing changes or scale-up programs during phase 3 clinical trials, sponsors should consider that product comparability may have to be demonstrated (see ref. 23 and Section II.E.). This may or may not require additional clinical studies depending upon the adequacy of preclinical data (see ref. 23 and Section II.E.). Thus, it is suggested that scale-up programs and contemplated changes in product manufacture be anticipated prior to the initiation of phase 3 trials. Sponsors should study a number of separate product lots during drug development to demonstrate that a safe and effective product can be prepared reliably.

D. ADMINISTRATION OF RADIOLABELED ANTIBODIES

1. Dosimetry

Grade 3 and grade 4 organ toxicities have been reported with therapeutic radioisotopes. Therefore, dosimetry estimates for human subjects are required prior to the initiation of phase 1 studies. The dosimetry estimates should be developed with simulation models utilizing an appropriate diagnostic radioisotope label on the antibody. If no diagnostic radiolabel for the antibody is available for simulation, animal studies with the therapeutic radiolabel may be utilized for dosimetry estimates. The actual dosimetry data for the therapeutic radiolabeled antibody, itself, should be acquired concurrent with the initial phase 1 study and reported prior to the initiation of a phase 2 study.

For diagnostic radiolabeled antibodies, as for the therapeutic isotopes, the investigator should provide estimates of the organ dosimetry prior to the first phase 1 study. Final dosimetry calculations from human studies should be completed prior to the submission of the license application.

a. General considerations: Sufficient data from animal or human studies should be submitted to the IND, to allow a reasonable calculation of radiation-absorbed dose to the whole body and to critical organs upon administration to a human subject [21 CFR 312.23(a)(10)(ii)]. See Appendix III for a list of organs to be included in dosimetry estimates.

The amount of radiation delivered by internal administration of radiolabeled antibodies should be calculated by internal radiation dosimetry. The absorbed fraction method of radiation dosimetry is described in two systems [21 CFR 361.1 (b)(3)(iv)]:

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i. The Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine

ii. The International Commission on Radiological Protection (ICRP).

The investigator should specify which methodology is used. The mathematical equations used to derive the radiation doses and the absorbed dose estimates should be provided. Sample calculations and all pertinent assumptions should be listed and submitted.

Safety hazards for patients and health care workers during and after administration of the radiolabeled antibody should be identified, evaluated, and managed appropriately.

b. Calculation of radiation dose to the target organ

Investigators should determine the following, based on the average patient:

i. The amount of radioactivity that accumulates in the target tissue/organ.

ii. The amount of radioactivity that accumulates in tissues adjacent to the target tissue/organ.

iii. The residence time of the radioactive mAb in the target tissue/organ and in adjacent regions.

iv. The radiation dose from the radioisotope, including the free radioisotope and any daughter products generated by decay of the radioisotope.

v. The total radiation from bound, free, and daughter radioisotopes associated with the radioimmunoconjugate, based upon immediate administration following preparation and upon delayed administration at the end of the allowed shelf life.

c. Maximum absorbed radiation dose

The amount of radioactive material administered to human subjects should be the smallest radiation dose that is practical to perform the procedure without jeopardizing the benefits obtained.

i. The amount of radiation delivered by the internal administration of radiolabeled antibodies should be calculated by internal radiation dosimetry using both the MIRD and ICRP systems. The higher estimate of the absorbed dose determined from either of these systems should be used in the radiation dosimetry safety assessment.

ii. Because of known or expected toxicities associated with radiation exposure, dosimetry estimates should be obtained as delineated in IV.D.1.a and b.

iii. Calculations should be provided that anticipate changes in dosimetry that might occur in the presence of diseases in organs that are critical in radioimmunoconjugate metabolism or excretion (e.g., renal dysfunction causing a larger fraction of the administered dose to be cleared via the hepatobiliary system or vice versa).

iv. Possible changes in dosimetry resulting from patient to patient variations in antigen mass should also be considered in dosimetry calculations (e.g., a large tumor mass may result in a larger than

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expected radiation dose to a target organ from a radiolabeled anti-tumor mAb).

v. The mathematical equations used to derive the estimates of the radiation dose and the absorbed dose should be provided. Sample calculations and all pertinent assumptions should be listed.

vi. Calculations of dose estimates should be done assuming freshly labeled material to account for maximum amount of label as well as at the maximum shelf life of the radiolabeled antibody to allow for the upper limit of radioactive decay contaminants and should: (a) Include the highest amount of radioactivity to be administered; (b) Include the radiation exposure contributed by other diagnostic procedures such as roentgenograms or nuclear medicine scans that are part of the study; (c) Be expressed as Gray (Gy) per megaBequerel (MBq) or per millicurie of radionuclide; and (d) Be presented in a tabular format and include doses of individual absorbed radiation for the target tissues/organs and the organs listed in Appendix III.

2. Early clinical development of therapeutic radiolabeled mAb

a. Evaluations that should be conducted prior to Phase 1 studies.

Prior to phase 1 studies, investigators of therapeutic applications of radiolabeled antibodies should conduct the following evaluations for the average adult to be entered into the study:

i. The therapeutic radiolabeled antibody should be evaluated for *in vitro* stability and composition of the radioactive material to be administered. The expected and acceptable levels of the percent of free radioisotope, the percent of radioisotope bound to immunoreactive antibody, and the percent of bound radioisotope to non-immunoreactive antibody should be established. Calculations of the estimates should be at the maximum planned shelf life of the radiolabeled antibody to allow for the upper limit of radioactive decay contaminants and should be based upon the maximum dose of radioactive material to be administered to patients.

ii. The expected biodistribution and routes of clearance of the administered radiolabeled antibody dose fractions in tissues/organs should be defined.

iii. The expected biodistribution and routes of clearance that might occur in the presence of diseases in organs that are critical in radioimmunoconjugate metabolism or excretion should be described.

iv. The expected biodistribution and routes of clearance that might occur in the presence of immune responses (e.g., HAMA, HAHA, HARA) should be described.

With reference to the radioactive fractions of the administered radiolabeled antibody dose and the patterns of biodistribution, the following issues should be addressed:

v. From the biodistribution estimation, the expected residence time of the radiolabeled antibody fractions in the target tissues/organs and non-target tissues/organs should be determined.

vi. Based on the estimated residence times in each organ, the radiation exposure for each tissue/organ should be estimated.

vii. Based on the radiation exposure for each tissue/organ, the potential toxicity should be

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described.

viii. Based on the potential radiation toxicity to tissues/organs, toxicity monitoring protocols should be developed and incorporated into the clinical trial.

ix. If the study has increasing doses of radioactive materials (e.g., a study of the maximum tolerated dose), the radiation exposure for tissues/organs and the associated potential toxicities should be estimated for each radiation dose level.

b. Selection of patients for phase 1 trials of therapeutic radiolabeled mAb.

Patients should be entered into phase 1 trials with therapeutic radiolabeled antibodies only after consideration of the following:

i. To reduce the potential for alterations in biodistribution, patients enrolled in early studies should not have prior or concurrent exposure to investigational or approved antibodies.

ii. Patients should be evaluated for immune response to the appropriate species of monoclonal antibody (e.g., HAMA, HARA). Patients demonstrating evidence of immune response should generally be excluded from phase 1 studies.

iii. For proper evaluation of potential adverse events in early studies, patients should be in adequate health to allow follow-up for three months without adjunctive chemotherapy or radiation therapy. Generally, patients should have a Karnofsky score greater than 70.

iv. Consultation with CBER is strongly recommended when considering the inclusion of pediatric patients in early radiolabeled antibody trials.

c. Study design issues for phase 1 studies of therapeutic radiolabeled mAb

Phase 1 studies should be designed to address the following points:

i. The prepared therapeutic radiolabeled antibody should be evaluated for *in vivo* stability. The previously estimated expected and acceptable levels of the percent of free isotope, the percent of bound radioisotope to immunoreactive antibody, and the percent of bound radioisotope to non-immunoreactive antibody should be confirmed and demonstrated to be reproducible. Calculations should be with the routine shelf life of the prepared radiolabeled antibody and based upon the maximum dose of radioactive material administered to patients.

ii. The pharmacokinetics and biodistribution in the patient population should be studied.

iii. The residence times with radiation dosimetry for tumor (when applicable), tissues, and organs should be determined.

iv. The pattern of toxicity, its relationship to administered dose and the organs of concern for acute and delayed radiation injury should be established.

v. Any apparent evidence of response of tumor to the administration of the radiolabeled antibody should be documented.

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vi. The trial design should incorporate patient imaging with a diagnostic radiolabel on the antibody to confirm the expected biodistribution and residence times prior to the administration of the therapeutically radiolabeled antibody.

3. Adverse events for patients enrolled in trials of therapeutic radiolabeled mAb

The mechanism for follow-up of patients and reporting of adverse events should be described in the protocol prior to initiation of the trial. Complete evaluation and reporting of the adverse events potentially associated with the therapeutically radiolabeled antibody should be assured. If patients are referred to their attending physicians during the follow up phase, the investigator should plan and control the follow up of the treated patients for complete and timely reporting of adverse events potentially associated with the administration of the therapeutic radiolabeled antibody.

Patients removed from a trial should be continued in follow up for three months. All adverse events during that time interval should be reported, even if they are not thought to be related to the administered radiolabeled antibody.

4. Clinical development of radiolabeled mAb used as imaging agents

a. Prior to the initiation of phase 3 studies

Investigators of diagnostic applications of radiolabeled antibodies should collect stability, safety and pharmacokinetic information for the average adult expected to be entered into phase 3 studies.

i. The expected percent of free radioisotope, the expected percent of radioisotope bound to immunoreactive antibody, and the percent of the radioisotope bound to non-immunoreactive antibody should be determined. If unlabeled antibody is to be administered in conjunction with radiolabeled antibody, the ratio and amounts of the labeled and unlabeled antibody should be defined.

ii. The biodistribution of the administered radiolabeled antibody dose fractions in tissues/organs should be delineated, and from the biodistribution, the expected residence time of the radiolabeled antibody fractions in the target tissues/organs and non-target tissues/organs should have been estimated. The routes of clearance of the radiolabeled antibody should be determined.

iii. The changes in pharmacokinetics of the radiolabeled antibody with organ impairment, antigen load in the circulation, and tumor burden should be evaluated. The potential for clearance artifact to degrade patient imaging should be explored.

iv. Estimates of appropriate imaging times and techniques should be developed. Adjunctive imaging aids (e.g., enemas, emptying of the urinary bladder) should be evaluated.

v. Evidence of image quality should be gathered. The ability of the radiolabeled antibody to image known and/or occult disease should be documented. These data should be compared to imaging data obtained using standard diagnostic techniques whenever possible.The incidence of false positive localization of the radiolabeled antibody and the incidence of misinterpretation of the images to produce false positive and false negative interpretations should be explored. Disease specific factors (e.g., stage of disease, tumor burden, and co-morbid illness) should be evaluated for impact on technical procedures in the imaging protocol (e.g., time of imaging).

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vi. Phase 2 trials should be designed to define the appropriate patient populations for phase 3 trial(s), to define the technical procedures used for imaging in the anticipated patient populations and to identify potential clinical utility of the test to be further explored in later studies.

vii. Multiple clinical sites should be employed in phase 2 studies to assess the reproducibility of the imaging techniques, and of the preparation and administration of the radiolabeled antibody.

b. Pivotal efficacy studies of radiolabeled mAb used as imaging agents

CBER staff should be consulted for review of and comments on Phase 3 protocol(s), prior to the initiation of the phase 3 study(ies). The following elements should be incorporated into each clinical protocol:

i. A prospectively defined and detailed primary efficacy endpoint and analytical plan.

ii. A study population consisting of those patients for whom the imaging agent is intended after licensure. The performance and utility of an imaging test may vary substantially based on the stage, extent, or severity of the disease, determined in part by the results of other diagnostic tests. Therefore, the study population and subpopulations to be analyzed should be carefully defined in terms of stage of disease as well as in terms of diagnostic tests performed and test results prior to study imaging. The protocols should be designed to assess the imaging performance and the utility in the populations.

iii. A plan for acquisition and storage of imaging data for radiolabeled antibody in the confirmatory studies.

iv. A prospective plan for evaluation of imaging performance:

(a). On-site image interpretation and reporting should be defined and documented. To the extent possible, the information available to the on-site reader should be defined by protocol and recorded on the case report forms.

(b). The off-site image interpretation should be the basis of the principal analysis of imaging performance in the phase 3 clinical trial. The off-site image interpretation and reporting of all radiolabeled antibody image findings and all confirmatory imaging should be defined and documented prospectively. The information available to the off-site reader should be defined by protocol and recorded on the case report form. In general, the off-site reader should have little information beyond the entry criteria of the study and specifically should not be aware of the on-site reading, the results of other diagnostic tests, or patient outcome data.

(c). Planned sample size should be sufficient to determine imaging performance measures to a predetermined precision (i.e., 95% confidence interval width). Imaging performance may vary with the stage, extent, and/or site of disease as defined by pre-imaging evaluations, and this should be accounted for prospectively in planning analysis of imaging performance. To determine performance, imaging results should be compared with another indicator of disease, usually results of standard imaging, biopsy, exploration, patient follow-up or some combination of these.

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(d). If the planned use of the test is in conjunction with other diagnostic tests, its imaging performance should be determined and reported in groups of patients defined by the results of the other tests. For example, in some cases it will be important to know the imaging performance in patients with positive CT scans and the imaging performance in patients with negative CT scans.

v. A prospective plan for evaluation of clinical utility:

(a).When an agent has a significant incidence of false negative and false positives or significant toxicities, it is particularly important that the clinical utility be assessed to determine whether the value of the diagnostic information outweighs the potential adverse consequences of incorrect information or the toxicities. In this context, clinical utility means the extent to which information obtained by use of the mAb agent in a defined clinical setting can be expected to contribute to outcome, to contribute to the convenience or appropriateness of patient management, or to provide accurate prognostic information.

(b). Based on phase 2 and other data, the protocol should indicate the specific manner in which clinical utility is to be explored. The following issues should be addressed:

(1) The stage and severity of the disease in which the test is to be indicated should be specified.

(2) The protocol should specify whether the test is to be used in conjunction with or in lieu of other diagnostic tests. Because radiolabeled mAb image by a mechanism distinct from that of radiopharmaceuticals or diagnostic devices, the information obtained from a monoclonal antibody image may be complementary to that obtained by those means. For example, a mAb agent may not be as sensitive overall as an accepted standard test, but may be able to detect disease accurately under conditions where the standard technique fails.

(3) How the various results of the test are hypothesized to be clinically useful (for management or prognosis) should be clearly delineated. For example: positive results together with a positive CT scan are sufficiently diagnostic to avoid further diagnostic evaluation including biopsy; positive results are useful to guide biopsy; positive uptake is predictive of response to a specific therapeutic modality.

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V. APPENDIX I: NORMAL HUMAN TISSUES USED IN CROSS-REACTIVITY TESTING

1. Adrenal
2. Bladder
3. Blood cells
4. Bone Marrow
5. Breast
6. Cerebellum
7. Cerebral cortex
8. Colon
9. Endothelium
10. Eye
11. Fallopian tube
12. Gastrointestinal tract
13. Heart
14. Kidney (glomerulus, tubule)
15. Liver
16. Lung
17. Lymph node
18. Ovary
19. Pancreas
20. Parathyroid
21. Pituitary
22. Placenta
23. Prostate
24. Skin
25. Spinal cord
26. Spleen
27. Striated muscle
28. Testis
29. Thymus
30. Thyroid
31. Ureter
32. Uterus (cervix, endometrium)

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VI. APPENDIX II: MOUSE ANTIBODY PRODUCTION TEST

The following tests for murine viruses (mouse antibody production test (27) should be performed on any MCB and EPC derived from murine cell lines and on all lots of mAb derived from mouse ascites fluid:

1. Ectromelia
2. EDIM
3. GD VII virus
4. Hantaan virus
5. LCM virus, including challenge for non-lethal strains
6. LDH-elevating virus
7. Minute virus of mice
8. Mouse adenovirus
9. Mouse encephalomyelitis
10. Mouse hepatitis
11. Mouse salivary gland (murine CMV)
12. Pneumonia virus of mice
13. Polyoma
14. Reovirus type 3
15. Sendai
16. Thymic virus

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VII. APPENDIX III: ORGANS TO BE CONSIDERED IN DOSIMETRY ESTIMATES

1. all target organs/tissues
2. bone
3. bone marrow
4. liver
5. spleen
6. adrenal
7. kidney
8. lung
9. heart
10. urinary bladder
11. gall bladder
12. thyroid
13. brain
14. gonads
15. gastrointestinal tract
16. adjacent organs of interest

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Can Immunoglobulin C_H1 Constant Region Domain Modulate Antigen Binding Affinity of Antibodies?

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Abstract

Although the switch process is frequently associated with affinity maturation, the constant region is not assumed to play a role in Ag–Ab binding. In the present work, we demonstrate that two clonally related human monoclonal Igs sharing identical V_H and V_L sequences, but expressing different isotypes (IgA1κ^{PER} and IgG1κ^{PER}), bind tubulin with significantly different affinities. This difference was mainly accounted for by a disparity in the association rate constants. These results suggest that affinity maturation of this clone could be achieved through class switching in the absence of further somatic mutations. Since the differences observed were found at the Fab level, they also suggest a role for the C_H1 domain in structuring the Ag-binding site into a more kinetically competent form. (*J. Clin. Invest.* 1996. 98:2235–2243.) Key words: immunoglobulins • isotype • switching • C_H1 • affinity

Introduction

Ig are heterodimeric proteins composed of two H and two L chains. Each L chain contains either a C_κ or a C_λ and a V_L domain that helps define the Ag-binding site of the Ab. In addition to a variable domain, each H chain contains between three and four constant domains that specify the effector function of the Ab. Multisequence comparisons of variable domains have shown that each variable domain contains three regions of extensive sequence variability, termed the complementary determining regions (CDRs),[1] and four regions of relative sequence stability, termed framework regions (FRs). The three L chain CDRs and the three H chain CDRs are juxtaposed to form the Ag-binding site of the Ab, as classically defined. In turn, the FRs create a scaffold that surrounds, supports, and influences the conformation and structure of the CDRs (1–4).

There are five major classes of C_H domains in a mammalian genome (μ, δ, γ, ϵ, and α). B cells are endowed with the ability to switch from one set of constant domains to another (1). This class switch allows the B cell to change the effector function of the Ab that it produces while maintaining the Ag specificity of the original Ab (5). The sequence and structure of the Ag binding site can be altered through a process of somatic hypermutation that is targeted at the V domain, leading to a change in the binding affinity and specificity of the Ab (2, 3, 6, 7). Both class switching and somatic hypermutation of the variable domains are processes that occur in the germinal center B cells that are undergoing Ag selection and affinity maturation of their Ab. It has long been assumed that the constant domains do not play a role in the maturation of Ag–Ab interactions. However, recent studies have reported the influence of Fc on apparent binding strength, particularly among IgG subclasses, which do not depend on multimerization, but on subtle Fc–Fc cooperative interactions (8, 9).

We have previously described in a single individual, the presence of four different clonally related MIg in the serum of a patient suffering from an immunocytic sarcom (10). Three of these MIg were found to bind tubulin. Among these, IgG1κ^{PER} was found to bind tubulin and to display binding with actin, myosin, and ssDNA, whereas IgA1κ^{PER} and IgG4κ^{PER} were found to exclusively bind tubulin. In the present work, we have further studied two of these (IgG1κ^{PER} and IgA1κ^{PER}) and demonstrated that, despite sharing identical variable H and L sequences, these Ab and their Fab fragments bind tubulin with significantly different affinities, suggesting that the C_H1 constant domain may play a role in defining the Ag–Ab affinity.

Methods

Purification of MIg

MIg^{PER} were purified from patient's serum by ammonium sulfate precipitation, followed by ion-exchange chromatography on a DE-52 column (Whatman, Maidstone, UK). Two different IgG of the γ_1 and γ_2 subclasses were eluted from the DE-52 column at the same molarity (0.005 M), and were further isolated by preparative isoelectrofocusing with a Rotafor apparatus (Bio-Rad Laboratories, Richmond, CA). Monomeric and dimeric IgA1κ^{PER} were eluted in 0.03 and 0.05 M fractions, respectively. Purity, homogeneity, and M_r of the IgG1κ^{PER} and IgA1κ^{PER} fractions were then assessed by ELISA and by HPLC, as described (10). The pI of the different MIg was measured by isoelectrofocusing.

Preparation of Fab and F(ab')2 fragments from IgG1κ^{PER} and IgA1κ^{PER}

To obtain purified Fabγ fragments, 3 mg of monoclonal IgG1κ^{PER} were digested for 24 h at 37°C with 50 μg of papain in the presence of the reducing agent cystein. The reaction was terminated by adding crystalline iodoacetamide to a 0.03 M final concentration. Separation of Fab from Fc fragments was assessed by ion-exchange chromatography in a Trisacryl-DEAE column equilibrated with 0.01 M Tris-HCl, pH 8.6, and eluted with a linear gradient from 0 to 0.5 M NaCl in the

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1. *Abbreviations used in this paper:* CDR, complementary determining region; CNBr, cyanogen bromide; FR, framework region; LC/ESMS, liquid chromatography/electrospray mass spectrometry; R, replacement; S, silent.

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starting buffer. The different peaks were analyzed by SDS-PAGE. $F(ab')_2$ fragments were obtained by digesting 3 mg of $IgG1\kappa^{PER}$ for 24 h at 37°C with 50 μg of pepsin in acetate buffer, pH 4.5, followed by a gel filtration chromathography (Superose 12 column; Pharmacia Fine Chemicals, Uppsala, Sweden).

To obtain purified Fabα fragments, 50 mg of monomeric $IgA1\kappa^{PER}$ were digested overnight at 37°C with 100 μg of IgA1-protease (Boehringer Mannheim, Mannheim, Germany) and subsequently purified by DEAE-Sepharose and gel filtration chromatography. $F(ab')_2$ fragments were obtained by digesting 10 mg of monomeric $IgA1\kappa^{PER}$ for 48 h at 37°C with a 100 μg pepsin solution in acetate buffer, pH 4.5, followed by a gel filtration chromatography (Superose 12 column).

Sample preparation for Edman degradation and mass spectrometry analyses

Ig heavy and light chain purification. Ig were denaturated in 6 M guanidine-HCl, 1 mM EDTA, 0.4 M Tris, pH 8.2, and reduced by incubating for 2 h at 37°C with 8 mM dithiothreitol. Cysteine residues were carboxymethylated with 20 mM iodoacetic acid for 1 h at 37°C followed by overnight at 4°C. Separation of heavy and light chains was performed by gel filtration through a Sephadex G-100 column in 1 M acetic acid and 8 M urea. The purified proteins were dialyzed against 0.5% acetic acid and lyophilized.

Cyanogen bromide cleavage. The reduced-alkylated heavy chains were dissolved in 70% trifluoroacetic acid. Cyanogen bromide (CNBr) was added to this solution (CNBr/protein = 50 wt/wt) and the reaction was allowed to proceed at 4°C for 20 h in the dark, and was terminated by addition of 10 vol water before lyophilization.

Enzymic digestion. Lysine endopeptidase digestion of reduced-alkylated entire light chains and of CNBr peptides of reduced-alkylated heavy chains was performed at 37°C in 100 mM Tris-HCl, pH 8.2, 1 mM EDTA, at an enzyme/substrate ratio of 1:50 (wt/wt). The IgG_1 light chain peptide P1-117 was obtained by reverse-phase HPLC of a low molecular weight fraction of the pepsin digest performed for F(ab')2 preparation. Tryptic digestion of P1-117 was performed at 37°C in 100 mM Tris-HCl, pH 8.2, 5 mM $CaCl_2$ at an enzyme/substrate ratio of 1:50 (wt/wt). Lysis times (4–24 h) were adjusted by reverse-phase HPLC study of small aliquots during digestion.

Reverse phase HPLC. Separation of peptides generated by CNBr or enzymic cleavage was carried out using a system (130A; Applied Biosystems, Inc., Foster City, CA). The C8 column (Aquapore RP-300, 100 × 2.1 mm; Applied Biosystems, Inc.) was eluted with a linear gradient of 5–80% acetonitrile (0.1% trifluoroacetic acid) for 45 min at 200 μl/min.

Mass spectrometry

Liquid chromatography/electrospray mass spectrometry (LC/ESMS) was performed using a Sciex AP I/III triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) equipped with an atmospheric pressure ionisation source and a Sciex IonSpray interface. A Carlo Erba (Pheonix 20, Fisons Instruments Laboratory Systems, Altrincham, UK) liquid chromatograph, equipped with a homemade reverse-phase capillary column (Nucleosil C8, 0.25 × 150 mm, fused silica; Macherey Nagel, wDüren, Germany), was coupled directly to the mass spectrometer. Separation of digests were performed with a linear gradient of 5–80% acetonitrile (0.1% trifluoroacetic acid) in 60 min at a flow rate of 5 μl/min. The spectra were recorded in the 500–1,500 range of mass-to-charge ratio using dwell times of 2 ms.

Amino acid sequence determination

Automated Edman degradation was performed using a gas-phase Sequencer (477 A; Applied Biosystems, Inc.) with on-line analysis of the phenylthiohydantoin derivatives. Repetitive yields varied between 90 and 95%, depending on the peptide sequence.

V_H and V_L nucleotide sequence of $IgA1\kappa^{PER}$

cDNA preparation. RNA was isolated from 10^6 PBMC by the guanidinium thiocyanate-phenol-chloroform method (11). cDNA synthesis was performed in a 20-μl reaction volume at 37°C for 60 min using an oligo dT primer 15-mer (Boehringer Mannheim), 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Bethesda, MD), 40 U of RNAse inhibitor (Boehringer Mannheim) and 0.5 mM of each deoxyribonucleotide.

V_H and V_L amplification by PCR. PCR amplification was carried out from 1 μg of single-stranded cDNA using *Taq* DNA polymerase (Perkin-Elmer Cetus Instruments, Emeryville, CA). According to the amino acid sequence the 5′ primer used for V_H gene amplification was designed to anneal to the leader region of the V_H3 family (5′ L_H3 fow: GTA TCG ATG GAG TTT GGG CTG AGC TGG), and an antisense oligonucleotide specific to C_H1 domain of Cα region was used as the 3′ primer (3′ Cα-rev: ATC TGG CTG GGT GCT GCA GAG GCT). The 5′ primer used for $V_\kappa II$ gene amplification was designed to anneal specifically to the framework 1 (FR1) region of this kappa light chain family (5′ $V_\kappa II$-fow: CTA GAG TCG ACG ATA TTG TGA TGA CTC AGT CTC C), and in 3′, the consensus C_κ primer (3′ C_κ-rev: CAG ATG GCG GGA AGA TG) was used. In all cases, 30 cycles of amplification (95°C 1′, 62°C 1′, and 72°C 1′) were carried out.

V_H and V_L cloning and sequencing. Purified PCR fragments were ligated in blunt ends into the SmaI site of pBluescript KS+ phagemide vector. XL-1 blue *Escherichia coli* were transformed and selected in culture medium containing ampicillin. Recombinant plasmids were purified from transformed bacteria and selected by restriction analysis. Nucleotide sequencing was performed by the dideoxy chain termination method (Promega Corp., Madison, WI). All inserts were sequenced from two strands and from three independent clones. Nucleotide sequence data were analyzed and comparisons were carried out with the GCG software package and the GenBank/EMBL/DDBJ database.

Establishing germinal counterparts of H and L chains from $IgA1\kappa^{PER}$ and $IgG1\kappa^{PER}$

V_H3 (V_H germ) and $V_\kappa II$ (V_κ germ) germline nucleotide sequences obtained from autologous genomic DNA of the patient's T cells were compared to the 3-73 (clone MTGL) (12) and $V_\kappa IIA3$ germline genes (13), and to the nucleotide sequence obtained from the cDNA of the patient's peripheral blood B cells expressing $IgA1\kappa^{PER}$. T cells from the patient's PBMC were cultured for 4 wk in the presence of PHA plus IL-2. Genomic DNA was extracted from T lymphocytes of the patient and nonrearranged V_H3 genes were amplified with the same consensus 5′ L_H3 fow and an antisense heptamer-spacer 3′ primer (V_H3-rev: CAC AGT GAG GGG AGG TCA GTG TGG TCG ACC T). The amplified products were cloned and sequenced as described (11). Identification of the germline counterpart was achieved using a specific CDR2 probe (ACG CGA CAG CAT ATG CTG CGT CG). $V_\kappa II$ nonrearranged genes were amplified using the same consensus 5′ $V_\kappa II$ family primer and a 3′ $V_\kappa II$ heptamer-spacer-nonamer reverse primer ($V_\kappa II$-rev: GTA TCG ATG TTT CTG TTA GGG GTT GTA CCA CTG TG). Amplified fragments were cloned, selected by a specific reverse 3′$V_\kappa II$-FR3 probe (TGT AAA ATC TGT GCC TGA TCC ACT) and sequenced.

Affinity and kinetic constants determination by surface plasmon resonance (14)

Equilibrium and kinetic constants for the interactions between MIg and tubulin were determined by the BIAcore™ system (Pharmacia Biosensor AB, Uppsala, Sweden). Immobilization of tubulin via primary amines to the Sensorchip CM5 was performed using amine coupling kit (Pharmacia Biosensor AB). Activation of the carboxymethylated dextran matrix was obtained by injecting a mixture of *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide.

Tubulin was prepared at a concentration of 25 μg/ml in 100 μl of a 10 mM acetate buffer, pH 4.1. Deactivation of remaining esters was obtained by injecting a solution of ethanolamine-HCl. In all runs, PBS was used as driving eluent. 100 μl of different mAb fractions at the following concentrations: $IgA1\kappa^{PER}$, 135, 13.5, and 1.35 nM;

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Table I. Equilibrium and Kinetic Constants for MIg^{PER} Binding to Tubulin

	k_{DISS} (s^{-1})	k_{ASS} (M^{-1} s^{-1})	K_D (M)
$IgA1\kappa^{PER}$	4.19×10^{-4}	1.47×10^{4}	2.85×10^{-8}
$F(ab')2$ $IgA1\kappa^{PER}$	4.64×10^{-4}	1.41×10^{4}	3.29×10^{-8}
Fab $IgA1\kappa^{PER}$	7.01×10^{-4}	1.16×10^{4}	6.04×10^{-8}
$IgG1\kappa^{PER}$	1.59×10^{-3}	1.56×10^{3}	1.02×10^{-6}
$F(ab')2$ $IgG1\kappa^{PER}$	1.51×10^{-3}	*	—
Fab $IgG1\kappa^{PER}$	2.20×10^{-3}	*	—

Affinity and kinetic constants determination by surface plasmon resonance. *Association rate constants for $IgG1\kappa^{PER}$ F(ab')2 and Fab fragments were too slow and thus could not be calculated.

$IgG1\kappa^{PER}$, 10 and 2.5 μM, and 500 and 100 nM; F(ab')2 and Fab $IgA1\kappa^{PER}$, 1 μM and 100 nM; F(ab')2 and Fab $IgG1\kappa^{PER}$, 2.3 and 1 μM were allowed to bind to immobilized tubulin at a flow of 5 μl/min, and binding events as well as the dissociation in buffer flow were recorded in sensorgrams at 20°C. The amount of Ab bound (R_{max}) to the tubulin as well as the reaction rate (d*R*/d*t*) were calculated using the Bialogue™ software. Kinetic rate constants (k_{ASS} and k_{DISS}) as well as apparent equilibrium affinity constants ($K_D = k_{DISS}/k_{ASS}$) were determined using Bialogue™ Kinetics Evaluation software (Table I). Background binding was determined by analyzing the sensorgram of the dextran surface–MIg interaction. Surface reconstitution was performed by eluting with a 1 M NaCl solution.

To investigate the role of temperature on association and dissociation of $IgA1\kappa^{PER}$ with tubulin, binding assays in the same above described conditions were carried out at 15, 20, 28, 31, and 37°C.

Measurement of the affinity constant in solution by ELISA (15)

Various concentrations of tubulin were individually incubated in solution with $IgA1\kappa^{PER}$ and $IgG1\kappa^{PER}$ at constant concentration until equilibrium was reached (15 h at 20°C). The concentration of free Ab was then measured by an indirect ELISA after incubating at 20°C for 15 min (incubation time was determined to avoid equilibrium displacement). The correlation between enzymatic activity and the concentration of free Ab in solution was first established by adding various known amounts of Ab in coated wells, under experimental conditions identical to those used in the binding experiment. This calibration allows the determination of the free Ab concentration at

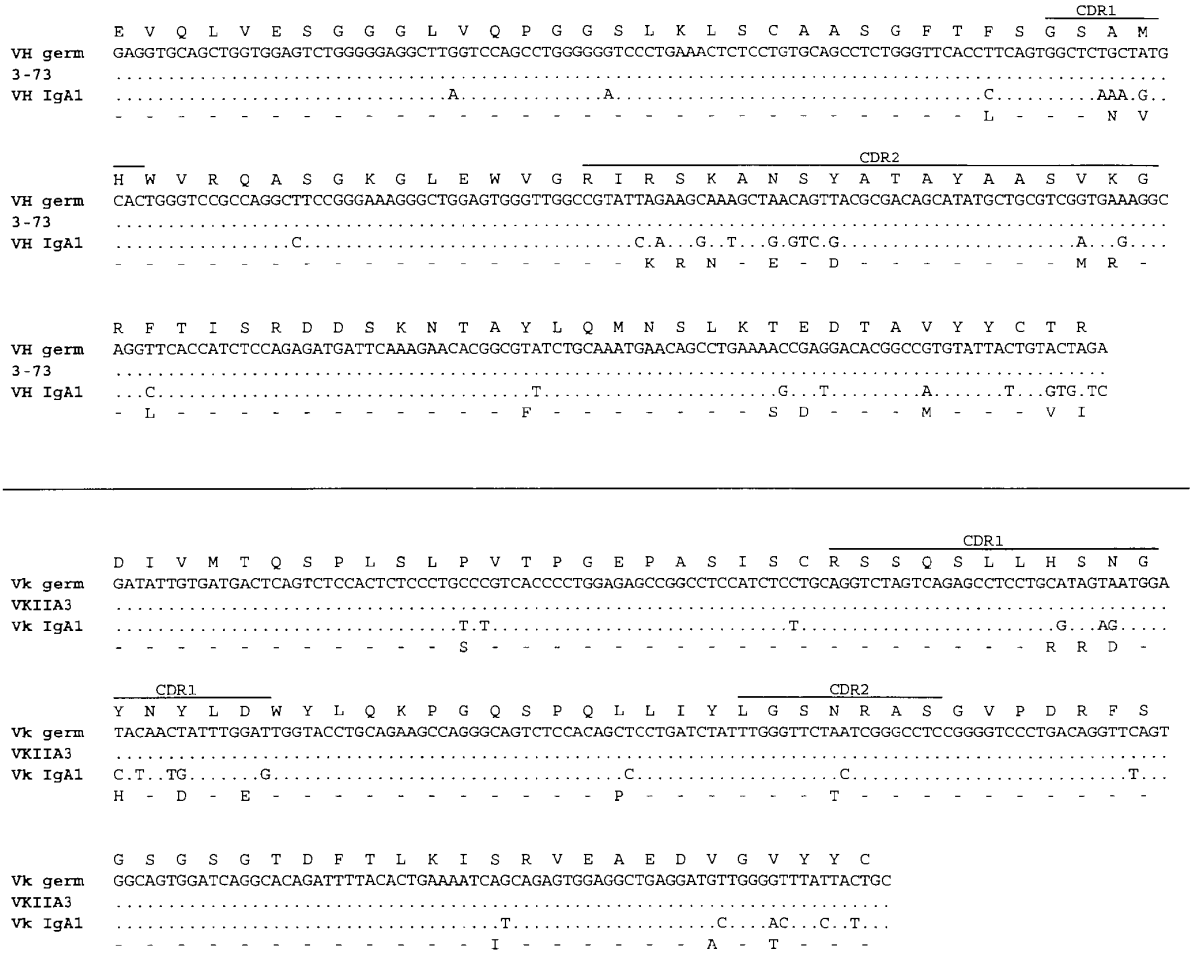


Figure 1. Determination of V_H and V_L nucleotide sequence and germline counterpart of $IgA1\kappa^{PER}$. Amino acid and nucleotide sequences of V_H and V_L segments from $IgA1\kappa^{PER}$ and their germinal V_H and V_L counterparts (V_H and V_κ *germ*), compared with the most closely matching germline genes (*3-73* and *VKIIA3*). Nucleotide sequence and amino acid identity are respectively indicated by dots and dashes. Replacements are depicted by bottom case letters. CDR1 and CDR2 domains are indicated.

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equilibrium, provided that the total Ab concentration is known. The affinity constant was determined by Scatchard plot analysis.

Results

Determination of V_H and V_L nucleotide sequence and germline counterpart of IgA1κ^{PER}. In agreement with cytometric results showing that almost all peripheral blood cells from patient PER were expressing IgAκ (10), PCR amplification products could only be obtained for this MIg. In a first step, the cDNA nucleotide sequences encoding the heavy and light chain V domains of IgA1κ^{PER} were determined. This MIg expressed a V_H3 family member gene (Fig. 1) associated to a DXP'1 D segment and to a JH4 segment (Fig. 2) and a $V_κII$ family member gene rearranged to a $J_κ5$ (Figs. 1 and 2).

To evaluate the importance of the somatic mutation process in the generation of these autoantibodies and to avoid misinterpretation related to V gene polymorphisms, we have determined the V_H and V_L germline counterparts of the MIgs.

Fig. 1 shows the germline nucleotide V_H and V_L sequences obtained from autologous T cell genomic DNA. By parsimony, it was determined that the H chain variable domain most likely derived from the V_H3 gene segment 3–73 (12), and the L chain variable domain from the $V_κII$ A3 gene segment (reference 13 and Fig. 1). In the heavy chain, there were eight and four codons that respectively underwent replacement (R) and silent (S) mutations in the frameworks (R:S ratio of 2), and there were nine replacement and three silent mutations in CDRs 1 and 2 (R:S ratio of 3). Similarly, in the light chain, there were five and four codons that respectively underwent replacement and silent mutations in the frameworks (R:S ratio of 1.25), and there were seven replacement mutations and only one silent mutation in CDRs 1 and 2 (R:S ratio of 7.0). Mutations and replacements predominated in CDR of both H and L chains and induced a significant hydrophobicity shift in these domains (data not shown). Interestingly, in the middle of the H chain CDR2 occurs an unmutated region displaying a palindromic sequence (CAGCATATGCTG) with a potential stem-loop structure. Similar structures have been found in other V region genes and could act as entry sites for a repair mechanism favoring local mutation (16).

Somatic mutations resulting in multiple replacement mutations in the CDRs with preservation of the peptide sequence of the frameworks are typical of Abs that have undergone selection by Ag. In addition, mutations in FRs predominated in the FR3 domain of both H and L chains, a region that may itself contribute to Ag recognition (17).

Amino acid sequence of the IgG1κ^{PER} light chain. Comparison with the IgA1κ^{PER} light chain sequence. IgG1κ^{PER} and IgA1κ^{PER} light chains were prepared in the carboxymethylated form by Sephadex G-100 gel filtration (not shown). Their lysine endopeptidase digests were compared by reverse-phase HPLC. Interestingly, the two chromatographic profiles (Fig. 3) showed identical retention times for all the peptides. Sequencing of the separated lysine endopeptidase peptides of the IgG1^{PER} light chain was performed and permitted the determination of about 80% of the aminoacids (Fig. 4).

A peptide (P1-117) was isolated from a pepsin digest of the unreduced IgG1κ^{PER} molecule and subdigested with trypsin before separation by reverse-phase HPLC (not shown). Edman degradation of the purified peptides of P1-117 permitted the completion of the amino acid sequence of the variable region (Fig. 4).

Finally, except for residues 189–195 and 213–219 (underlined in Fig. 4 and taken from Kabat et al., reference 1) of the constant region, the almost complete amino acid sequence of the IgG1κ^{PER} light chain was determined by Edman degradation. The NH_2-terminal part of this sequence, including all the variable domain, showed a perfect identity with the determined part of the IgA1κ^{PER} light chain (Figs. 1, 2, and 4). In addition, NH_2-terminal amino acid sequencing of IgA1κ^{PER} light chain was performed (data not shown). The determined sequence (residues 1–24) covered the region corresponding to the FR1 primer (residues 1–8) and was identical to the deduced sequence.

Mass spectrometry analyses (LC/ESMS) of the carboxymethylated entire IgG1κ^{PER} and IgA1κ^{PER} light chains, of their lysine endopeptidase digests, and of the tryptic digest of IgG1-117, were performed. The expected mass of the carboxymethylated IgG1 light chain, calculated from its amino acid sequence, is 24,275.0 D. The measured masses of the IgG1κ^{PER} and IgA1κ^{PER} carboxymethylated light chains are 24,272±5 and 24,271±5D, respectively (not shown). The measured mass of all the peptides issued from the IgG1κ^{PER} light chain digests corresponded well with their expected masses (Fig. 4). Finally, the measured masses (not shown) of the peptides issued from the lysine endopeptidase digest of the IgA1κ^{PER} light chain corresponded exactly with the masses of the corresponding

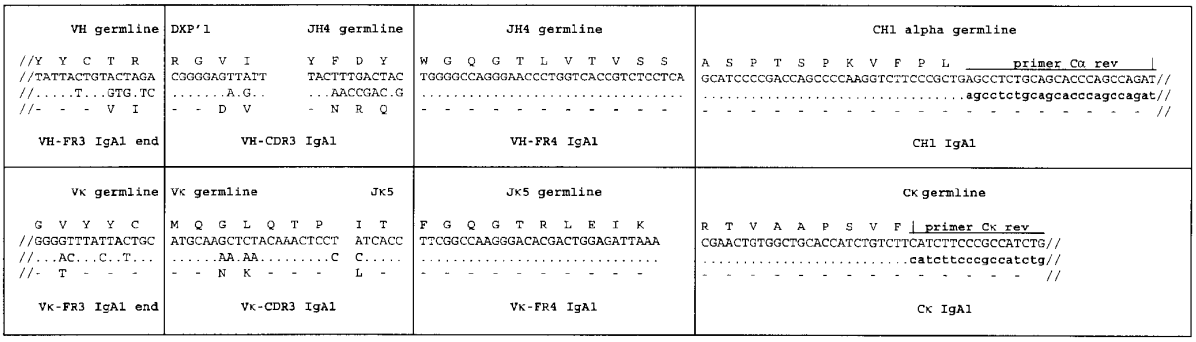


Figure 2. CDR3 and FR4 regions from IgA1κ^{PER} H and L chains. Amino acid and nucleotide sequence of H and L CDR3 regions are compared with the closest germinal counterpart. Replacements are depicted by bottom case letters. $C_α$ and $C_κ$ reverse primers are indicated by lower case letters.

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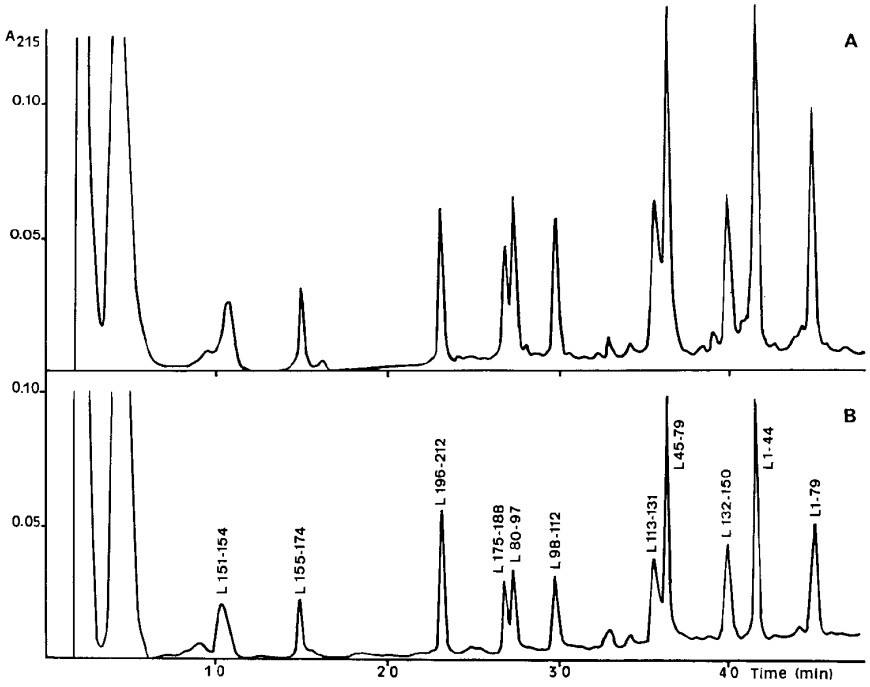


Figure 3. Reverse phase HPLC separation of peptides generated by lysine endopeptidase digestion. (*A*) IgA1κ^{PER} and (*B*) IgG1κ^{PER} light carboxymethylated chains. The peptides (Lx-x) were identified by Edman degradation and mass spectrometry (see Fig. 4).

peptides issued from the IgG1κ^{PER} light chain. Mass spectroscopy analyses excluded the possibility of amino acid insertions in the junctions between identified peptides.

Hence, these results unambiguously prove the complete identity of the IgG1κ^{PER} and IgA1κ^{PER} kappa light chains.

Amino acid sequence of the NH_2-terminal part of the IgG1κ^{PER} heavy chain. Comparison with the IgA1κ^{PER} heavy chain sequence. Carboxymethylated IgG1κ^{PER} and IgA1κ^{PER} heavy chains purified by Sephadex G-100 gel filtration were submitted to CNBr cleavage and reverse-phase HPLC (not shown). The chromatographic profiles of the two digests were different except for a few fractions. Compared mass spectrometry (LC/ESMS) analysis of the two heavy chain digests showed identity of the measured masses for three well defined peptides, showing identical retention times (data not shown). The three peptides isolated by HPLC chromatography of the IgG1κ^{PER} digest were submitted to Edman degradation. These studies permitted determination of the partial or complete sequence of these three socalled peptides CB1-66, CB67-85, and CB67-95. The NH_2-terminal sequence of a high mass peptide (CB96. . .) was also established (Fig. 5). All these determined amino acid sequences of the IgG1κ^{PER} heavy chain corresponded exactly with sequences of the variable region of the IgA1κ^{PER} heavy chain (Figs. 1 and 2).

Peptide CB1-66, and higher mass fractions of the IgG1 heavy chain CNBr digest, were submitted to lysine endopeptidase cleavage, Edman degradation, and mass spectrometry analyses. A set of peptides permitted to define the complete sequence of the variable region (amino acids 1–119) and to determine a large part of the CH1 constant domain (Fig. 5). The peptides L90-123, L150-207, and L150-212 were only partially sequenced, but their measured masses corresponded well with their expected masses deduced from partial sequencing and from the data of Kabat et al. (1). Mass spectroscopy analyses excluded the possibility of amino acid insertions in the junctions between identified peptides. The mass of the so called peptides CB96. . .could not be measured, but its NH_2-terminal sequence (amino acids 96–120) was confirmed and completed by analysis of smaller peptides.

Overall, comparison of the complete IgG1κ^{PER} heavy chain NH_2-terminal sequence (Fig. 5) with the corresponding IgA1κ^{PER} heavy chain sequence (Figs. 1 and 2) proves the complete identity of the variable regions and shows the expected differences in the CH1 constant domain.

In a previous work (10), a difference was reported in position 6 of the NH_2-terminal amino acid sequence of the heavy of the four clonally related MIg, including the two compared in this work. Careful reexamination (data not shown) of these NH_2-terminal sequences was performed and unambiguously showed that the amino acid residue in position 6 was always a glutamic acid (*E*) and never a glutamine (*Q*).

Affinity and kinetic constant determination against tubulin for IgA1κ^{PER} and IgG1κ^{PER}. In a previous study, both MIg^{PER} were found to bind tubulin (10), even after dissociation and cross-reassociation of H and L chains. In addition, after limited enzymatic proteolysis of tubulin, both MIg^{PER} were shown to recognize overlapping epitopes in the carboxy-terminal region of both α and β tubulin subunits, with minor differences in the specificity. However, in spite of sharing identical variable domain sequences, having identical light chain constant domains and recognizing overlapping epitopes on the Ag, both Ab significantly differed in the K_D for tubulin as determined by measuring surface plasmon resonance (2.85×10^{-8} M for IgA1κ^{PER} and 1.02×10^{-6} M for IgG1κ^{PER}) (Table I) or by Friguet's assay (6.9×10^{-9} M for IgA1κ^{PER} and 5.0×10^{-6} M for IgG1κ^{PER}). To determine whether these differences were due

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         10        20        30        40        50        60
DIVMTQSPLSLSVTPGEPASISBRSSQSLLRRDGHNDLEWYLQKPGQSPQPLIYLGSTRA
         70        80        90       100       110       120
SGVPDRFSGSGSGTDFTLKIIRVEAEDAGTYYBMQNKQTPLTFGQGTRLEIKRTVAAPSV
        130       140       150       160       170       180
FIFPPSDEQLKSGTASVVBLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL
        190       200       210
SSTLTLSKADYEKHKVYABEVTHQGLSSPVTKSFNRGEB
```

Fragment	Sequence (Edman degradation)	Expected mass (Da)	Measured mass (Da)
L1-44	DIVMTQSPLSLSVTPGEPASISBR---	inc.seq.	4974.0
L45-79	PGQSPQPLIYLGSTRASGVPDRFSGS---	inc.seq.	3582.0
L80-97	IIRVEAEDAGTYYBMQNK	2162.44	2162.5
L98-112	QTPLTFGQGTRLEIK	1688.96	1689.0
L113-131	RTVAAPSVFIFPPSDEQLK	2102.43	2102.0
L132-150	SGTASVVBLLNNFYPREAK	2127.41	2128.2
L151-154	VQWK	559.68	559.5
L155-174	VDNALQSGNSQESVTEQDSK	2136.18	2136.0
L175-188	DSTYSLSSTLTLSK	1502.65	1502.2
L196-212	VYABEVTHQGLSSPVTK	1877.11	1876.8
P1-117	DIVMTQSPLSLSVTPGEPA---	inc.seq.	12735.0
T1- 24 / T80-97	DIVMTQSPLSLSVTPGEPASISCR / IIRVEAEDAGTYYCMQNK	4591.28	4591.2
T1- 24 / T83-97	DIVMTQSPLSLSVTPGEPASISCR / VEAEDAGTYYCMQNK	4208.78	4208.4
T25-31	SSQSLLR	789.90	789.5
T25-32	SSQSLLRR	946.08	n.obs.
T32-59	RDGHNDLEWYLQKPGQSPQPLIYLGSTR	3269.64	3269.4
T33-59	DGHNDLEWYLQKPGQSPQPLIYLGSTR	3113.45	3113.1
T60-79	ASGVPDRFSGSGSGTDFTLK	1986.13	1985.5
T67-79	FSGSGSGTDFTLK	1303.40	1302.0
T98-108	QTPLTFGQGTR	1205.35	1205.0
T109-112	LEIK	501.63	n.obs.
T109-113	LEIKR	657.82	657.5

Figure 4. Amino acid sequence of the IgG1κ^{PER} light chain determined by Edman degradation and mass analyses (LC/ESMS). Lx-x, lysine endopeptidase peptides obtained from the reduced-alkylated entire light chain; B, carboxymethylated cysteine; P1-117, NH_2-terminal peptide of the light chain purified from a pepsin digest of the unreduced IgG1 molecule; Tx-x, tryptic peptides derived from P1-117; inc. seq., incompletely sequenced peptide; n. obs., nonobserved peptide. Underlined amino acid residues 189–195 and 213–219 have not been determined in this work and are from Kabat et al. (1). The expected masses of peptides L1-44, L45-79, and P1-117 could be calculated after analysis of the tryptic digest and are 4,974.57, 3,581.96, and 12,734.34 D, respectively.

to interactions between the Fc portions of the Ab, F(ab')2 and Fab fragments were prepared (Fig. 6). The relative differences in the affinity constants between IgG1κ^{PER} and IgA1κ^{PER} were preserved at the Fab level (Table I). These values were of 3.29×10^{-8} M for F(ab')2 and 6.04×10^{-8} M for Fab from IgA1κ^{PER}, but could not be calculated for IgG1κ^{PER} fragments since association rates were too slow to be measured, even after running the sample for 120 min. To obtain measurable K_D in these conditions would require an exposition time of several hours (18). Thus, this difference in affinity could be attributed to the C_H1 domain.

Kinetic analysis demonstrated that this difference in affinity was mainly accounted for by a disparity in the association rate constants (1.47×10^4 M^{-1} s^{-1} for IgA1κ^{PER} and 1.56×10^3 M^{-1} s^{-1} for IgG1κ^{PER}), with only slight differences in the dissociation rate constants (4.19×10^{-4} s^{-1} for IgA1κ^{PER} and 1.59×10^{-3} s^{-1} for IgG1κ^{PER}, Table I). The differences in the association rate constants would suggest however, that the pathway leading to the formation of a stable complex might differ. We analyzed the effect of changing the temperature on complex formation of IgA1κ^{PER} and tubulin (Table II and Fig. 7). The amount of Ab bound at equilibrium decreased with increasing temperature from 15 to 37°C. Although the dissociation rate steadily increased across this temperature range, the association rate decreased. In addition, similar results were observed with the Fab fragment from IgA, where the association constant at 37°C was 5.72×10^3 M^{-1} s^{-1} as compared with 1.16×10^4 M^{-1} s^{-1} at 20°C and the dissociation rate constants of 7.89×10^{-3} s^{-1} (7.01×10^{-4} s^{-1} at 20°C). Similar changes were observed in the association process for Fab and complete IgA. However, a more rapid dissociation could be observed for the monovalent Fab. It remains unclear whether these differences could be related to the bivalency of the complete IgA.

Discussion

Our results show that two different Igs unambiguously sharing identical V_H and V_L domains, but expressing different heavy constant regions bind tubulin with significant differences in affinity. The observation that even Fab fragments from these Ab reproduce differences in binding suggests that structural differences in the C_H1 domain underlie the differences in the affinity constants, and thus the differences in Ag binding.

As for affinity studies, a number of data are now available

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        10         20         30         40         50         60
EVQLVESGGGLVQPGGSLKLSBAASGFTLSGSNVHWVRQASGKGLEWVGRIKRNAESDAT
        70         80         90        100        110        120
AYAASMRGRLTISRDDSKNTAFLQMNSLKSDDTAMYYBVIRGDVYNRQWGQGTLVTVSSA
       130        140        150        160        170        180
STKGPSVFPLAPSSKSTSGGTAALGBLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
       190        200        210
LYSLSSVVTVPSSSLGTQTYIBNVNHKPSNTK
```

Fragment	Sequence (Edman degradation)	Expected mass (Da)	Measured mass (Da)
CB1-66	EVQLVESGGGLVQPGGSLKLSBAASGFTL---	inc.seq.	6788.0
CB67-85	RGRLTISRDDSKNTAFLQm'	2161.42	2161.0
CB67-95	RGRLTISRDDSKNTAFLQ(MO)NSLKSDDTAm'	3240.58	3240.0
CB96...	YYBVIRGDVYNRQWGQGTLVTVSSA---	inc.seq.	n.obs.
L1-19	EVQLVESGGGLVQPGGSLK	1854.10	1854.0
L20-43	LSBAASGFTLSGSNVHWVRQASGK	2521.80	2521.5
L44-52	GLEWVGRIK	1057.27	1057.0
L53-78	RNAESDATAYAAS(MO)RGRLTISRDDSK	2859.10	2859.0
L79-89	NTAFLQ(MO)NSLK	1282.49	1282.0
L90-123	SDDTA(MO)YYBVIRGDVYNRQWGQGTLVTV---	3847.22*	3847.0
L124-135	GPSVFPLAPSSK	1186.38	1186.0
L136-149	STSGGTAALGBLVK	1322.51	1322.0
L150-207	DYFPEPVTVSWNSGALTSGVHTFPAVLQ---	6189.88*	6190.0
L150-212	DYFPEPVTVSWNSGALTSGVHTFPAVLQ---	6717.45*	6717.0

Figure 5. Amino acid sequence of the NH_2-terminal region of the IgG1κ^{PER} heavy chain determined by Edman degradation and mass analyses (LC/ESMS). CBx-x, CNBr peptides obtained from the reduced-alkylated heavy chain; Lx-x, lysine endopeptidase peptides obtained by digestion of CNBr peptides; m′, homoserine lactone; (MO), methionine sulfoxide; B, carboxymethylated cysteine; inc. seq., incompletely sequenced peptide; n. obs., nonobserved peptide. Underlined amino acid residues 121–123 and 178–212 have not been determined in this work and are from Kabat et al. (1). *Expected masses calculated from the sequences partially determined by Edman degradation and completed by the data from Kabat et al. (1). The expected mass of peptides CB1-66 could be calculated after analysis of the lysine endopeptidase digest and is 6,788.55 D.

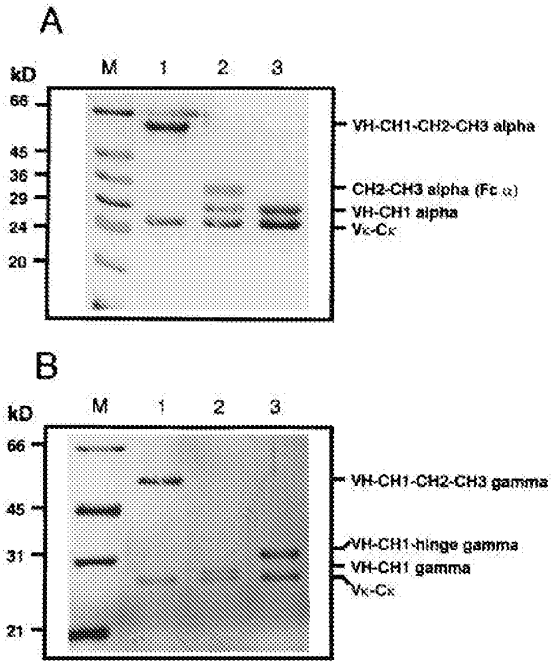


Figure 6. SDS-PAGE of reduced purified Ig and Fab fragments from IgA1κ^{PER} and IgG1κ^{PER}. (*A*) Lane *1*, monomeric IgA1κ^{PER}; lane *2*, after digestion with IgA1 protease; and lane *3*, purified Fab after ion-exchange chromatography. (*B*) Lane *1*, IgG1κ^{PER}; lane *2*, purified Fab; and lane *3*, purified (Fab′)2.

on kinetic measurements of antigen-antibody interactions assessed by surface plasmon resonance (8, 19–22). These studies show that equilibrium constants of these interactions vary from 10^6 to 10^{11} with k_{ass} values ranging from 10^3 to 10^6 and k_{diss} ranging from 10^{-3} to 10^{-5}. Our results for IgA1κ^{PER}-tubulin binding are consistent with a conventional Ag–Ab interaction.

In a previous work (23), we studied 135 different IgA MIgs. The pattern of binding to tubulin of this particular IgA1κ^{PER} was unique, thus excluding the possibility that the interaction between Ag and Ab occurred via a particular interaction between a C_H1 domain and tubulin or via a superantigen-like mechanism.

The smooth temperature profile of the dissociation rates for IgA suggests that dissociation is occurring from the same stable complex within the temperature range studied. In con-

Table II. Equilibrium and Kinetic Constants for IgA1κ^{PER} Binding to Tubulin as a Function of Temperature

	k_{DISS} (s^{-1})	k_{ASS} ($M^{-1}s^{-1}$)	K_D (M)
15°C	3.94×10^{-4}	1.70×10^4	2.32×10^{-8}
20°C	4.19×10^{-4}	1.47×10^4	2.85×10^{-8}
28°C	4.51×10^{-4}	1.25×10^4	3.61×10^{-8}
31°C	3.94×10^{-4}	1.31×10^4	3.01×10^{-8}
37°C	7.84×10^{-4}	6.01×10^3	1.30×10^{-7}

Temperature dependence of IgA1κ^{PER} binding constants. Affinity measurements were carried out at 15, 20, 28, 31, and 37°C. Dissociation rates increase with increasing temperature, whereas the reverse occurs for association rate constants.

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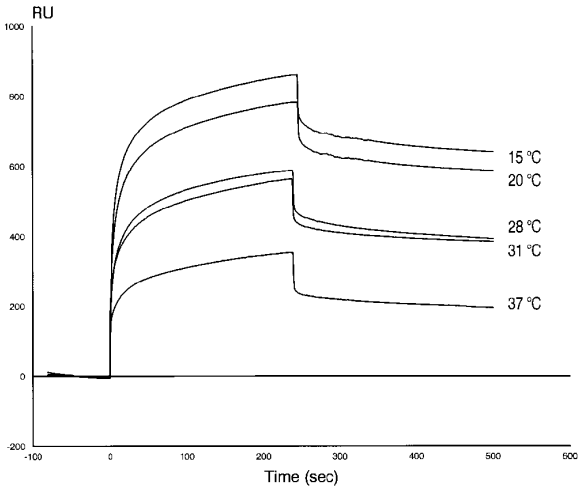


Figure 7. Role of temperature on association and dissociation of IgAκ^{PER} with tubulin. Sensorgrams obtained with IgAκ^{PER}, when kinetic measurements were carried out at 15, 20, 28, 31, and 37°C. Results are expressed as Resonance units (*RU*) as a time (sec) function.

trast, there is an unusual change seen in the association rate as a function of temperature. At 37°C there is weak discrimination in favor of the stable complex, whereas at low temperature a stable complex is favored. There is a change in a rate-limiting step on a sequential pathway leading to this stable complex. Differences in the C_H1 domain could lead to the formation of different structural isomers that attenuate the rates at which the variable domains undergo the necessary conformational changes required for formation of a stable Ag–Ab complex. No measurable changes in the weak association rates for IgG1κ^{PER} with tubulin as a function of temperature could be obtained. This suggests that at high temperature the IgA1κ^{PER}-tubulin reaction approaches IgG1κ^{PER}-tubulin reaction and that the formation of the final stable complex in both cases may be impeded by some conformational rearrangement subsequent to initial binding. Thus, these results suggest that the same antigen-binding site displays different specificities when juxtaposed with different heavy chains. This could explain the differences in binding of these two MIg (discrete polyreactive binding including tubulin for IgG1κ^{PER}, exclusive binding to tubulin for IgA1κ^{PER}) (10).

It has long been assumed that the constant domains do not play a role in the maturation of Ag–Ab interactions. Indeed, Fabs are pseudosymetric dimers where V_H contacts with C_H1, whereas no contact is observed between V_L and C_L (24). Although crystallographic studies have shown that amino acids ensuring the link between Fv and C_H1 and C_L are flexible and allow spatial arrangements facilitating binding of epitopes by Fabs, there is controversy concerning the possiblity that elbow bending could facilitate signal transduction from the Ag-binding site to the Fc domain (25–28). However, recent work has demonstrated that changes in the C_H domains can affect the functional affinity and specificity of Abs, through a complex mechanism involving Ab flexibility and cooperative interactions in the case of Abs bound to multivalent Ags (8, 9). Schneider et al. (29) showed that segmental flexibility is controlled by the C_H1 domain and hinge regions of Abs, and Horgan et al. (30) reported that removal of the hinge region of an IgG4 resulted in a gain of relative affinity that approximated that observed with the same Ab expressing an IgG1 isotype. As concerns functional activity of Abs, Cavacini et al. (31) found that Ab isotype can strongly influence virion neutralization of the HIV-1 virus. Although the association of C_L and C_H1 necessarily increases the reach of the Ab and the probability of an appropriate quaternary interaction between V_L and V_H (32, 33), Bhat et al. (34) demonstrated that Fv and Fab fragments make very similar contacts with the Ag. However, Eigenbrot et al. (35) found contact differences in Fv and Fab fragments from the same Ab and Takahashi et al. (36) have shown that constant domains can affect the fluctuation of V_L domain. One difficulty in substantiating this by standard crystallography may arise from the fact that the role of molecular mobile components in the interaction with another molecule may not be fully captured by this method (27). This may emphasize the importance of kinetic studies on the elucidation of structural problems by allowing us to study the nature of transitions characterizing the formation of a physiologically relevant interaction (37).

It is presently unclear whether this observation could refer to an unusual mechanism and further work in different established Ag–Ab systems is warranted to establish how often this mechanism is employed by the immune system. Whatever the frequency with which this phenomenon could occur, these results favor the view that affinity maturation of this particular tumoral clone could be achieved through class switching in the absence of further somatic mutations, probably because the C_H1 domain plays a role in structuring the Ag-binding site into a more kinetically competent form.

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Can isotype switch modulate antigen-binding affinity and influence clonal selection?

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Four different monoclonal Ig (MIg) (IgA1κ, IgG1κ, IgG2κ and IgG4κ) displaying anti-tubulin activity were detected in the serum from a lymphoma patient. The complete sequence of three of these MIg showed identical V_H and V_L domains and the presence of mutations compatible with an antigen-driven process. Surprisingly, despite complete homology in their variable domains, IgA1κ, IgG1κ, or their Fab fragments bound to a common motif recognized in β tubulin, with significant differences in affinity (IgA1κ 1.52×10^{-8} M, and IgG1κ 2.09×10^{-7} M). To substantiate these results, the V_H and V_L domains from IgA1κ were cloned and introduced into expression vectors containing the constant κ exon and either the μ or the γ1 constant exon, and complete recombinant IgMκ and IgG1κ were obtained. Like the IgA1κ, the IgMκ construction bound to the tubulin epitope with consistent affinity (7.7×10^{-9} M), whereas the IgG1κ construction displayed a significantly lower affinity (3.28×10^{-7} M). These results provide definitive evidence that isotype can influence binding affinity to antigen and suggest that malignant transformation occurred at the germinal center once the mutational process was achieved and the switch process was still active.

Key words: Isotype switch / Antigen-driven selection / Anti-tubulin activity / Immunocytic lymphoma / Multiple monoclonal Ig

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1 Introduction

Immunoglobulins (Ig) are heterodimeric proteins composed of two heavy (H) and two light (L) chains. Each H and L chain contains a variable domain, which defines the antigen-binding site of the antibody, and a constant domain, which is assumed to play effector functions without intervening in the antigen-binding specificity of the antibody. Multisequence comparisons of variable domains have shown that each variable domain contains three regions of extensive sequence variability, termed complementary determining regions (CDR), and four regions of relative sequence stability, termed framework regions (FR). The three L chain CDR and the three H chain CDR are juxtaposed to form the antigen-binding site of the antibody, as classically defined. In turn the FR create a scaffold that surrounds, supports, and influences the conformation and structure of the CDR [1–4].

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Abbreviations: MIg: Monoclonal Ig **CDR:** Complementary determining regions **FR:** Framework regions

There are five major classes of H constant domains in a mammalian genome (μ, δ, γ, ε and α). B cells are endowed with the ability to switch from one set of constant domains to another [1]. This class switch allows the B cell to change the effector function of the antibody that it produces, while maintaining the antigen specificity of the original antibody [5]. The sequence and structure of the antigen-binding site can be altered through a process of somatic hypermutation that is targeted at the V domain, leading to a change in the binding affinity and specificity of the antibody [2, 3, 6, 7]. Both processes, class switching and somatic hypermutation of the variable domains, occur in the germinal center, where B cells undergo antigen selection and affinity maturation of their antibodies. However, except antibody multimerization leading to increased functional affinity, it is usually assumed that the constant domains do not play a role in the maturation of antigen-antibody interactions.

Molecular studies of Ig gene rearrangements in B cell tumors provides better understanding of the malignant transformation process, by allowing definition of the site where malignant transformation has occurred and assessing the role played by antigenic stimulation. We

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have previously described [8, 9] the presence of four different clonally related monoclonal Ig (Mig) with anti-tubulin activity (IgA1κ^{PER}, IgG1κ^{PER}, IgG2κ^{PER} and IgG4κ^{PER}) in a single individual suffering from an immunocytic lymphoma, and shown that two of these human monoclonal Ig (IgA1κ and IgG1κ), sharing identical variable H and L sequences but expressing different isotypes, bound tubulin with significantly different affinities (2.85×10^{-8} M for IgA1κ^{PER} and 1.02×10^{-6} M for IgG1κ^{PER}) [9]. Since this difference was also observed at the Fab level, we have hypothesized that the C_H1 domain could play a role in modulating the binding affinity of these antibodies. To provide definitive evidence of this phenomenon in the present work, we have: (1) cloned the V_H and V_L domains of the IgA1 and transfected them into expression vectors where the V_H domain was associated with the human γ1 or μ isotypes and the V_L domain with the human κ constant domain; (2) expressed complete recombinant IgMκ and Igκ antibodies by transfecting V_H and V_L constructions into the X-63 myeloma line, (3) mapped a C-terminal epitope recognized by these antibodies in β tubulin, and (4) measured affinity constants of the interaction between these molecules and the synthetic peptide containing the epitope. Our results provide definitive evidence that the C_H1 domain can play an important role in the antigen-antibody interaction, and suggest that malignant transformation of this clone probably occurred at the level of the germinal center at a moment when the somatic mutation process was achieved and the switch process was still active. They also provide original evidence for an unusual antigen-selection mechanism, since predominance of the α1 clone in this patient is probably explained by the C_H1 domain playing a role in structuring the antigen-binding site into a more kinetically competent form.

2 Results

2.1 Characteristics of the four Mig present in serum PER

The total quantity of IgA1 in serum of the patient (PER) was 40 mg/ml (30 mg of dimer and 10 mg of monomer), whereas total IgG was 13.8 mg/ml (roughly 5.7 mg IgG1^{PER}, 5.7 mg IgG2^{PER} and 2.4 mg IgG4^{PER}). Isoelectric points of pH 8.9, 7.9 and 6.5 were found for IgG1^{PER}, IgG2^{PER} and IgG4^{PER}, respectively, whereas for both IgA1^{PER} monomer and dimer this value was of 4.9. In addition, the previously described murine monoclonal IgG1κ anti-idiotypic antibody 3.2/9B11 [8], was found to bind a private idiotope located in the κ chains of the four Mig (Table 1). We have previously reported that IgA1κ^{PER} and IgG1κ^{PER} bound to tubulin with significantly different affinities (2.85×10^{-8} M for IgA1κ^{PER}, 1.02×10^{-6} M for IgG1κ) when studied by surface plasmon resonance measurements. Interestingly, despite this difference in the binding to tubulin, affinity studies of IgA1κ^{PER} and IgG1κ^{PER} against anti-idiotypic antibody 3.2/9B11 carried out by plasmon resonance did not show significant differences between these two antibodies. Dissociation constants were $K_D = 0.99\times10^{-9}$ M ($k_{ASS} = 2.60\times10^{5}$ $M^{-1}s^{-1}$ and $k_{DISS} = 2.57\times10^{-4}$ s^{-1}) for IgA1κ^{PER}, and $K_D = 1.09\times10^{-9}$ M ($k_{ASS} = 3.11\times10^{5}$ $M^{-1}s^{-1}$ and $k_{DISS} = 3.39\times10^{-4}$ s^{-1}) for IgG1κ^{PER}.

Amino acid and nucleotide sequences were determined for IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}, and were found to be identical at the variable regions (Fig. 1; Gene Bank accession nos: AF184762; AF184763; AF184764; AF184765; AF184766; AF184767). In addition, when compared to their germinal counterparts they displayed numerous somatic mutations, predominating in CDR regions and leading to an important change in the hydropathicity profile, compatible with an antigen-driven process (Fig. 2A, B).

Table 1. Characteristics of Mig from patient PER

Mig	Molecular mass (kDa)	Serum level mg/ml	Ig (H+L) (pI)	L chains (pI)	Binding to anti-id 9B11
IgG1κ^{PER}	150	5.7	8.9	8.5	+
IgG2κ^{PER}	150	5.7	7.9	8.5	+
IgG4κ^{PER}	150	2.4	6.5	8.5	+
IgA1κ^{PER}	160 330	10 30	4.9	8.5	+

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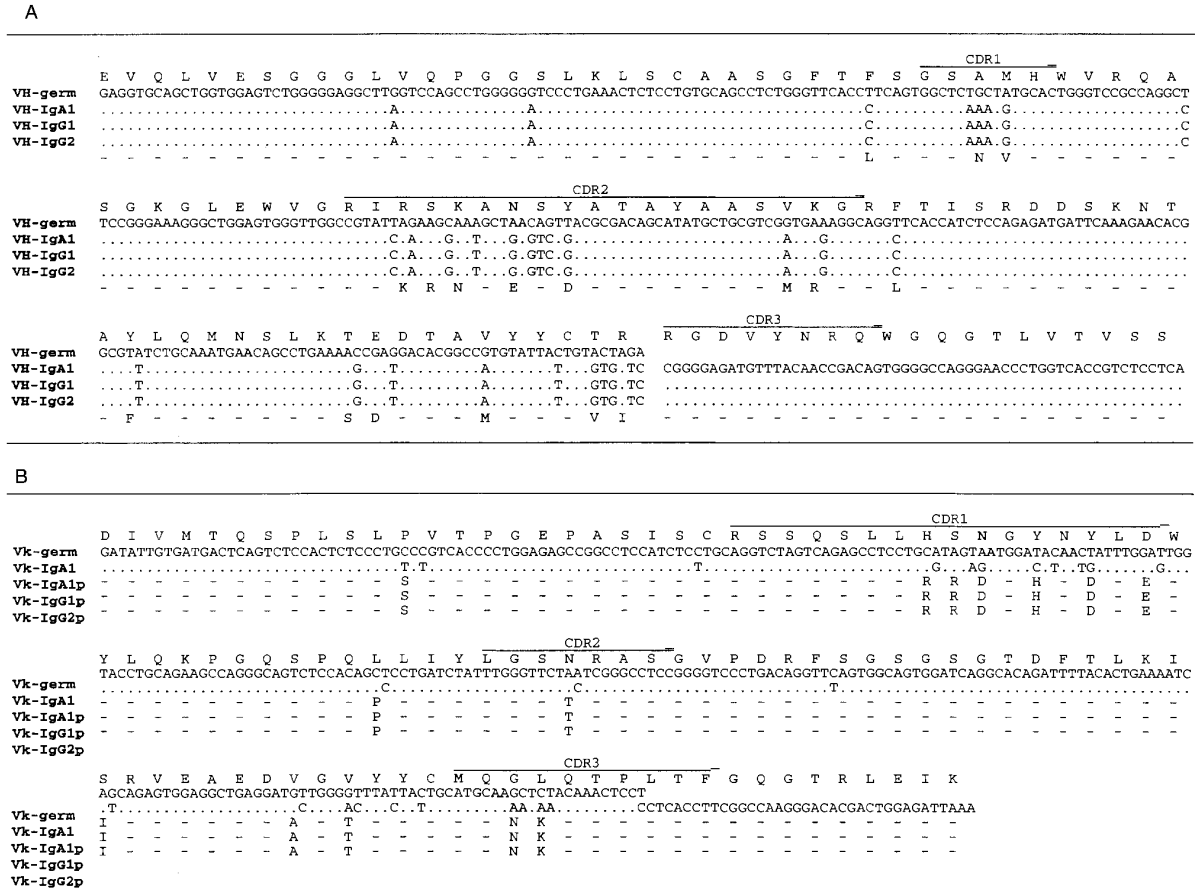


Fig. 1. Determination of V_H (A) and V_L (B) nucleotide and amino acid sequences from IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}. For V_H sequences nucleotide and amino acid sequences were obtained. Since nucleotide sequencing does not allow to assign the V_L corresponding to each isotype, V_L sequences were determined by amino acid sequence from purified IgA1κ^{PER}, IgG1κ^{PER} and IgG1κ^{PER}. Nucleotide sequence and amino acid identities are, respectively, indicated by dots and dashes. Replacements are depicted by the letters beneath the germ-line counterpart; CDR1, 2 and 3 are indicated.

2.2 Definition of the epitope recognized on β tubulin by PER antibodies

In a previous study, Houdayer et al. [8] demonstrated, after limited enzymatic proteolysis of purified tubulin, that IgA1κ^{PER} recognized C-terminal peptides of tubulin. For a better definition of the epitope recognized on tubulin by this antibody, we amplified and expressed a complete β tubulin chain, as well as a C-terminal fragment described in Sect. 4. Results of these experiments are depicted in Fig. 3A. It is shown that IgA1κ^{PER} recognizes the complete (lane 3) and C-terminal fragments of the β tubulin chain (lane 1).

To further define the epitope recognized by these antibodies, we sequenced the 450-bp C-terminal fragment from β tubulin (data not shown) recognized by both IgA1κ^{PER} and commercial anti-β tubulin antibody. On the basis of sequence data and using a peptide structure program, we designed eight tentative immunogenic peptides of 15 amino acids each corresponding to the C-terminal region of β tubulin. Fig. 3B depicts the results of these experiments. IgA1κ^{PER} (lane A) and commercial anti-β tubulin antibody (lane B) both bound to peptide 7. However, IgA1κ^{PER} was also found to bind to peptide 8. On the basis of these results, overlapping peptides were synthesized and tested for reactivity with these two monoclonal antibodies. As shown in Fig. 3C, IgA1κ^{PER}

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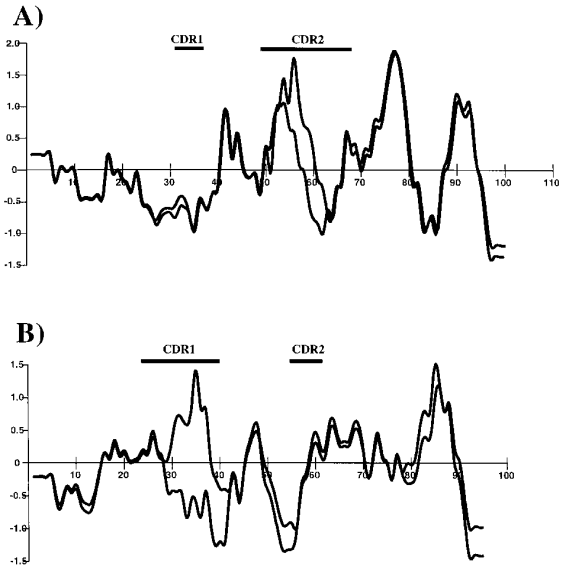


Fig. 2. Hopp and Woods hydropathicity plot of VH^{PER} and $V\kappa^{PER}$. Comparison of the V_H (A) and V_L (B) amino acid sequence of $IgA1\kappa^{PER}$ with their germinal counterparts [9] show the presence of high numbers of replacement mutations predominating in the V_H CDR2 region and V_L CDR1, which induce important changes in the Hopp and Woods hydropathicity plot.

recognized a TAEEEEDFGE peptide number 6 (lane A) in the C-terminal region of β tubulin. In contrast, commercial anti-β tubulin bound to peptides 4 and 5 (lane C) located immediately upstream of the β tubulin chain (Fig. 3C). The same epitope was also recognized by $IgG1\kappa$. Interestingly, a closely related acidic motif was present in the C-terminal region of α tubulin, which could explain recognition of both α and β tubulin by these antibodies.

2.3 Construction of two recombinant $IgG1\kappa$ and $IgM\kappa$ transfectomes containing the heavy and light variable domains of $IgA1\kappa^{PER}$

Our previous results showed that $IgA1\kappa^{PER}$ and $IgG1\kappa^{PER}$, sharing identical V_H and V_L domains but expressing different constant regions, bound tubulin with significant differences in affinity [9]. The observation that even Fab fragments from these antibodies reproduced differences in binding excluded a role for Fc interactions (including dimerization) and suggested that structural differences in the C_H1 domain accounted for the differences in the affinity constants.

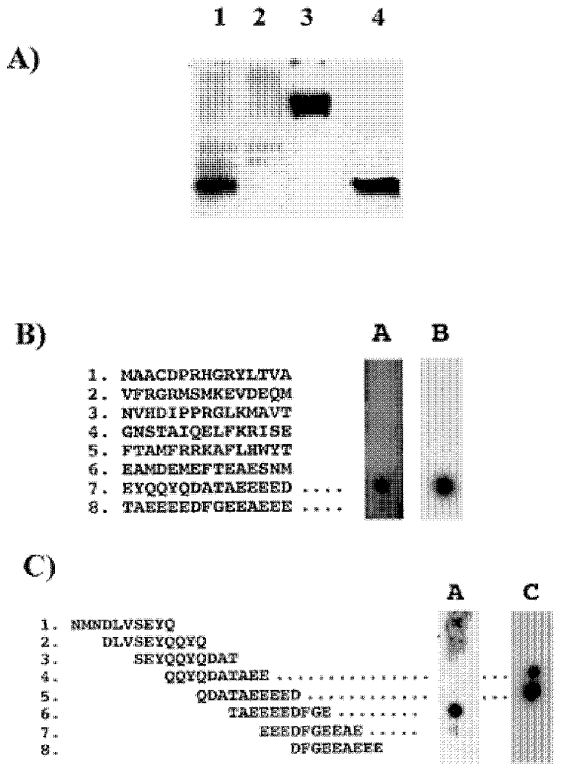


Fig. 3. Mapping of β-tubulin epitope. (A) Western blot experiments comparing the reactivities of anti-tubulin antibodies with the different expressed β tubulin fragments: binding of $IgA1\kappa^{PER}$ with the expressed C-terminal peptide derived from the 450-bp C-terminal fragment of β tubulin (lane 1) and the absence of binding with the control derived from the same bacteria not induced by IPTG (lane 2), or with brain tubulin (lane 3). Lane 4 represents the binding of commercial anti-β tubulin antibody to the 450-bp C-terminal fragment of β tubulin. (B) Both $IgA1\kappa^{PER}$ (lane A) and commercial anti-β-tubulin antibody (lane B) bind to peptide 7; $IgA1\kappa^{PER}$ also binds to peptide 8 (lane A). (C) $IgA1\kappa^{PER}$ recognizes a TAEEEEDFGE motif (lane A), whereas commercial anti-β tubulin binds to a QDATAEEEED motif (lane C).

To obtain definitive evidence, we cloned the V_H and V_L domains of $IgA1\kappa^{PER}$ and expressed them associated to human μ and γ1 heavy chain constant domains and human κ chain domains. The expression vectors for human μ (pRTM1μ-PER), γ1 (pcDNA1γ1-PER) and κ (pSVκ-PER) chains were constructed and co-transfected into the X63 cell line [10, 11]. Transfected clones were selected on the basis of their resistance to neomycin, and supernatants were tested by ELISA and Western blot (data not shown) for secretion of complete $IgM\kappa$ and $IgG1\kappa$. Tranfectomas secreting complete human $IgM\kappa$ and $IgG1\kappa$ were expanded; culture supernatants were

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Table 2. Equilibrium and kinetic constants for serum (S) and recombinant (R) MigPER binding to synthetic peptide

	k_{DISS} (s^{-1})	k_{ASS} ($M^{-1}s^{-1}$)	K_D (M)
IgA1-PER (S)	3.00×10–4	1.98×104	1.52×10–8
IgG1-PER (S)	6.32×10–4	3.02×103	2.09×10–7
IgG1-PER (R)	8.99×10–4	2.74×103	3.28×10–7
IgMκ^{PER} (R)	4.23×10–4	5.50×10^4	7.70×10–9

concentrated and purified by affinity chromatography. As analyzed by gel filtration HPLC, secreted IgG1κ corresponded to monomeric and IgMκ corresponded to pentameric molecules (data not shown).

2.4 Affinity and kinetic constants determination by surface plasmon resonance

To investigate the role of the isotype in the binding to tubulin, we tested the binding affinity against the peptide motif of serum monomeric IgA1κ^{PER}, serum IgG1κ^{PER} and recombinant IgMκ^{PER}, and IgG1κ^{PER} against the TAEEEEDFGE peptide motif by surface plasmon resonance. Affinity constants were 1.52×10^{-8} M for serum IgA1κ^{PER}, 2.09×10^{-7} M for serum IgG1κ^{PER}, whereas it was, respectively of 7.7×10^{-9} M for recombinant IgMκ^{PER} and of 3.28×10^{-7} M for recombinant IgG1κ^{PER} (Table 2). Although the BIAcore system takes into account the valency of the molecule to calculate affinity constants, the possibility exists that the density of the peptide in the surface is so high that it allows multipoint binding. We examined this last eventuality by studying affinity constants on Sensor Chips with decreasing concentrations of the peptide. Results showed a decrease in bound IgM to the Sensor chip consistent with a decrease in peptide density, without revealing differences in affinity constants (data not shown).

3 Discussion

In this work we have cloned the Vκ domain and the V_H domain of the PER IgA1κ anti-tubulin antibody and transfected them into expression vectors allowing the expression of the Vκ and V_H domains from IgA1κ^{PER} associated to γ1 and μ C_H regions. Next, we have obtained the expression of the complete antibody by transfecting H and L chain constructions into the X-63 myeloma line. A C-terminal epitope recognized on tubulin by IgA1κ^{PER} could be defined for β tubulin. A motif close to this epitope also exists in α tubulin. Finally, we demonstrated that the recombinant antibodies resulting from the association of the V_H from IgA1κ^{PER} with γ1 and μ C_H domains bind this epitope with significantly different affinities.

These results provide additional evidence indicating that changes of the isotype can significantly influence the binding affinity interactions of the antigen-antibody complex. Our previous work, indicating that Fab fragments from these antibodies reproduce differences in binding, suggests that structural differences in the C_H1 domain underlie the differences in the affinity constants, and thus the differences in antigen binding.

It has long been assumed that the constant domains do not play a role in the maturation of antigen-antibody interactions. Indeed, Fab are pseudosymetric dimers where V_H contacts with C_H1, whereas no contact is observed between V_L and C_L [12]. Although crystallographic studies have shown that amino acids ensuring the link between Fv and C_H1 and C_L are flexible and allow spatial arrangements facilitating binding of epitopes by Fab, there is controversy concerning the possibility that elbow bending could facilitate signal transduction from the antigen-binding site to the Fc domain [13–16]. However, recent work has demonstrated that changes in the C_H domains can affect the functional affinity and specificity of antibody, through a complex mechanism involving antibody flexibility and cooperative interactions in the case of antibodies bound to multivalent antigen [17, 18]. Schneider et al. [19] showed that segmental flexibility is controlled by the C_H1 domain and hinge regions of antibodies, and Horgan et al. [20] reported that removal of the hinge region of an IgG4 resulted in a gain of relative affinity that approximated to that observed with the same antibody expressing an IgG1 isotype. Concerning the functional activity of antibodies, Cavacini et al. [21] found that antibody isotype can strongly influence virion neutralization of HIV-1 virus. One difficulty in substantiating this using standard crystallography may arise from the fact that the role of molecular mobile components in the interaction with another molecule may not be fully captured by this method [22].

The observed differences in antigen-binding affinities for antibodies with identical V_H/V_L domains may arise in part from multivalent antigen binding (isotype-dependent avidity), since IgM and IgA have a larger number of antigen-binding sites than the IgG subclasses. However, multivalency cannot explain our observations, since we have carried out our BIAcore studies with monomeric IgA1κ^{PER} isolated from the 0.030 M fraction and also IgA1κ^{PER} Fab fragments. Both monomeric IgA1κ^{PER} and its Fab fragments displayed comparable binding affinities for tubulin [9] and significant affinity differences when compared to IgG1κ^{PER} ([9] and present work). From

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a structural point of view, two possible hypothesis spring to mind: (1) conformational signaling through the elbow angle, and (2) distinct antigen-binding sites in each isotype. The second hypothesis appears less plausible, since it implies that the high-affinity binding sites in the IgA and IgM molecules are not defined by the CDR of the variable domains from the light and heavy chains (otherwise, IgG should also bind the antigen with high affinity). Instead, we favor a mechanism by which specific differences in the C_H1 domains could impose structural and/or kinetic constraints on the antigen-binding site through the extended contacts at the V_H/C_H1 interface. Some structural evidence is compatible with this mechanism. In their comparisons of murine C_H1 domains from five different isotypes, Sheriff et al. [23] noted that the IgA C_H1 domain displays significant conformational changes and specific insertions/deletions with respect to IgG subclasses. We have constructed a model of the V_H-C_H1 interface of IgA1 and IgG1, using crystallographic data derived from homologous proteins. It is found that one of the C_H1 loops directly involved in V_H-C_H1 contacts displays a significantly different conformation for each isotype (Fig. 4). Such a different pattern of contacts could affect the relative orientation of the variable domains, which in turn might modulate antigen-antibody affinity.

Although, there is a consistent body of evidences on antigen-antibody interactions, demonstrating that the high-affinity antigen-binding sites are defined by the CDR of the variable domains, the results presented in this work provide evidence favoring the hypothesis that specific C_H1 differences can allosterically affect the antigen-combining site of an antibody. Whatever the frequency with which this phenomenon may occur, these results favor the view that affinity maturation of this particular tumoral clone could be achieved through class switching in the absence of further somatic mutations, probably because the C_H1 domain plays a role in structuring the antigen-binding site into a more kinetically competent form.

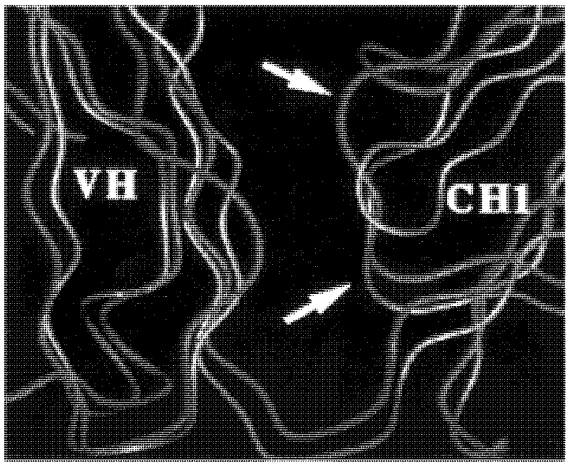


Fig. 4. Model of V_H-C_H1 interface. Schematic model of the V_H-C_H1 interface. The backbone models are shown for IgG1 (in blue) and IgA1 (in red). Note the different conformation of a C_H1 loop (indicated with arrows) that is directly involved in V_H-C_H1 contacts.

The mutation profile observed for the PER clone is compatible with a selective process driven by the antigen. Although in this work we have studied the pentameric soluble form of recombinant IgM and membrane IgM is monomeric, it can be hypothesized that the μ isotype allows good binding to the antigen. At this stage this clone may have been positively selected during the switch process.

Since all the four different clones isolated from PER (γ1, γ2, γ4 and α1) shared identical V_H and V_L sequences, it can be assumed that malignant transformation occurred once the mutational process was achieved, as previously reported in chronic lymphocytic leukemia B cells [24]. It is plausible to assume that the malignant transformation probably took place at the moment when the switch process was still active and probably rescued the three IgG clones, which, owing to the low affinity of their binding to tubulin, should in normal conditions be negatively selected. In contrast, the α1 clone predominated probably because the C_H1 domain of IgA allows the B cell receptor to establish higher affinity interactions with the antigen.

4 Materials and methods

4.1 Purification of Mig

Patient PER, affected by an immunocytic lymphoma expressed four different clonally related Mig in his serum [8]. Mig^{PER} were purified from serum by ammonium sulfate precipitation, followed by anionic-exchange chromatography on a DEAE-Sepharose column. Two different IgG of the γ1 and γ2 subclasses were eluted from the DEAE-Sepharose at the same molarity (0.005 M), and were further isolated by cationic-exchange chromatography on a CM-Sepharose column. $IgG4\kappa^{PER}$, monomeric and dimeric $IgA1\kappa^{PER}$ were eluted in the 0.015 M, 0.03 M and 0.05 M fractions, respectively. Purity, homogeneity and M_r of the $IgG1\kappa^{PER}$ and $IgA1\kappa^{PER}$ fractions were then assessed by ELISA and by HPLC, as described [8]. The isoelectric charge (pI) of the different Mig was measured by IEF.

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4.2 V_H and V_L nucleotide sequence of IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}

4.2.1 cDNA preparation

RNA was isolated from 10^6 PBMC by the guanidinium thiocyanate-phenol-chloroform method [9]. cDNA synthesis was performed in a 20-μl reaction volume at 37°C for 60 min using an oligo(dT) primer 15-mer (Boehringer, Mannheim, Germany), 200 U Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, Bethesda, MD), 40 U RNase inhibitor (Boehringer) and 0.5 mM of each deoxyribonucleotide.

4.2.2 V_H and V_L nucleotide sequence determination

PCR amplification was carried out from 1 μg of single-stranded cDNA, using Taq DNA polymerase (Cetus, Perkin Elmer, Emeryville, CA). According to the amino acid sequence the 5'primer used for V_H gene amplification was designed to anneal to the leader region of V_H3 family (5' L_H3 foward: GTA TCG ATG GAG TTT GGG CTG AGC TGG), and antisense oligonucleotides specific to C_H1 domain of Cα region, hinge of γ1 and γ2 regions were used, respectively, as 3' primers (3' Cα-reverse: ATC TGG CTG GGT GCT GCA GAG GCT; 3' hinge γ1: GGC ACG GTG GGC ATG TGT GAG and 3' hinge γ2: TGG GCA CGG TGG GCA CTC GAC). The 5' primer used for $V_κII$ gene amplification was designed to anneal specifically to the framework 1 (FR1) region of this κL chain family (5' $V_κII$-foward: CTA GAG TCG ACG ATA TTG TGA TGA CTC AGT CTC C), and in 3', the consensus $C_κ$ primer (3' $C_κ$-reverse: CAG ATG GCG GGA AGA TG) was used. In all cases, 30 cycles of amplification were carried out. V_H and V_L cloning and sequencing were carried out as previously reported [9].

4.3 Amino acid sequence determination

Since nucleotide sequencing does not allow the assignment of the VL corresponding to each isotype, V_L and V_H sequences were determined by amino acid sequence followed by mass spectroscopy analysis from purified IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}, as reported previously [9].

4.4 Construction of two recombinant IgG1κ and IgMκ expressing lines displaying IgA1κ^{PER} V_H and $V_κ$ domains

4.4.1 PBMC and genomic DNA isolation

Genomic DNA was isolated from patients' IgAκ expressing lymphocytes [9].

4.4.2 Construction of μ chain expressing vector (pRTM1μ-PER)

Genomic DNA was amplified using a 5' primer annealing to the leader region (5'L_H3-*Cla*I: ATG CAT CGA TAA TCA CCA TGG AGT TTG GGC TGA GCT GG) and 3'consensus primer annealing to J_H region (J_H-*Sal*I: TGT GTC GAC TCA CCT GAG GAG ACG GTG ACC AGG G) primers. The amplified fragment was cloned in a pKS plasmid and sequenced to discard Taq infidelities and to confirm identity with original PER sequence. Next, it was cloned into a plasmid containing μ constant exons (pRTM1) kindly provided by T. Kipps [10, 25].

4.4.3 Construction of γ1 chain expressing vector (pcDNA1γ1-PER)

An L_H-J_H fragment was obtained by PCR amplification from pRTM1μ-PER plasmid by using 5' leader specific L_H3 *Hind*III (TGC AAG CTT ATC ACC ATG GAG TTT GGG CTG) and 3' J_H-*Xho*I (GAG CTC GAG ACT CAC CTG AGG AGA CGG TGA CC). Following a cloning step as above mentioned, nucleotide sequence was confirmed and the restriction fragment was ligated into the pcDNA1γ1 vector kindly provided by L. Silberstein [26].

4.4.4 Construction of κ chain expressing vector (pSVκ-PER)

An Lκ-Jκ PER fragment was obtained by genomic DNA amplification with 5' leader specific LκII-*Cla*I (CAT ATC GAT TTC TCA CAA TGA GGC TCC CTG CTC AGC) and 3' Jκ5-*Not*I (AGA GCG GCC GCA AAT GCT TAC GTT TAA TCT CCA GTC GTG TCC C) primers. A fragment containing a confirmed Vκ sequence was ligated into pSVκ vector [10].

4.4.5 Construction of IgG1κ^{PER} and IgMκ^{PER} secreting transfectomas

Both H and L chain expression vectors were transfected into the non-secreting murine myeloma P3X63Ag8.653 cell line by electroporation. Briefly, 5×10^6 X-63 cells in RPMI medium without FCS were co-electroporated with 10 μg of linearized DNA from pRTM1μ-PER or pcDNA1γ1-PER and pSVκ-PER, using a 250 V/1,500 μF pulse in an EasyJect+ device (Eurogentec, Belgium). Transfected cells were cultured in RPMI medium containing 20% FCS, selected with neomycin (G418, Gibco, France) at 1 mg/ml final concentration, and then, cloned by limit dilution. Expression of complete engineered IgMκ^{PER} and IgG1κ^{PER} molecules was analyzed by Western blot and ELISA, as previously reported [10]. Purification of recombinant IgM and IgG molecules was proceeded by affinity chromatography on an activated anti-human κ CNBr immunoadsorbent.

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4.5 Cloning and expression of β tubulin fragments

The complete β-tubulin chain was amplified from murine cDNA, following sequence data from Villasante et al. [26] by: a 5' *Hind*III (GAC AAG CTT ATG AGG GAA ATC GTG CAC ATC CAG GCC GG) and a *Not*1 (CTT GCG GCC GCG GCC TCC TCT TCT GCC TCC TCT CCG AAA TCC) in 3'. In addition the 450-bp C-terminal fragment was amplified using: a 5' *Hind*III (GAC AAG CTT GAT GCC AAG AAC ATG ATG GCC G) and a *Not*1 (CTT GCG GCC GCG GCC TCC TCT TCT GCC TCC TCT CCG AAA TCC) in 3'. Amplified DNA fragments were cloned into the pET-22b(+) (Novagen, R&D System, Europe Ltd., Abington, GB) vector and transformation was carried out on NovaBlue (DE3) competent cells (Novagen, R&D System). Transformed clones were selected and expanded. Protein expression was induced by IPTG and the produced peptides were tested by Western blot for reactivity with IgA1κ^{PER} and with a commercial anti-β tubulin monoclonal antibody (Sigma-Aldrich chimie, St Quentin Fallavier, France). Blots were developed using the enhanced chemiluminescence detection system (ECL-Amersham International plc, GB) [27].

4.6 Peptides

Solid-phase synthesis of peptides was performed using SPOTs technology (Genosys Biotechnologies, Cambridgeshire, GB) on derived cellulose membranes and subsequently assayed for immunological reactivity [28]. Sequences of peptides are depicted in Fig. 4.

4.7 Affinity and kinetic constants determination by surface plasmon resonance

Equilibrium and kinetic constants for the interactions were determined by the BIAcore™ system (Pharmacia Biosensor AB Uppsala, Sweden). Immobilization of proteins via primary amines to the Sensor Chip CM5 was performed using an amine coupling kit. Activation of the carboxymethylated dextran matrix was obtained by injecting a mixture of N-ethyl-N'-(dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). A biotinylated-peptide: Biot-Q-19-E (biotinyl QQYQDATAEEEEDFGEEAE) was synthesized (Neosystem Laboratoire, Strasbourg, France) for affinity measurements. Immobilization of biotinylated peptide was achieved using a Sensor Chip SA containing streptavidin covalently coupled to a carboxymethylated dextran surface. Kinetic rate constants (k_{ASS} and k_{DISS}) as well as apparent equilibrium affinity constants ($K_D = k_{DISS}/k_{ASS}$) were determined using Bialogue™ Kinetics Evaluation software [29].

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Ca^{2+} sensitivity of the bilayer channel varies markedly between experiments. It remains to be determined whether the channels in the patch and the bilayer are distinct, or whether the apparent differences in some of their properties result simply from differences in their membrane environment.

The bilayer channel is closed virtually all of the time in 10^{-8} M Ca^{2+} at +40 mV (Fig. 4*a*), but is open for a significant time when the Ca^{2+} concentration is raised to 10^{-6} M (Fig. 4*b*). This degree of Ca^{2+} sensitivity is in the middle of the wide range that we observed. There is no effect on channel activity after prolonged treatment (in this case 13 min) with dithionitrobenzene (DTNB)-inactivated or heat-inactivated CS (Fig. 4*c*). However, the opening and closing kinetics of the channel change dramatically within 20 s of the addition of active CS, with a large increase in open probability (Fig. 4*d*). The latency for the onset of the response ranged from too fast to measure (that is, <5 s) to as long as 7 min. The *p* values to the right of each trace in Fig. 4 demonstrate the magnitude of the probability change at +40 mV produced by phosphorylation with active CS; such changes were observed in all the experiments ($n = 11$) carried out with either the large planar bilayers or the 'tip-dip' technique. Investigation of the change in probability elicited at various voltages by CS treatment produced results (not shown) entirely consistent with a shift in channel affinity for Ca^{2+}.

Previous studies from our own and other laboratories have provided evidence that protein phosphorylation can regulate the membrane properties of excitable cells. The present results extend these findings by demonstrating directly that the activity of individual Ca^{2+}-dependent K^+ channels can be modulated by cAMP-dependent protein phosphorylation. In addition, the results put the phosphorylation site considerably closer to the channel; indeed, from the reconstitution experiments, in which individual channels are effectively at infinite dilution in the bilayer, we conclude that the phosphorylation target is either the K^+ channel itself or something intimately associated with the channel. Finally, clues have been provided about those aspects of channel function which are modulated by phosphorylation, and it will be of great interest to extend these approaches to investigate the detailed molecular mechanisms of ion-channel regulation.

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Drastic change in idiotypic but not antigen-binding specificity of an antibody by a single amino-acid substitution

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In proliferating B lymphocytes, somatic mutation of rearranged antibody variable (V)-region genes occurs at high frequency and may have a key role in the selection of these cells[1–3]. It is of interest in this context to learn in which way single mutations can affect antigen binding and/or idiotypic specificity of an antibody. Previous investigations have analysed spontaneous mutants of myeloma and hybridoma cells in which the mutation affected the antigen-binding specificity of the antibody[4–8]. Here we describe an antibody mutant that has fully retained antigen-binding specificity but has lost or drastically changed all V-region antigenic determinants (idiotopes) of the wild type as defined by monoclonal anti-idiotope antibodies. The mutant phenotype is generated by a glycine to arginine exchange in the middle of the diversity (D) element, at position 103 of the heavy chain.

The mutant was derived from the monoclonal antibody B1-8.δ1, which binds the hapten 4-hydroxy-3-nitro-5-iodophenylacetyl (NIP) with high affinity[9,10]. From a series of monoclonal anti-idiotope antibodies raised against antibody B1-8 (refs 11, 12), two were used for mutant selection. Antibody Ac38 defines an idiotope (idiotope Ac38) outside the hapten-binding cleft. In contrast, idiotope Ac146, recognized by antibody Ac146, is presumably located close to the hapten-binding site because hapten and antibody Ac146 compete for binding to the B1-8 V region. Spontaneously arising idiotope loss mutants of B1-8.δ1 were isolated by fluorescence-activated cell sorting. It has been shown previously that an $Ac146^-Ac38^+$ mutant has lost hapten-binding specificity together with the binding site-related idiotope Ac146[4]. The present experiments describe a mutant selected for the expression of the latter idiotope and against expression of idiotope Ac38.

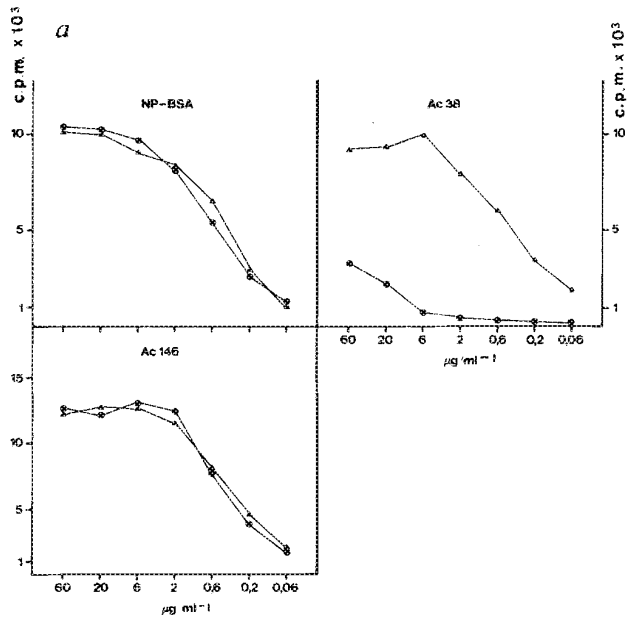
Table 1 Affinity and fine specificity of hapten binding to wild-type and mutant antibody

	IC_{50} NIP-cap (M)	IC_{50} NP-cap (M)	Fine specificity
B1-8.δ1	1×10^{-7}	2×10^{-5}	NNP = NIP > NCLP = NP
B1-8.δ1V3	1.1×10^{-7}	1.8×10^{-5}	NNP = NIP > NCLP = NP

The IC_{50} value is the molar hapten concentration resulting in 50% inhibition of binding of antibody (60 μg ml^{-1}) to N^{125}IP-cap (4×10^{-8} M), determined by Farr assay as described in ref. 9. For unknown reasons, IC_{50} values for NP-cap differed reproducibly by an order of magnitude from those published previously[10]. The order of haptens according to affinity was: NNP, (4-hydroxy-3,5-dinitrophenyl)acetyl; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; NCLP, (4-hydroxy-5-chloro-3-nitrophenyl)acetyl; NP, (4-hydroxy-3-nitrophenyl)acetyl.

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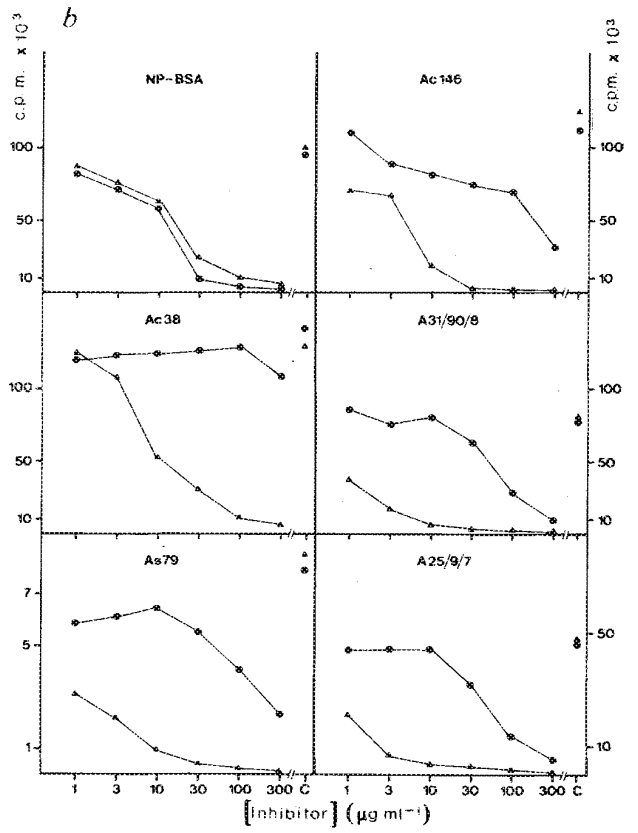


Fig. 1 Serological analysis of B1-8.δ1 (▲) and B1-8.δ1V3 (●). *a*, Direct binding in a solid-phase radioimmunoassay[26]. Plastic plates were coated with NIP-BSA or anti-idiotope antibody at a concentration of 50 μg ml^{-1}. After saturating the plastic surface with BSA, B1-8.δ1 or B1-8.δ1V3 antibody was added to the coated wells at the concentrations indicated on the abscissa, and bound antibody detected with ^{125}I-labelled monoclonal anti-mouse λ1-chain antibody Ls136 (0.5 μg ml^{-1}; refs 11, 27). Results are shown for NIP-BSA and the anti-idiotopes Ac146 and Ac38. The anti-idiotope antibodies Ac106, As79, A25.9.7 and A31.90.8 behaved like antibody Ac38 in that they failed to bind the mutant B1-8.δ1V3 (data not shown). *b*, Binding inhibition. The binding of iodinated antibody B1-8.δ1 to NIP-BSA or anti-idiotope-coated plates was inhibited by graded concentrations (abscissa) of unlabelled antibody B1-8.δ1 (▲) or B1-8.δ1V3 (●). Control binding without inhibitor is indicated (C).

B1-8.δ1 cells were incubated with fluoresceinated antibody Ac146 (50 μg ml^{-1}) in the presence of a 100-fold excess of unlabelled antibody Ac38. In these conditions, only cells that have retained the expression of idiotope Ac146 but lost that of idiotope Ac38 are stained. Stained cells were then enriched in eight successive rounds of sorting, expanding the separated cells after each sort. The mutant cells were finally isolated by cloning and identified by a radioimmunoassay of culture supernatant. Fifteen clones produced antibodies of mutant phenotype, that is, antibodies binding to antibody Ac146 but not to antibody Ac38. The frequency of the variant cells in the original B1-8.δ1 population is presumably $>2\times10^{-8}$, as about 5×10^{7} cells were used in the initial sorts. On the other hand, Ac146^{+}Ac38^{-} cells were not detected in the original population by fluorescence microscopy, staining the cells simultaneously with the two anti-idiotope antibodies coupled to different fluorochromes[4] and repeatedly screening 1×10^{5} cells. These results are consistent with previous data[4] and indicate that the frequency of a given idiotype variant in B1-8.δ1 cells is at least one order of magnitude lower than the frequency of variants of the myeloma S107 which have lost the property of antigen binding.

Because of the rare occurrence of the variants and the fact that three of the variant cell clones behaved identically when tested with a variety of other anti-idiotopes, it seemed unlikely that we had isolated a series of independent mutants. Therefore, we chose only one clone, B1-8.δ1V3, for a detailed characterization. The antibody produced by this clone (antibody B1-8.δ1V3) was assayed for binding to a NIP-conjugate of bovine serum albumin (NIP_{14}-BSA) and the anti-idiotope antibodies

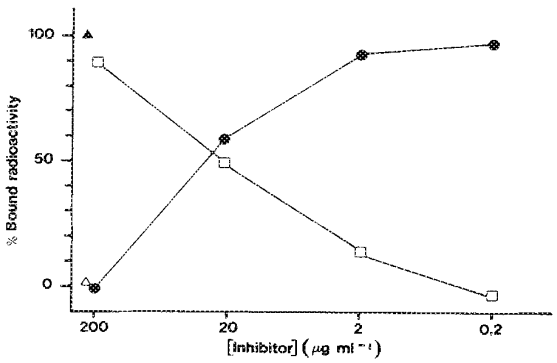


Fig. 2 Binding of λ1 light chain to variant and wild-type heavy chain. The myeloma protein HOPC1 (IgG2a, λ1) and IgD from B1-8.δ1 and B1-8.δV3 were mildly reduced with 10 mM dithioerythrol (150 min, room temperature, in the dark) and light and heavy chains separated on a Sephadex G-150 column (Pharmacia) in 1 M acetic acid with 10 mM dithioerythrol. The various fractions were dialysed first against 10 mM sodium acetate *p*H 5.5 and then against phosphate-buffered saline (PBS). When tested on a 7.5% SDS-polyacrylamide electrophoresis gel, the separated chains were >95% pure. HOPC1 λ1 chains were iodinated[27], re-associated at 20 μg ml^{-1} in PBS with 1% BSA at 4 °C overnight to either B1-8.δ1 (▲) or B1-8.δ1V3 (△) heavy chains (20 μg ml^{-1}) and tested for binding to an Ac38 (30 μg ml^{-1}) anti-idiotope-coated plastic plate. The λ1 chains can bind to the anti-idiotope only when associated with a B1-8.δ1 chain. Bound λ1 chains were counted in a γ-counter. The re-association of λ1 to B1-8.δ1 or B1-8.δ1V3 heavy chains was inhibited by increasing amounts (abscissa) of B1-8.δ1V3 (●) or B1-8.δ1 (□) heavy chains, respectively. The two curves cross each other at roughly 50% binding, indicating a similar affinity of the two heavy chains for the λ1 light chain.

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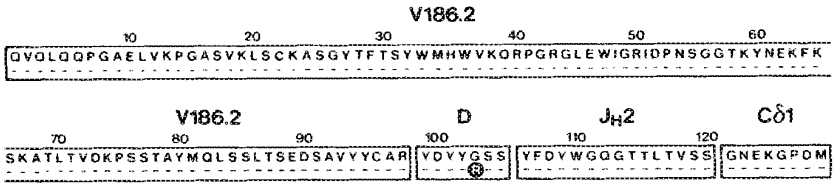


Fig. 3 Amino-acid sequence of the B1-8.δ1V3 heavy-chain variable region. The B1-8.δ1V3 antibody was cleaved by cyanogen bromide and the three fragments of the heavy-chain variable region isolated as described for B1-8.δ1 and B1-8.δ1V3 (refs 5, 28). Because H1 (residues 1–34) and H3 (residues 82–128) are blocked by the presence of N-terminal pyroglutamic acid[8], only H2 (residues 35–81) was sequenced directly by automated Edman degradation in a spinning-cup sequencer. Fragments H1, H2 and H3 were cleaved with trypsin or/and thermolysine and the fragments isolated by reverse-phase HPLC. The peptides were identified by amino-acid analysis and compared with the B1-8.δ1 sequence (upper line, single-letter code). The peptides covering residues 20–34 and 99–128 were sequenced in a gas-phase sequencer[29]. The amino-acid compositions of the peptides covering residues 1–21 and 82–98 isolated from B1-8.δ1 and B1-8.δ1V3 were indistinguishable, suggesting that for these parts both heavy chains are identical. The sequence of B1-8.δ1V3 (lower, dashed line) shows at position 103 an arginine residue instead of the glycine found for B1-8.δ1. The region of the variable regions encoded by the *D* gene (D), J_H2 gene (J_H2) and the J_H2 border to the first constant domain ($C_\delta1$) are indicated by vertical lines.

Ac146, A25.9.7, Ac38, A31.90.8, A6.24, As79 and Ac106. Efficient binding was observed only in the case of NIP-BSA and antibody Ac146 (Fig. 1*a*). The Ac146 determinant, however, is also affected by the mutation, as the B1-8.δ1V3 antibody was about 100-fold less efficient than the wild type in inhibiting the binding of radiolabelled antibody B1-8.δ1 to antibody Ac146 (Fig. 1*b*). In the case of the anti-idiotopes Ac38, A25.9.7, As79 and A31.90.8, binding inhibition by the mutant was even less efficient.

Despite its drastic idiotypic alteration, the mutant antibody inhibited the binding of the B1-8.δ1 wild type to NIP-BSA with the same efficiency as the wild-type antibody itself (Fig. 1*b*), and the affinities of both antibodies to NIP and some related haptens were identical within the limits of the analysis (Table 1).

The mutation in B1-8.δ1V3 was localized to the heavy chain. In chain re-association experiments, mutant and wild-type heavy chains, when re-associated to λ1 chains of the myeloma HOPC1, gave rise to mutant and wild-type immunoglobulin, respectively. The mutation does not interfere with the capacity of the heavy chain to bind light chain, as the two heavy chains competed with equal efficiency for re-association to λ1 (Fig. 2). Isoelectric focusing of Fv fragments revealed a small but distinct difference between the C-terminal heavy-chain variable-region fragments (relative molecular mass 19,000) of mutant and wild-type immunoglobulin (data not shown).

The amino-acid sequence of the V_H, D and joining (J) segments of the B1-8.δ1V3 heavy chain, determined by the strategy developed previously for the sequencing of B1-8.δ1V1 (ref. 5), revealed a single amino-acid difference between variant and wild type; at position 103 a glycine residue is changed to an arginine residue (Fig. 3).

In the previously isolated mutant, B1-8.δ1V1 (ref. 4), recombination (conversion) between the expressed and a non-rearranged V_H gene had generated the mutant phenotype[5,6]. Although we cannot exclude such an event in the case of B1-8.δ1V3, it seems more likely that in this antibody a point mutation has occurred, changing the glycine-encoding triplet GGT at position 103 (ref. 13) into CGT, encoding arginine. Point mutation is also the simplest explanation for the generation of mutants of the S107 myeloma line with changed antigen-binding specificity[8,14].

The observations that single amino-acid substitutions can profoundly affect idiotypic and/or antigen-binding specificity and that the D element has a key role in determining idiotypic specificity are not new[3,15–20]. The main point of the present analysis is the demonstration that a single amino-acid exchange in D can essentially eliminate the reactivity of an antibody with the majority of the anti-idiotopes raised against it, while leaving hapten-binding specificity intact. The conservation of NIP-binding specificity in the mutant, its reactivity with the NIP group on a macromolecular carrier (BSA), and the chain re-association data argue that the mutation has left the overall structure of the antibody and the hapten-binding cleft intact and selectively changed a surface structure (arginine 103 is a surface residue according to ref. 21) of key importance for idiotypic recognition. This structure may be directly involved in the interaction of the various anti-idiotope antibodies with the B1-8 V region. Like other protein interactions, idiotypic interactions seem in general to involve extended areas on the protein surface[3,19]. In the present system it has been shown that, in addition to D, residues in V_H and the V region of the light chain all contribute to the reactivity of antibody B1-8 with various anti-idiotopes[3,4,22]. It is therefore not surprising that, as far as analysed, the anti-idiotope antibodies compete with each other in binding to antibody B1-8 (ref. 11). Nevertheless, the points of contact differ for the various antibodies, as none of the target idiotopes is necessarily co-expressed with any other (refs 12, 23, 24 and our unpublished observations). Only some of the idiotopes are intimately associated with the hapten-binding site[11,12].

In biological terms, the B1-8.δV3 mutant demonstrates that a single amino-acid exchange can make an antibody essentially invisible for a variety of anti-idiotypic antibodies. Thus, somatic mutation can easily mediate escape from idiotypic control[25].

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REVIEW

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Complement-targeted therapeutics

Daniel Ricklin & John D Lambris

The complement system is a central component of innate immunity and bridges the innate to the adaptive immune response. However, it can also turn its destructive capabilities against host cells and is involved in numerous diseases and pathological conditions. Modulation of the complement system has been recognized as a promising strategy in drug discovery, and a large number of therapeutic modalities have been developed. However, successful marketing of complement-targeted drugs has proved to be more difficult than initially expected, and many strategies have been discontinued. The US Food and Drug Administration's approval of the first complement-specific drug, an antibody against complement component C5 (eculizumab; Soliris), in March 2007, was a long-awaited breakthrough in the field. Approval of eculizumab validates the complement system as therapeutic target and might facilitate clinical development of other promising drug candidates.

When the bactericidal properties of certain heat-labile blood components were initially described more than a century ago[1], no one could have imagined the considerable impact the complement system would have on both immunology and our understanding of many disease processes. Even the name "complement," originally coined by Paul Ehrlich[2], implied only a supplementary role of this system in the defense against microbial intruders. However, continued progress in research has led to a dramatic change in our knowledge about the complement system and moved it into the spotlight of basic and applied life sciences.

Today it is clear that complement is a key player of the innate immune system. However, this integral position in the maintenance and regulation of immune and inflammation reactions also makes it a trigger point for a variety of pathologic conditions. Erroneous activation or insufficient regulation of the complement cascade may turn its destructive actions against the host's cells. As a consequence, many inflammatory and autoimmune diseases are thought to be caused, or at least supported, by unleashed complement. Inhibition or modulation of complement activity has therefore been recognized as a promising therapeutic strategy for many years.

Although many creative attempts to design complement-specific drugs have been made in recent decades, their development from experimental concept to clinical product has faced many obstacles. In light of these complications, most of the major pharmaceutical companies seem to have abandoned their initial efforts to develop drugs that target complement. Despite its bitter taste, this decision has encouraged a series of small startup companies in the biotechnology field to take up the challenge. The first results of this commitment are now visible and may well lead to a revival of this difficult but promising area of drug discovery. With its 2007 approval of an antibody against complement component C5 (eculizumab; Alexion, Cheshire, CT, USA), the US Food and Drug Administration (FDA) has now authorized the first complement-specific drug[3]. Many other promising drug candidates and therapeutic strategies are currently in the pipeline of various companies. The aim of this review is to provide an overview of current therapeutic strategies, with a focus on drug candidates that are in clinical trials (**Table 1**) or late preclinical development (**Table 2**).

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The complement system in innate and adaptive immunity

With an estimated age of 600–700 million years, the complement system is one of the most ancient defense strategies[4], yet it represents far more than merely a simple protection mechanism against microbial infections. As an essential connection between adaptive and innate immunity, complement components are able to orchestrate immune reactions by communicating with multiple immune cells (**Fig. 1**). In addition, complement contributes to the maintenance of homeostasis by recognizing and eliminating apoptotic and necrotic cells. Furthermore, through its activity, the solubility of circulating immune complexes is maintained, thereby facilitating their elimination[5–9].

As a first line of defense against pathogens, complement has to quickly detect, tag, destroy and eliminate microbes. This task can be accomplished only by a complex and tightly regulated assembly of proteins involving various soluble and surface-bound complement components, receptors and regulators (**Fig. 2**). Complement activation is usually initiated by the interaction of several pattern-recognition receptors with foreign surface structures. Depending on the activation trigger, the complement cascade follows one of three pathways: the classical, lectin or alternative pathway.

The recognition of antibody complexes on the surface of pathogens, mediated by the multimeric collectin C1q, was early recognized as initiator of an enzymatic cascade that was later termed classical pathway. In the related lectin pathway, complement activation is initiated by an interaction of mannose binding lectin (MBL) with high-density arrays of terminal mannose on bacterial surfaces. In both cases, these pattern-recognition proteins form complexes with serine proteases (C1r/C1s and MASP, respectively) that lead to the cleavage of C4 to its fragments C4b and C4a. After binding of C2 to C4b, the same protease complexes are responsible for generating the active C2a fragment, which is part of the classical pathway C3 convertase (C4bC2a; **Fig. 2a**). To guarantee instant response to pathogens, the cascade is constantly kept at a low level of

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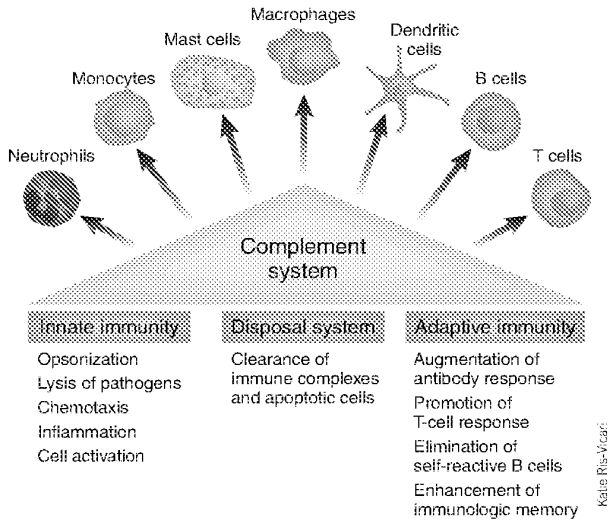


Figure 1 The complement system as a bridge between innate and adaptive immunity. Although the complement system has traditionally been considered part of the innate immune system, research in recent decades has revealed that complement is able to activate cells involved in both the adaptive and innate immune response. Complement triggers and modulates a variety of immune activities and acts as a linker between the two branches of the immune response. In addition, the complement system maintains cell homeostasis by eliminatiing cellular debris and immune complexes.

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activity—'tick-over'—by the alternative pathway. Spontaneous hydrolysis of C3 to C3w induces a conformational change that enables binding to factor B and formation of the initial alternative pathway C3 convertase (C3wBb; **Fig. 2a**). The alternative pathway may represent up to 80% of complement activation in some instances and can also be triggered by various reactions with foreign surfaces.

All three pathways converge in the cleavage of C3 into its active fragments, C3a and C3b, by the C3 convertases. Formation of C3b exposes a previously hidden thioester group that covalently binds to patches of hydroxyl and amino groups on the pathogen surface. This process, termed opsonization, is critical for all subsequent steps in the complement cascade and the elimination of pathogens and apoptotic host cells by phagocytes. In an amplification loop, binding of factor B to surface-bound C3b and its cleavage by factor D generates the alternative pathway C3 convertase C3bBb, which causes a gradual acceleration of C3b production. Surface-tethered C3b also plays a central role in the formation of the two C5 convertases (C4bC2aC3b and C3bC3bBb), which cleave C5 into C5a and C5b. C5b initiates the assembly of the membrane attack complex (MAC), a pore that is formed by components C5b, C6, C7, C8 and multiple units of C9 and ultimately leads to cell lysis. The anaphylatoxins C3a and C5a, generated as a result of proteolytic cleavage of C3 and C5, respectively, are potent inflammatory mediators that trigger a plethora of processes, which culminate in the stimulation of immune cells and the elimination of the pathogen[5].

Despite years of intensive research in this field, our picture of complement activation seems to be far from complete. Very recently, additional activation pathways have been identified: for example, MBL-associated protease 2 (MASP-2) of the lectin pathway seems to directly attack and cleave C3 without formation of the corresponding C3 convertase (C2 bypass pathway; **Fig. 2a**)[10]. Similarly, direct cleavage of C3 and C5 by a series of extrinsic proteases such as kallikrein or thrombin (extrinsic protease pathway; **Fig. 2a**), has also been proposed. This activity points to an interesting connection to other essential plasma cascades such as the coagulation system[11]. Finally, properdin seems to not only stabilize the C3 convertase as a positive complement regulator but may also act as a pattern-recognition molecule and actively induce convertase formation on foreign surfaces[12,13].

One of the most fascinating aspects of complement is the sophisticated regulation mechanism that allows a rapid reaction to pathogenic intruders while protecting host cells from its destructive potential. After activation, the opsonization rates often reach several thousand C3b molecules per cell per minute. In principle, this attack is not specifically directed against foreign cells. However, interplay of time, location and molecule-based regulation mechanisms generally prevents the complement cascade from attacking host tissues. Both the thioester group of C3b and the C3 convertases have short half-lives, limiting their action to the site of activation. Whereas the convertase is actively stabilized on foreign cells by properdin (**Fig. 2a**), a set of soluble and membrane-bound negative regulators inactivates and degrades active molecules on host cells (**Fig. 2b**). These structurally related regulators of complement activation include factor H, decay accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46), C4-binding protein (C4BP) and complement receptor 1 (CR1/CD35). These regulators either induce an accelerated decay of the convertases (DAF, CR1, factor H) or act as cofactors for factor I, which degrades C3b to its inactive form iC3b (MCP, CR1, factor H). An additional regulator of complement activation, CD59, prevents the formation of MAC by intercalating between C8 and C9 subunits (**Fig. 2b**)[5,14,15].

Complement's involvement in disease

The central role of complement in the orchestration of immune reactions inevitably renders it a target for immune evasion or attack by pathogens and also a contributor to many diseases. Even small disruptions in the delicate balance of activation and regulation can lead to an overreaction of complement that triggers inflammation and cell lysis. Many inflammatory, autoimmune, neurodegenerative and infectious diseases have been shown to be associated with excessive complement activity (**Fig. 3**). Complement involvement is usually complex and may include both an inappropriate initiation of the cascade and deficiencies in specific components or regulators[6,16,17].

Ischemia/reperfusion (I/R) injury is a severe consequence of conditions that lead to a deprivation and subsequent restoration of the tissues' blood supply; I/R injury can occur in a number of clinical conditions, including acute myocardial infarction, stroke, and hemorrhagic and septic shock, and may also be a complication of coronary artery bypass graft surgery. Paradoxically, it appears that the tissue reperfusion and not the ischemia activates complement and leads to inflammation-induced damage. Even though the exact involvement of complement activation in I/R injury is still unclear, several experimental studies have indicated a connection between complement and the pathogenesis of I/R injury, and have suggested complement inhibition as a potential therapy[18].

Complement seems to be a major contributor to a number of autoimmune diseases. For example, deposition of immune complexes during systemic lupus erythematosus initiates the classical pathway by binding of C1q (**Fig. 2b**) to these complexes[19]. A similar mechanism may also be involved in the pathogenesis of rheumatoid arthritis, and C5a (**Fig. 2b**) seems to have a major role in psoriasis and complement-mediated inflammatory diseases including asthma[20]. Complement activation by C1q, which acts as a pattern-recognition receptor for amyloidal plaques, has been correlated with the pathology of Alzheimer's disease[21]. Although complement also seems to be involved in other neurodegenerative diseases such as Huntington's disease or Parkinson's, the exact connection is less clear[21].

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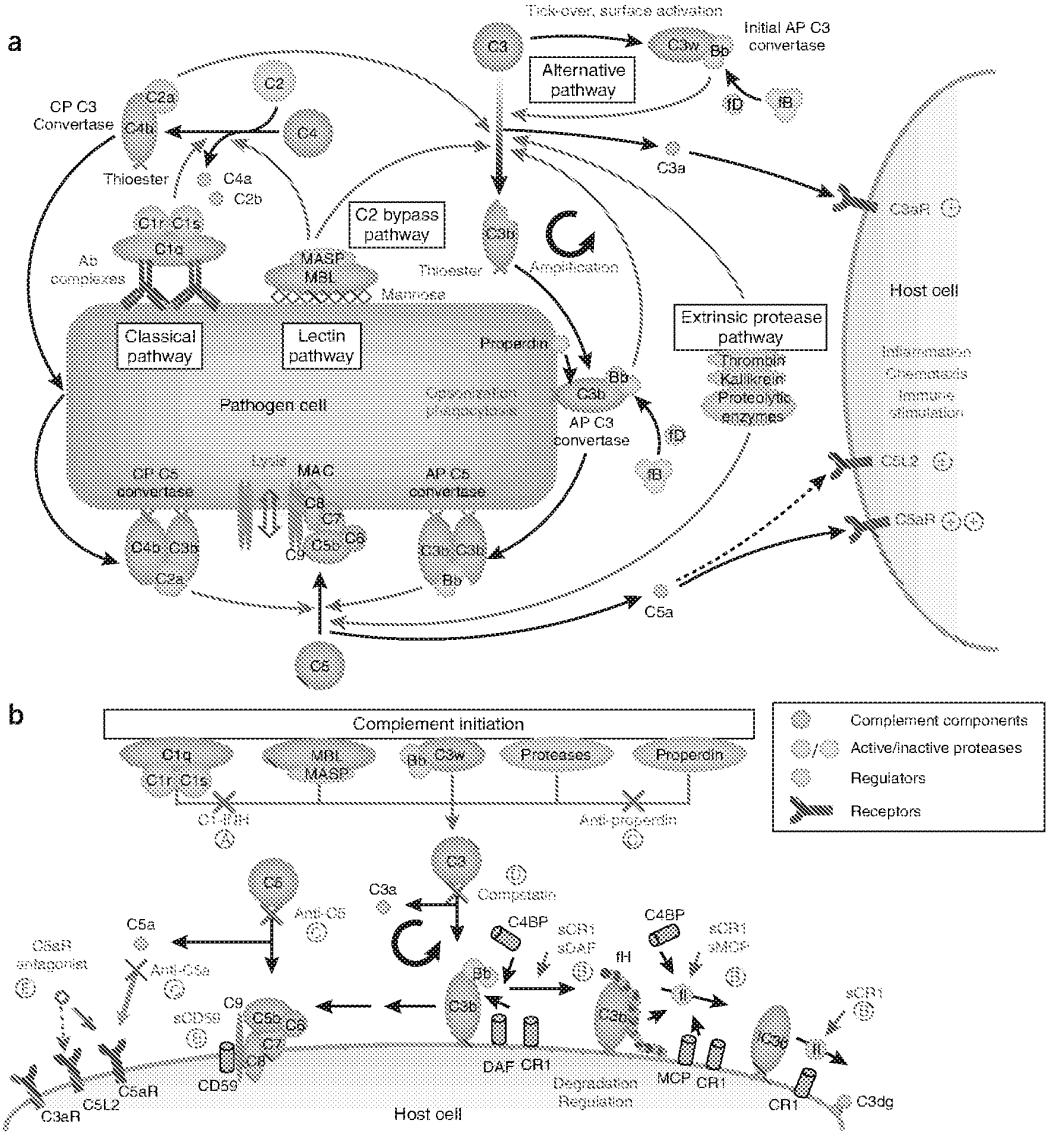


Figure 2 Activation, regulation and therapeutic modulation of the complement system. (**a**) The complement cascade after activation by pathogens. In addition to a low level of constant activation by the alternative pathway (AP; tick-over via hydrolyzed C3 (C3w) results in the formation of the initial C3 convertase C3wBb), the complement cascade is usually activated by antibody complexes (classical pathway) or high-density mannose (lectin pathway) on the surface of pathogens. This activation leads to the formation of the C3 convertases C4bC2a and C3wBb, which cleave native C3 to C3b and C3a. Deposition of C3b on cell surfaces via its thioester group initiates the cleavage of more C3 (the amplification loop via the final AP C3 convertase C3bBb), opsonization and phagocytosis, as well as lysis as a result of the formation of the membrane attack complex (MAC). In addition, the anaphylatoxins C3a and C5a are released and trigger further immune reactions upon binding to their receptors (C3aR, C5aR, C5L2). These combined actions of complement lead to the elimination of pathogenic cells. Recently, studies have shown that some steps of the cascade can be directly initiated by certain proteolytic enzymes (the extrinsic protease pathway) or by MBL/MASP (the C2 bypass pathway). Furthermore, the regulatory protein properdin may propagate and stabilize the formation of C3 convertases on the surface of the cell. Protein conversions are shown as black arrows and enzymatic reactions as green arrows. (**b**) Regulation, deactivation and inhibition of the complement cascade on host cells by natural regulators and complement-specific therapeutics. Several pathogenic processes and diseases are the result of an erroneous activation or insufficient downregulation of the complement cascade. Under normal conditions, any host-associated C3 convertase (C3bBb) undergoes an accelerated decay mediated by complement receptor 1 (CR1), decay accelerating factor (DAF), C4b-binding protein (C4BP) or factor H (fH). C3b is degraded to inactive iC3b by factor I in a reaction that requires as cofactor CR1, fH, C4BP or membrane cofactor protein (MCP). In addition, CD59 prevents the formation of the MAC. Some of the therapeutic interventions focus on increasing this downregulation by using soluble forms of these regulators (that is, sCR1, sDAF, sMCP, sCD59). Other approaches involve the substitution of the natural C1 inhibitor (C1-INH), the inhibition of the central conversion of C3 to C3b and C3a (compstatin), blockage of C5 or C5a by antibodies, and the suppression of anaphylatoxic signaling by C5a receptor antagonists. For clarity, only the regulation of the alternative pathway, which may contribute up to ~80% of all complement activity, is shown here. The C3 convertase of the classical pathway (C4bC2a), as well as the C5 convertases (C4bC2aC3b and C3bC3bBb), can be modulated by the same regulators and drug compounds. Therapeutic modulators in clinical trials or late preclinical development are indicated in red, with the circled letter referring to the corresponding drug class in **Tables 1** and **2** and the main text.

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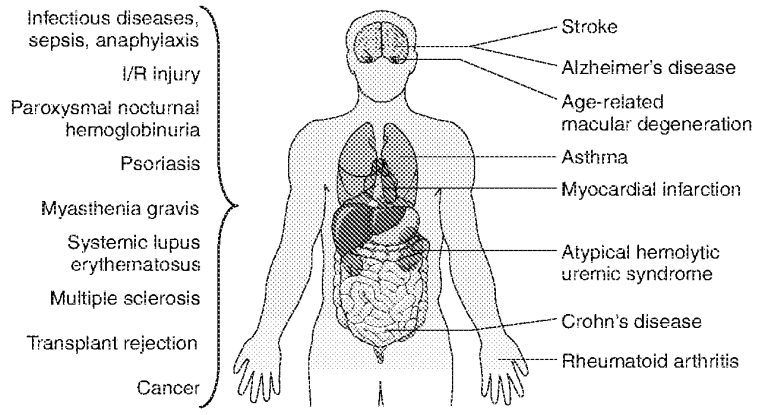


Figure 3 Examples of pathological conditions involving the complement system. Erroneous activation or insufficient regulation of the complement cascade may lead to an attack by the immune system against self-tissue. Many autoimmune, inflammatory and ischemia/reperfusion (I/R) injury-related diseases are therefore connected with complement. Whereas some of these pathological conditions are localized to specific organs and tissues, many of them are systemic. In addition, some pathogens have found ways to evade or even misuse the complement system, thereby contributing to infectious diseases and their consequences.

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Alterations in the expression and functions of complement regulators may be another cause of complement-related diseases. For example, polymorphism and mutations in genes encoding factor H (**Fig. 2b**) are associated with age-related macular degeneration (AMD), the major cause of blindness in industrial nations, and atypical hemolytic uremic syndrome (aHUS), respectively[22]. Low plasma levels of C1 inhibitor have been found to be responsible for hereditary angioedema (HAE)[23,24], and a lack of CD59 (**Fig. 2b**) on erythrocyte surfaces causes paroxysmal nocturnal hemoglobinuria (PNH)[25].

Finally, a series of pathogens have found ways to evade or even hijack complement proteins. Microbial proteins that either inhibit complement components or mimic regulators have been identified in both viruses (e.g., HIV, smallpox, Epstein-Barr virus) and bacteria (e.g., *Staphylococcus aureus, Streptococcus pneumoniae*)[5,26–30].

Druggability of complement

The central role of complement in the pathophysiology of major diseases makes it an interesting target for the pharmaceutical industry. However, despite numerous attempts in recent years to inhibit or modulate complement therapeutically[17,31–34], the success rate has been disappointingly low. Before the recent approval of complement-specific drugs, many promising candidates encountered insuperable obstacles while in clinical development. Inevitably, this high attrition rate raises questions about what renders complement drug discovery such a difficult task.

The multifaceted nature of both the cascade and its disease involvement may be one central problem. Many questions about the exact disease-related mechanisms of complement, both at the molecular and clinical level, are still unresolved, and this lack of clarity complicates specific targeting. "Druggability" (**Box 1**) is another major issue. Traditional drug discovery aims for small molecular entities with the potential for oral administration, yet many critical steps in the complement cascade are fundamentally based on large protein-protein interactions, which are challenging to influence with small molecules[35]. Anaphylatoxin receptors and serine proteases are the only complement targets with an auspicious druggability potential. This limitation may also explain the exceptionally high number of biopharmaceuticals (proteins, antibodies, peptides, nucleotides) and biotechnology startups that are involved in the current drug development process (**Tables 1** and **2**). Finally, the optimal target within the complement cascade (**Fig. 2b**) and the extent of inhibition has to be critically defined for each indication. Suppressing pathogenic complement activity without compromising its defensive and immunomodulatory functions might be one of the most delicate challenges when treating chronic conditions. Intervention at the activation level may require a highly specific approach, for example, inhibiting the recognition of amyloid plaques by C1q in the case of Alzheimer's disease. However, inhibition of a single pathway may be insufficient for many diseases and can still put pathogen defense at risk. Inhibition of C3 and its convertase potentially has the broadest effect, because it blocks all terminal activity of complement as well as the amplification loop. However, this nearly complete blockage may also lead to adverse effects and therefore favors local or targeted applications. Prevention of terminal complement action, for example, by inhibiting C5a formation and binding, is highly specific but requires the most information about the target involved. Finally, supplementation or inhibition of complement regulators may offer a more physiologic and specific way to modulate complement activity, especially in the treatment of chronic diseases.

Current complement therapeutics

A number of promising approaches for the clinical substitution, inhibition or modulation of complement have been developed and are presented in the following sections. We have classified complement therapeutics that either have been in clinical trials (**Table 1**) or are in the late steps of preclinical development (**Table 2**) into five major categories according to the mechanism of action (labeled **a–e** in **Tables 1** and **2**, and **Fig. 2b**).

Serine protease inhibitors. The consecutive cleavage and activation of several proteases constitutes the driving force behind complement function. Eight serine proteases are integral elements of the complement cascade itself (C1r, C1s, C2a, MASP-1, MASP-2, factor D, factor B, factor I)[36], whereas others are involved through the extrinsic protease pathway (**Fig. 2**)[11]. Because proteases are among the most druggable targets in the complement system (**Box 1**), it is not surprising that early drug development efforts focused on protease inhibitors[37]. Although screening- and structure-based approaches resulted in drug candidates for factor D (BCX-1470, BioCryst, Birmingham, AL, USA)[38] and C1s (C1s-INH-248, Knoll/Abbott, Abbott Park, IL, USA)[39], short half-lives and a lack of specificity have thus far prevented the successful development of small-molecule protease inhibitors for the treatment of complement-related disorders.

The only complement-associated protease inhibitor currently on the market is C1-inhibitor (C1-INH), a heavily glycosylated plasma protein that is used in the treatment of HAE[23,24,40,41]. This disease is characterized by recurrent episodes of severe skin and mucous membrane edema and is caused by either an insufficient production of C1-INH (type 1) or a mutation around its binding site that leads to inactivity (type 2). In both types, therapeutic supplementation of this protein has proven to be an effective and safe treatment for HAE and helps prevent severe disease

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relapses and life-threatening complications[42,43]. A third HAE type of unknown etiology and low prevalence does not benefit from supplementation, because it is not dependent on C1-INH activity[44]. Although C1-INH gained its name from its ability to block the serine esterase activities of C1r and C1s[45], its specificity includes other proteases, such as MASP, kallikrein and coagulation factors XI and XII. Although plasma levels of C2 and C4 are clearly affected by HAE, the primary mechanism of C1-INH seems to be more closely associated with bradykinin-kallikrein than with the complement system[23]. However, there is increasing evidence that therapeutic administration of C1-INH may also be beneficial in other disorders with a direct connection to complement, such as I/R injury[41,46–48].

Whereas pasteurized C1-INH concentrates for the treatment of HAE have been available since 1985, a lack of licensed products still limits the accessibility of this treatment in several countries, including the United States[43]. Fortunately, annotation of orphan drug status by the FDA (**Box 2**) and licensing agreements with established providers have boosted the development and registration of this important drug, and its approval in the United States is imminent[49]. Current C1-INH formulations are still being improved[50], for example, by the use of nanofiltered concentrates that feature better safety with regard to viral contamination. The high production costs and extensive quality control requirements for plasma-derived C1-INH still makes it an expensive treatment option and will certainly limit its availability in poorer countries. Development of a recombinant form, which is isolated as a glycosylated protein from the milk of transgenic animals, may be an important step in overcoming these limitations[51]. Experience with C1-INH in the clinic and its good safety profile are expected to lead to an extension of its indications. For example, clinical trials regarding the use of C1-INH for the prevention of I/R injury after acute myocardial infarction are currently planned.

Table 1 Complement therapeutics on the market or in clinical trials

Product (company)	Activity	Stage of development
Protease inhibitors (A)		
C1-INH (Cetor/Sanquin, BerinertP/CSL Behring, Lev Pharma)	Purified plasma protein; inhibition of C1r/C1s, kallikrein and other proteases	Marketed (HAE)[a]; clinical phase 3 (HAE)[a]; preclinical/phase 1 for other indications (AMI, CABG)
Rhucin/rhC1INH (Pharming Group N.V.)	Recombinant human C1-INH from transgenic rabbits	Clinical phase 3 (HAE)
Soluble complement regulators (B)		
sCR1/TP10 (Avant Immunotherapeutics)	Extracellular part of CR1; decay accelerator, factor I cofactor	Clinical phase 2 (CABG)[a]
CAB-2/MLN-2222 (Millenium Pharmaceuticals)	Chimera of DAF and MCP; decay accelerator, factor I cofactor	Clinical phase 1 (CABG)[a]
Therapeutic antibodies (C)		
Eculizumab/ Soliris (Alexion Pharmaceuticals)	Humanized long-acting mAb against C5	Marketed (PNH), preclinical for other indications
Pexelizumab (Alexion Pharmaceuticals)	Humanized short-acting mAb against C5	Clinical phase 3 (AMI, CABG)[a]
Ofatumumab (Genmab A/S)	Humanized anti-CD20 mAb	Clinical phase 2
Complement component inhibitors (D)		
Compstatin/POT-4 (Potentia Pharmaceuticals)	Peptidic C3 inhibitor; no generation of C3a/C3b	Clinical phase 1 (AMD)
Receptor antagonists (E)		
PMX-53 (Peptech Ltd.)	Peptidic C5aR antagonist	Clinical phase 2 (RA, psoriasis)
Other		
rhMBL (Enzon Pharmaceuticals)	Recombinant human MBL as substitution therapy	Clinical phase 1b (MBL deficiency)

[a]C1-INH preparation are only approved in some countries, whereas they are in clinical phase 3 in the USA. [b]Clinical trials have recently been performed but no product development has been undertaken since then, or the development was officially discontinued. AMD, age-related macular degeneration; AMI, acute myocardial infarction; CABG, coronary artery bypass grafting; HAE, hereditary angioedema; PNH, paroxysmal nocturnal hemoglobinuria; RA, rheumatoid arthritis.

Soluble complement regulators. Because regulators of complement activation are natural modifiers of complement activities and prevent a host cell from being attacked by its own defense system, they have been considered for therapeutic use since the early stages of complement drug discovery[52]. A first breakthrough was reached with the expression of a soluble form of complement receptor 1 (sCR1)[53]. This molecule featured both decay accelerator and cofactor activity and had a high potency in inhibiting both the classical and alternative pathways. sCR1 showed promising results in the treatment of I/R injury and various other conditions in experimental animal models. Based on these encouraging results, sCR1 was developed as a therapeutic (TP10; Avant Immunotherapeutics, Needham, MA, USA) for use after coronary artery bypass graft surgery. TP10 is expressed as a 240-kDa glycoprotein in Chinese hamster ovary cells, has a plasma half-life of ~55 hours and is safe and well-tolerated in both adult and infant patients[54]. In a large placebo-controlled phase 2 trial, comprising 564 high-risk patients undergoing cardiac surgery, a single intravenous bolus of TP10 immediately before surgery was found to inhibit complement activation for up to 3 days postoperatively. Whereas TP10 treatment resulted in a significant improvement of the clinical endpoints in males, no such benefits were observed in female participants. This surprising gender specificity was confirmed by a subsequent phase 2b study comprising 297 women. Whereas the mechanism governing these differences is still unclear, effects other than complement inhibition are likely to be involved. As a consequence, a male-only indication of TP10 for bypass surgery was considered for clinical phase 3. TP10 also showed promising results in smaller studies of adults undergoing lung transplants and infants undergoing coronary artery bypass graft surgery[54]. However, Avant Immune has recently decided not to continue investing in clinical trials of TP10 (ref. 55). It remains to be seen whether this development will mean the end of sCR1 as a therapeutic compound. Improvements in the structure (e.g., a reduction of its size) and formulation of sCR1 or an extension of its indications may well lead to its resurrection. Although the first steps in this direction have already been taken, neither TP20 (sCR1 with sialyl Lewis^X-glycosylation for targeting sites of inflammation[56], Avant Immune) nor Mirococept/APT070 (a truncated sCR1 with a membrane-tethering motif[57], Inflazyme Pharmaceuticals, Richmond, BC, Canada) has been tested in clinical trials.

Soluble forms of MCP, DAF and CD59 have also been considered as therapeutics. Whereas DAF and MCP each offer only a single regulatory activity, a recombinant chimera of their extracellular parts has been developed[17]. The resulting sDAF-sMCP hybrid was initially named "complement activity blocker 2" (CAB-2; Xoma, Berkeley, CA, USA) but entered clinical trials with a new name and licensing partner (MLN-2222; Millennium, Cambridge, MA, USA). However, no further studies have been initiated recently. Like Mirococept/APT070, a membrane-tethering sCD59 has been developed by Inflazyme Pharmaceuticals. Although

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Table 2 Complement therapeutics in pre-clinical development

Product (company)	Activity	Stage of development
Protease inhibitors (A)		
Factor D inhibitors[a] (BCX1470 etc.)	Small molecule serine protease inhibitors	Mostly discontinued (due to lack of specificity and/or short half-life)
Soluble complement regulators (B)		
sCR1-sLe^x/TP-20 (Avant Immunotherapeutics)	sCR1 with sLe^x-rich glycosylation to target sites of inflammation	Development/preclinical (stroke, heart attack)
Mirococept (Inflazyme Pharmaceuticals)	sCR1 with lipopeptide membrane linker	Preclinical (AMI, inflammatory diseases)
Therapeutic antibodies (C)		
TNX-234 (Tanox)	Humanized antibody against factor D	Preclinical (wet AMD)
TNX-558 (Tanox)	Humanized antibody against C5a	Development/preclinical (inflammatory diseases)
TA106 (Taligen Therapeutics)	Antibody against factor B	Development/preclinical
Neutrazumab (G2 Therapies)	Antibody blocking the C5a receptor	Development/preclinical (RA, stroke)
Anti-properdin (Novelmed Therapeutics)	Antibody against properdin	Development/preclinical
HuMax-CD38 (Genmab A/S)	Humanized anti-CD38 mAb that triggers complement	Preclinical studies (cancer, multiple myeloma)
Complement component inhibitors (D)		
ARC1905 (Archemix)	Aptamer-based C5 inhibitor (PEG-ylated)	Preclinical (AMD)
Receptor antagonists (E)		
JPE-1375, JSM-7717 (Jerini[b])	Small molecule/peptidomimetic antagonists for C5a receptor	Preclinical (inflammation, renal and ocular diseases[b])

[a]Currently, there are no complement-specific serine protease inhibitors listed in the pipelines of pharmaceutical companies. [b]Ophthalmology compounds are developed further by Jerini Ophtha m.c.

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not listed in the company's pipeline, experimental studies were recently performed to investigate its potential therapeutic usefulness as a treatment option for PNH[58]. This rare, genetic, life-threatening blood disorder leads to decreased expression of membrane-anchored proteins, including CD59 and DAF, on erythrocytes. Substitution and membrane tethering of recombinant CD59 may develop into a promising therapy for PNH[58].

Therapeutic antibodies. Following a general trend in the pharmaceutical industry, antibody-based therapeutics appear to be the most rapidly growing drug class against complement-related diseases. Crucial developments in their screening, production and humanization have led to a remarkable boost in the number of therapeutic antibodies[59]. By targeting specific components of the complement system, all stages from the initiation and activation process to single terminal actions can hypothetically be blocked in a selective manner. Current drug candidates in the pipeline focus primarily on the inhibition of downstream processes around C5 and its cleavage fragment, the anaphylatoxin C5a.

Selective inhibition of C5 using monoclonal antibodies (mAb) has been considered a promising therapeutic option for many years. A highly selective mAb against mouse C5 was introduced by Frei *et al.* two decades ago[60] and was later demonstrated to be effective in a mouse model of rheumatoid arthritis by Alexion Pharmaceuticals[61]. Continuous improvement and clinical testing finally led to the FDA approval of eculizumab[3], which is currently the only complement-specific antibody on the market[62]. Eculizumab is the first and only approved therapy for PNH, a rare but life-threatening disorder that is characterized by a chronic destruction of red blood cells. A mutation on the X chromosomes of hematopoietic stem cells prevents the proper biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, which leads to a deficiency in membrane-anchored proteins, including DAF and CD59. The lack of efficient inhibition of complement activation on red blood cells results in an increase in MAC formation on erythrocyte membranes and cell lysis. The survival of PNH erythrocytes is dramatically reduced in PNH patients, to 10% of normal red blood cells. On platelets, the absence of CD59 and increased MAC formation can lead to morphological changes of their surface. These platelets show enhanced susceptibility for activation, contributing to a high risk of thrombosis in PNH patients. Until very recently, only supportive therapies (blood transfusions, iron therapy, anticoagulation and others) or allogeneic stem cell transplantation had been available for the clinical management of this disease[25].

Given the complement-focused molecular mechanism of PNH, prevention of MAC activity evolved as the most promising treatment strategy. Two possible approaches were identified: substitution of defective CD59 or neutralization of complement proteins involved in MAC formation itself, as in the case of eculizumab. By tightly binding to C5 and preventing its cleavage to C5b, this new drug suppresses the formation of the MAC and reduces the clinical symptoms of PNH. Eculizumab (5G1.1-SC) is a humanized version of the anti-C5 antibody h5G1.1, which was first described in 1996 (ref. 63). Eculizumab received orphan drug status for PNH from both the FDA and the EU in 2003, and Alexion Pharmaceuticals subsequently initiated two phase 3 trials. In both studies, eculizumab was safe and well-tolerated, and no development of antibodies against the drug was detected. Because complement inhibition is expected to lead to a higher susceptibility to infection, particularly with *Neisseria meningitidis*, all patients had been vaccinated with a meningococcal vaccine before beginning treatment. The antibody was clearly effective: 51% of those who received eculizumab gained independence from blood transfusions (versus 0% in the placebo group). Both the endogenous erythrocyte mass and the hemoglobin levels were remarkably higher during treatment than before, and many symptoms related to quality of life (e.g., fatigue, abdominal pain) were attenuated. Whereas the preliminary data also indicated a lower risk for thrombosis, this benefit needs to be confirmed by ongoing clinical trials[25,64,65].

As an anti-C5 antibody that inhibits the generation of both C5b and the anaphylatoxin C5a, the potential indications for eculizumab are certainly not limited to PNH. Consequently, eculizumab has undergone several preclinical and clinical studies for a variety of conditions (e.g., psoriasis, rheumatoid arthritis, systemic lupus erythematosus and transplant rejection). Even though some of these paths seem to have been discontinued[65], Alexion Pharmaceuticals still performs preclinical development of eculizumab for autoimmune diseases and transplant rejection. Furthermore, nebulized eculizumab for the treatment of asthma and an intravitreal application to treat age-related macular degeneration are in the company's pipeline. In the case of pexelizumab, the short-acting sc-Fv fragment of eculizumab, several phase 3 trials have been done to test its potential for use in coronary artery bypass graft surgery and acute

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myocardial infarction[66]. Despite promising preclinical results, however, data from these clinical trials did not show significant improvement in the treatment group when compared to the placebo group[67,68]. Alexion Pharmaceuticals has therefore announced that it plans to focus its development efforts on eculizumab[69].

The concept of using humanized antibodies (and their fragments) to inhibit certain steps of the complement cascade seems to be a very productive strategy. Several companies list anticomplement antibodies in their pipelines (**Table 2**). Two of them target the various actions of the anaphylatoxin C5a. Neutrazumab (G2 Therapies, Darlinghurst, NSW, Australia) binds to extracellular loops of C5aR and thereby inhibits the binding of C5a to its major signaling receptor. G2 Therapies recently announced a partnership with Novo Nordisk (Bagsværd, Denmark) with regard to the development of their anti-C5aR antibodies. Another approach has been taken by Tanox (now part of Genentech, S. San Francisco, CA, USA) with their antibody TNX-558, which neutralizes C5a by binding to the anaphylatoxin itself. Interestingly, TNX-558 binds to a C5a epitope in native C5 but does not prevent its cleavage. Tanox has also developed an antibody directed against factor D (TNX-234). Together with the anti–factor B (TA106 for age-related macular degeneration and asthma[70]; Taligen, Aurora, CO, USA) and anti-properdin (Novelmed, Cleveland, OH, USA) antibodies in development, these biologics may offer additional approaches for inhibiting complement at the C3 convertase level.

Whereas inhibition of complement activity is the desired outcome in the vast majority of therapeutic approaches, its local stimulation may be beneficial in some malignant diseases, such as cancer. Experience with monoclonal antibodies in cancer therapy suggests that induction of cell death through antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity (CDC) may be a major driving force behind their effectiveness (e.g., in the case of the anti-CD20 mAb rituximab from Roche). However, relatively high antigen densities are required for an efficient initiation of the classical pathway by C1q. Genmab (Copenhagen) recently described anti-CD20 antibodies with exceptionally high CDC potency[71,72]. Both the location of the epitope as well as the dissociation rate are apparently responsible for this high level of effectiveness. Ofatumumab/HuMax-CD20 has been selected for clinical development and has recently been evaluated in terms of its effectiveness in treating acute rheumatoid arthritis (phase 2), B-cell chronic lymphocytic leukemia (phase 3) and follicular lymphoma (phase 3). Other antibodies with high CDC potency against multiple myeloma (HuMax-CD38) or colon, pancreatic and prostate cancer (HuMax-ZP3) are currently in preclinical development (http://www.genmab.com/). This strategy is a good example of how to make use of a more general, nonspecific property and select and optimize it to obtain the desired clinical effect. Because CDC may generally be induced by therapeutic IgGs (and IgMs), the CDC activity of these reagents may become a crucial parameter in the development of therapeutic antibodies.

Complement component inhibitors. Although antibodies may be considered the archetype of site-blocking compounds, smaller molecules such as peptides, nucleotides and synthetic molecules may also have the potential to interrupt protein functions by steric hindrance or the induction of conformational changes. Small functional inhibitors of complement activity are expected to have drug-like properties with enhanced pharmacokinetic profiles (**Box 1**). These advantages may make them suitable for drug development efforts related to oral bioavailability and better administration routes.

Compstatin is the most developed candidate in this class of substances and recently entered clinical trials. This cyclic tridecapeptide has been discovered through screening of combinatorial phage-display libraries[73] in the research group led by one of the authors of this review. It effectively prevents the cleavage of C3 to its active fragments C3a and C3b and therefore inhibits the most central step in the complement cascade. Although the exact mechanism of compstatin's inhibitory action has not yet been resolved, the recent publication of the cocrystal structure between compstatin and C3c suggests that a disruption of protein-protein interactions during convertase formation may be an important determinant[74]. This finding has led to the hypothesis that a conformational change or an interruption in protein-protein interactions is responsible for its activity. Compstatin has shown effective complement inhibition in a variety of experimental disease models[34]. Despite a narrow selectivity for primate C3 that may affect preclinical animal experiments[75], its high efficacy and rather small size make it a promising drug candidate. Its inhibitory efficacy has been increased by 264-fold[34,76] through the use of rational and combinatorial synthesis, structural analysis, and computational approaches to identify active analogs. Potentia (Louisville, KY, USA) has announced the inauguration of clinical trials for the treatment of neovascular age-related macular degeneration by intravitreal application of a compstatin analog (POT-4).

Combinatorial approaches have also been used to identify a short aptamer molecule directed against C5 (ref. 78). Aptamers are single-stranded nucleotide stretches that have molecular recognition properties similar to those of antibodies, but offer better options for derivatization. They can be selected in an automated high-throughput process known as systematic evolution of ligands by exponential enrichment (SELEX)[79]. More than 15 years after the initial description of this method, the first aptamer-based drug pegaptanib (Macugen; Eyetech Pharmaceuticals/Pfizer, New York) a PEG-ylated anti–vascular endothelial growth factor (VEGF) aptamer for the treatment of wet age-related macular degeneration, was approved by the FDA[80]. The optimized and heavily modified

Box 1 Drugs and druggability

For the pharmaceutical industry, the ideal drug is a small molecule featuring high potency, selectivity, few adverse effects, low production costs and administration in a single daily oral dose. However, identifying compounds with these properties is challenging, and drug companies have been confronted with an ever-growing drug attrition rate[97]. During the drug discovery process, molecules that fulfill a set of molecular guidelines for oral bioavailability, known as the "Rule of 5"[98] (RO5; e.g., a molecular weight below 500 Da or a partition coefficient [logP] below 5), are referred to as "drug-like" compounds. Similarly, this concept can be transferred to the targets of therapeutic intervention. Druggability refers to disease-related proteins that have the potential to be modulated by drug-like compounds[97]. Several attempts have been made to predict the druggability of protein families and to identify promising drug targets. Using the "druggable genome" approach, the targets of currently marketed drugs have been analyzed and mapped to protein families, assuming persistent druggability within the family[99]. Enzymes are the predominant class (nearly 50% of the total), followed by G-protein-coupled receptors (30%) and ion channels (7%). Within the complement cascade, the only targets with acceptable druggability potential are therefore the serine proteases and the anaphylatoxin receptors. Considering the large post-translational variability of protein targets and the increasing market for biopharmaceuticals, expansions of this model, such as the "pharmaceutically tractable genome," have recently been suggested[100].

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Box 2 Supporting the niches—the Orphan Drug Act

Development of a new drug compound is a challenging and expensive endeavor. The cost for a newly marketed drug is currently estimated to be in the range of $500 million to more than $2,000 million[101]. It is therefore understandable that pharmaceutical companies focus primarily on drugs to treat major diseases with a large market potential (that is, "blockbuster drugs"). This focus often leaves a painful therapeutic gap for rare diseases, niche markets or biopharmaceuticals with patent limitations (e.g., natural proteins). Compounds that are unprofitable to develop are usually referred to as "orphan drugs"[102]. In an effort to encourage the development of such drugs, several drug regulation authorities, including the US Food and Drug Administration (FDA), have initiated special programs such as the Orphan Drug Act (ODA) of 1983 (ref. 103). Designation of orphan drug status is coupled to a series of financial incentives, the most important of which is marketing exclusivity for 7 years after drug approval. In addition, orphan product development grants sponsor clinical trials and close the gap between basic research and clinical development. Undeniably, the ODA is one of the most successful recent United States legislative actions and has had a major impact on the drug market. Twenty years after its enactment, over 1,000 drugs had been accorded orphan drug status, and more than 200 had been approved for marketing, thereby improving the clinical situation of an estimated 11 million patients in the United States alone[102]. Meanwhile, these numbers have grown to 1,750 designated and 315 marketed orphan drugs (as of October 4, 2007, http://www.fda.gov/orphan/designat/list.htm). Similar initiatives also exist in other countries and regions (e.g., at the European Medicines Agency). In the complement field, eculizumab, for the treatment of paroxysmal nocturnal hemoglobinuria, and C1 inhibitor concentrate, for treatment of hereditary angioedema, are covered by the ODA. For more information, see http://www.fda.gov/orphan/.

form of the anti-C5 RNA aptamer (ARC1905; Archemix, Cambridge, MA, USA) features a subnanomolar binding affinity for human C5. It provides complete blockage of downstream complement activation by inhibiting the cleavage of C5 into C5a and C5b. A 40-kDa PEG group has been added to the 5′ end to improve its pharmacokinetic properties. Although ARC1905 was initially developed for use during coronary artery bypass graft surgery, the recent failure of pexelizumab for this indication has had a direct impact on the fate of ARC1905. Like compstatin (POT-4) and Macugen, the compound is now positioned as an intravitreal treatment option for wet and dry age-related macular degeneration and is awaiting clinical trials (P. Bouchard, Archemix Corp., personal communication).

Anaphylatoxin receptor antagonists. The proinflammatory activity of the anaphylatoxin C5a is the driving force behind many complement-associated disorders. After its liberation from C5, this hormone-like glycoprotein binds to two high-affinity receptors, the C5a receptor (C5aR) and C5L2 (**Fig. 2**)[81]. Most of the proinflammatory signaling seems to be induced by C5aR, a G-protein-coupled receptor that is expressed on numerous myeloid (e.g., neutrophils, macrophages) and nonmyeloid cells. Despite its similar structure and affinity for C5a, the C5L2 receptor produces a completely different signaling pattern. In plasma, the activity of C5a is controlled through the removal of the C-terminal arginyl residue by the plasma enzyme carboxypeptidase N. The resulting $C5a^{desArg}$ binds poorly to C5aR but with a high affinity to C5L2. Earlier reports about binding of C3a and $C3a^{desArg}$ to C5L2 have recently been questioned and require further investigation[81]. The differential binding pattern, as well as reports of the anti-inflammatory activity mediated by C5L2, has led to the hypothesis that the regulation of C5a involves a sophisticated feedback mechanism[20,64]. However, other studies have reported a proinflammatory or C5aR-supporting role for C5L2 (ref. 82), leaving its exact function a matter of scientific debate.

Selective inhibition of the binding of C5a to its receptors offers a very promising opportunity for dampening the inflammatory response without depleting the defensive potential of complement[64,83,84]. The macromolecular biopharmaceuticals we have previously discussed either inhibit C5a generation (anti-C5 antibodies and aptamers) or neutralize the C5a-C5aR interaction by shielding the relevant binding site. C5aR antagonists, on the other hand, are designed to bind to the receptor with high affinity without inducing any signaling activity (**Fig. 2b**). Because only one of the two proposed binding sites on C5aR has to be targeted for this purpose[81,84], these antagonists are expected to be rather small. Simultaneous efforts by many companies have focused on reducing the functional fragment of C5a to a C-terminal hexapeptide, while replacing individual residues to shift its activity from agonistic to antagonistic. Cyclization of these hexapeptides not only allows them to be locked into the desired turn-conformation but also gives them higher stability with regard to proteolytic degradation[84].

The most promising candidate to emerge, the cyclic peptidomimetic PMX-53, is currently under development by Peptech[85]. With a molecular size under 1 kDa and a high oral bioavailability, PMX-53 has many of the desired features of a designed drug. However, its short half-life of only 70 min may mean a requirement for frequent, if not continuous, administration. Although the drug showed high stability in blood, gastric and intestinal contents, it was rapidly degraded by proteolysis in intestinal mucosa washings. In a phase 1a/2b trial in 21 patients with rheumatoid arthritis, PMX-53 was orally administered and showed positive trends in many disease measures. An equally positive result was observed in a phase 1b/2a trial of topical application of the drug in ten people with psoriasis. In both studies, PMX-53 was found to be safe and well-tolerated[85]. Despite these encouraging results, the short half-life and rapid degradation in intestinal mucosa of this drug may severely limit its use. It remains to be seen if Peptech will further develop PMX-53 or initiate new trials with PMX-205, a similar yet more hydrophobic compound that shows high potency but a much lower susceptibility to proteolysis than that of PMX-53 (refs. 86,87).

Given the current uncertainty about the role of the C5L2 receptor, a critical evaluation of the binding specificity may be an important requirement for the development of successful C5aR antagonists. Several companies seem to have promising candidates in preclinical testing. For example, Jerini has a series of peptidomimetics and small organic molecules (JPE-1375, JSM-7717) for the treatment of inflammatory, renal and ocular diseases in preclinical stages (http://www.jerini.com/).

Other concepts. Whereas most of the previously discussed concepts are designed to inhibit or decrease complement activation, one compound is intended to boost low complement activity. Approximately 30% of the human population shows low plasma levels of MBL, making them more susceptible to infections under immunosuppressive conditions. Recombinant human MBL (rhMBL; Enzon, Bridgewater NJ, USA) restores the activity of the lectin pathway and has been found to be safe and well tolerated[88]. It is currently being tested in phase 1b trials as replacement therapy in people with multiple myeloma who are deficient

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in MBL and undergoing high-dose chemotherapy and stem cell transplants, and in MBL-deficient people undergoing liver transplantation. Additional approaches such as quenching of exposed thioester groups of C3b (thioester inhibitors)[17] or inhibition of the C1q-IgG interaction by anionic small molecules (e.g., steroid/triterpenoid sulfates) have been described[31,89] but have not appeared in preclinical settings thus far.

Perspectives and outlook

As is true for complement research itself, the quest for complement-directed therapeutics has made dramatic changes in direction and encountered difficult challenges; initial euphoria has given way to a more realistic view of strategies and aims. Whereas some first-generation approaches of complement drug discovery have either failed (e.g., small-molecule serine protease inhibitors) or are severely struggling (e.g., soluble regulators), there are many promising concepts on the horizon that represent a second generation of complement drugs. Current indications for complement drugs, such as HAE or PNH, certainly represent niche markets far away from blockbuster areas. Both eculizumab and the C1-INH concentrates are protected under the Orphan Drug Act (**Box 2**), and their approval is an important step in establishing the concept and accumulating clinical experience before expanding their indications.

Undeniably, the unsatisfactory results obtained for TP10 and pexelizumab have left a bitter taste and may have had a negative impact on the field. These disappointments underscore the limitations inherent in broadly applying complement-specific drugs in inflammatory diseases. Many of the compounds that have been discontinued were designed to target multifaceted disorders such as I/R injury. In these disorders, many parallel mechanisms are involved, and the influence of complement is still a matter of debate. For such complex indications, the key to success may be to use complement drugs not as single therapies but (in consonance with their name) as complementary therapies. Clearly, more basic research and additional tests are required in this context. In cases in which clear complement targets can be identified (as in HAE or PNH), complement therapeutics have already been successfully applied.

Several factors are expected to boost the development of complement therapeutics. In particular, our accumulating knowledge about the exact role of complement in various diseases will greatly facilitate the identification of relevant targets. Even more importantly, crystal structures of a number of crucial complement components (e.g., C3 (ref. 90), C3b[91,92], C3c[90], C3d[93], factor B[94], factor D[95]) have recently been solved and are available for structure-based design. Genentech has reported a co-crystal structure of C3b and C3c with the extracellular domain of the macrophage complement receptor CRIg[92]. The same group recently demonstrated that soluble murine CRIg selectively inhibits the alternative pathway by competing with C5 for convertase binding, and that it was effective in mouse models of arthritis[96]. Its high selectivity for the alternative pathway and the availability of a detailed crystal structure make CRIg an attractive lead structure and render its further clinical development highly likely. Along with the continuous development of established approaches, the quest for complement therapeutics may get help from an unexpected source: human pathogens. *Staphylococcus aureus* (*S. aureus*) alone offers several interesting candidates. Among them, the extracellular fibrinogen-binding protein[28] and the staphylococcal complement inhibitor[30] of *S. aureus* have been reported to inhibit the conversion of C3 to C3a and C3b. An understanding of their interaction sites and mechanisms could be applied to developing either potent complement inhibitors or new antibiotics targeting *S. aureus*. In addition, the chemotaxis inhibitory protein of *S. aureus*[29] has been found to antagonize C5aR and may therefore serve as a lead structure for novel antagonists.

In many aspects, complement drug discovery illustrates general trends that are observed in the pharmaceutical industry. Limitations in the development of small compounds (e.g., lack of specificity or inability to disrupt protein-protein interactions) and improved technology and safety in the production of natural macromolecules has led to a growing market for these biopharmaceuticals. An analysis of the compounds indicates that they almost exclusively represent large proteins and nucleotides, or that they have been developed from peptides. Furthermore, major pharmaceutical companies tend to outsource early drug discovery phases to academic institutions and small startup companies. Partnerships with major pharmaceutical companies are usually only sealed after clinical trials have begun (often after phase 1/2). Indeed, many of the promising complement drug candidates (e.g., compstatin and PMX-53) were initially developed in the academic field and further developed by biotechnology companies. Such partnerships have already been established in the case of ofatumumab (Genmab/Glaxo), pexelizumab (Alexion/Procter & Gamble, Cincinnati, OH, USA) and neutrazumab (G2 Therapies/Novo Nordisk).

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Effects of Protein Aggregates: An Immunologic Perspective

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ABSTRACT

The capacity of protein aggregates to enhance immune responses to the monomeric form of the protein has been known for over a half-century. Despite the clear connection between protein aggregates and antibody mediated adverse events in treatment with early therapeutic protein products such as intravenous immune globulin (IVIG) and human growth hormone, surprisingly little is known about the nature of the aggregate species responsible for such effects. This review focuses on a framework for understanding how aggregate species potentially interact with the immune system to enhance immune responses, garnered from basic immunologic research. Thus, protein antigens presented in a highly arrayed structure, such as might be found in large nondenatured aggregate species, are highly potent in inducing antibody responses even in the absence of T-cell help. Their potency may relate to the ability of multivalent protein species to extensively cross-link B-cell receptor, which (1) activates B cells via Bt kinases to proliferate, and (2) targets protein to class II major histocompatability complex (MHC)-loading compartments, efficiently eliciting T-cell help for antibody responses. The review further focuses on protein aggregates as they affect an immunogenicity risk assessment, the use of animal models and studies in uncovering effects of protein aggregates, and changes in product manufacture and packaging that may affect generation of protein aggregates.

KEYWORDS: aggregates, container closure system, immunogenicity, neutralizing antibody, tolerance

INTRODUCTION

The immune system has evolved to respond to invasive threats posed by microbial organisms. Such organisms have inherent "signatures" consisting of repetitive displays of proteins, polysaccharides, nucleic acids, or lipids on their external surfaces. In addition, pathogenicity of such organisms depends on complex protein structures critical for invasion and propagation such as enzymes (eg, neuraminidase). The immune system has both an innate response armamentarium, consisting of multiple cellular and humoral pattern receptors, including toll-like receptors (TLRs), which bind to conserved molecular patterns and trigger rapid defense responses, as well as an adaptive immune response arm that responds to unique microbial proteins. Such adaptive responses include pathogen-specific antibody, which may be generated independent of T-cell help (ie, a T helper independent [TI] response) or, less efficiently, may require collaboration with T-helper cells (Th) (ie, a T-helper dependent [TD] response).

An important element of the efficient response to microbial antigens is the rapid production of antibody by B cells that do not require T-cell help. The requirements for generation of antibody to such determinants have been examined in detail for both relatively simple polymers of peptides and polysaccharides, as well as for higher order structures such as viral capsids, composed of repetitive arrays of multiple protein components. Investigating the molecular requirements for TI antibody responses to polysaccharides, Dintzis et al[1] hypothesized that the B-cell stimulatory signal is "quantized" in that a minimum number of cell-surface-expressed antigen receptors must be connected together in a spatially contiguous cluster that they defined as an "immunon." They found that the molecular mass of polymers that successfully triggered TI antibody responses exceeded 100 kDa and that the valence of the hapten moieties exceeded 10. Successive work by many in the field indicated that the generation of a signaling complex leading to B-cell activation and antibody production depended on factors in addition to molecular mass and hapten valency, including hapten affinity, polymer rigidity, and binding kinetics.[2] More recently, Bachmann and Zinkernagel[3] extensively examined the effects of antigen organization on antibody responses by challenging transgenic mice, tolerant to a viral coat protein from vesicular stomatitis virus (VSVgp), with VSVgp in progressively more ordered arrays. Despite tolerance to the soluble monomeric protein, presentation of VSVgp in a loose aggregate was able to generate antibodies in a TD fashion, while its presentation in a highly ordered viral capsid generated antibody in the absence of T-cell help,[3] clearly demonstrating the potency of highly ordered structures in eliciting rapid and efficient immune responses.

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The central conclusion of this body of work is that high molecular weight (MW) arrays of antigen are highly efficient in eliciting an antibody response independent of T-cell help, whereas their less ordered counterparts are less immunogenic and may require T-cell help to generate an immune response.

The activation of antibody-producing B cells via the extensive cross-linking of B-cell receptor (BCR) by multivalent ligand involves activation of Bruton's tyrosine kinase/Tec kinase.[4] While this signaling apparatus is of itself sufficient to induce proliferation of B cells, antibody production appears contingent on delivery of a second signal, mediated by Th cells, or potentially by other signaling pathways including those mediated via TLR.[2]

Microbial pathogens express many enzymes and other structures necessary for viral invasion and proliferation. The activity of such pathogenic proteins depends critically on the 3-dimensional conformation of the active site. To protect the host, the immune system makes antibodies that are specific for the active site of the protein (ie, conformation-dependent antibody that neutralizes enzymatic activity). In fact, this preference for antibody to conformational rather than linear determinants of proteins appears to be a general property of the immune system. Thus, even when proteins pose no threat, antibodies are preferentially directed to the conformationally configured portions of the protein. For example, in a recent study by Ito et al,[5] it was found that in mice immunized with isolated hen egg lysozyme (HEL), a complex protein with significant tertiary structure, all of the mAbs elicited (15/15) reacted to native protein, with only 2 of the antibodies also recognizing linear epitopes present in the reduced form, implying that conformational determinants were the binding ligands. The group found that this was the pattern for 4 different nonmicrobial proteins that underwent similar scrutiny.

Additional evidence of the immune system's proclivity for conformation-dependent antibody is found in responses to endogenous proteins such as amyloid proteins. Thus, Nath et al[6] and O'Nuallain and Wetzel[7] found that patients with Alzheimer's disease mounted significant antibody responses to Aβ amyloid structures, but that such antibodies did not bind to the monomeric Aβ amyloid precursor protein. Intriguingly, O'Nuallain and Wetzel found that antibodies to Aβ amyloid structures cross-reacted significantly on amyloid structures from other proteins whose primary amino-acid sequences differed dramatically from that of the Aβ amyloid precursor. Thus, antibody responses to these proteins are specific for common higher-order structural elements. Finally, neutralizing antibody responses to therapeutic protein products appear to be dominated by conformation-specific antibody: serum from 12/13 patients with pure red cell aplasia (PRCA) resulting from antibodies to erythropoietin failed to bind to denatured erythropoietin containing only linear determinants[8]; and neutralizing antibody to human thrombopoietin (TPO) was not inhibited by short peptides of TPO that would have interfered with antibody binding to linear, but not conformational, determinants.[9]

So, how do protein aggregates relate to this "immunologic imperative"? First, we need to define what we mean by protein aggregates. For the purposes of this review, written from an immunologic and not a biochemical perspective, protein aggregates are defined very broadly as high MW proteins composed of multimers of natively conformed or denatured monomers. Such species may be soluble or insoluble (particulate), and reversible or irreversible, within the given environment. Moreover, in keeping with an immunologic focus on the consequences of such species, 3 types of protein aggregates are considered: the first, an assembly of native proteins in a polymeric structure; the second, an assembly of denatured protein irreversibly associated (within the given environment) and dependent on hydrophobic interactions; and the third, covalently linked proteins, which could be either in a native or denatured state.

The hypothesis is that the potency of protein aggregates and, particularly, of particulate protein aggregates in eliciting immune responses pertains to their resemblance to the microbial "signatures" to which the immune system has evolved. Thus, immunity to defined proteins can be enhanced by ensuring aggregation of native proteins in a rigid (eg, virus capsid) type of presentation,[10] an advantageous approach for vaccines. In contrast, minimization of immunogenicity of therapeutic protein products is best accomplished by ensuring stability of the native protein conformation and minimizing formation of high MW species. Our model, thus, envisions that antibody responses to high MW protein aggregates may be elicited in a similar fashion to those elicited by microbial pathogens, (ie, via multivalent ligand cross-linking of BCR).

There are 2 principal pathways by which multivalent ligand cross-linking of BCR enhances immune responses: (1) acceleration of antigen processing and presentation to Th cells,[11] and (2) activation of Bruton's tyrosine kinases mediating B-cell proliferation.[4] Thus, early and rapid IgM responses may be generated to aggregated protein in advance of the provision of T-cell help, but unlike polysaccharide antigens, which are not capable of recruiting T-cell help, protein aggregate-mediated BCR cross-linking would be expected to accelerate the recruitment of T-helper cells.

Induction of Immune Responses by Protein Aggregates: Qualitative Factors

Both qualitative and quantitative factors pertaining to the aggregate species per se, as well as factors independent of protein structure or amount are important in the ability of

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any particular aggregate species to induce an immune response. Among the qualitative factors critical in inducing antibody responses are MW and solubility. While low MW aggregates such as dimers and trimers appear inefficient in inducing immune responses, large multimers whose MW exceeds 100 kD are efficient inducers of immune responses. However, multimerization is key to immunogenicity, as larger-sized monomeric proteins are not necessarily more immunogenic than smaller monomeric proteins. Moreover, it has been long known that particulate (insoluble) antigens are very rapidly endocytosed by antigen-presenting cells, which initiate immune responses.[12] More recent work has shown that blood-borne dendritic cells ($CD11c^{lo}$ Mac1+) capture particulate antigens in the periphery, migrate into spleen, and induce TI antibody responses by activating and enhancing the survival of marginal zone (MZ) and B1 B cells.[13] Thus, it is possible that particulate protein aggregates introduced into the systemic circulation meet a similar fate, and traffic to MZ B cells, where the immune response proceeds independent of T-cell help.

Other critical factors bearing on immunogenicity of aggregates pertain to both product and host: product origin (foreign versus endogenous), the presence of product contaminants with immunomodulatory activity, the presence of neoepitopes (as may be created in fusion proteins), and glycosylation/pegylation may exert profound influences on either the formation of aggregates, or the generation of immune responses to aggregates. Host and protocol factors important in determining the potency of aggregate species in triggering immune responses include the frequency of administration, the route of administration, the immunomodulatory activity of the product itself, the host immune status, the activity of concomitant immunomodulators, and for therapeutic versions of endogenous proteins, the robustness of immunologic tolerance to the endogenous protein.

Thus, small amounts of aggregates would be expected to generate a robust response to foreign proteins, but not necessarily for therapeutic counterparts of endogenous proteins to which the immune system has been tolerized. However, the mammalian immune system is not equally tolerant to all endogenous proteins. Most notably, the works of Weigle[14] and of Goodnow[15] have demonstrated that the relative abundance and manner of presentation of the endogenous protein are essential in determining the extent to which the immune system is tolerized to that protein. Moreover, T cells are tolerized more readily by proteins at low abundance than are B cells, perhaps because of the active expression of tissue-specific antigens in the thymus by promiscuous gene expression.[16] Thus, aggregates of relatively low abundance proteins, to which we are not robustly tolerant, might potently trigger immune responses, whereas aggregates of high abundance proteins may have a limited ability to induce immune responses.

Risk Analysis of Protein Aggregates in Therapeutic Protein Products

Protein aggregates pose risk in terms of generation of immune responses to the therapeutic protein product. Of principal concern are those immune responses associated with adverse clinical effects: neutralizing antibody that inhibits the efficacy of the product or, worse, cross-reactively neutralizes an endogenous protein counterpart; and severe immediate hypersensitivity responses such as anaphylaxis.

Regarding immediate hypersensitivity responses, mediated by IgE, the role of protein aggregates in generating such responses is not known. In fact, speculation by Aalberse and Platts-Mills[17] is that aggregates, in increasing the "strength" of an immune stimulant, might deviate an IgE response to a far less devastating IgG4 response. Moreover, a response may be driven from IgE to IgG via TLR-mediated signaling.[18] Nonetheless, once an IgE response has been generated, aggregated allergen is highly efficient at triggering degranulation of mast cells via aggregating IgE receptors.

In considering the mechanism by which protein aggregates might generate neutralizing antibody responses in a highly efficient manner, it is clear that preservation of the conformation of the active site of the protein or proteins within the aggregate is required. This strategy is potentially useful for vaccines, where it is critical to neutralize the natively conformed structures responsible for invasion or toxicity. In contrast, this configuration might prove devastating for therapeutic proteins. Of great importance for avoiding the formation of natively conformed aggregates in therapeutic protein products is product formulation.[19-22] Thus, "structural" formulations that form a bilayer into which proteins can potentially insert, such as nonionic and ionic detergents and liposomes, are of interest. Insertion of proteins into such bilayers is feasible for membrane proteins, which contain clustered hydrophobic residues, whereas such a configuration is not common for soluble therapeutic proteins. Moreover, micelles of the nonionic detergent, polysorbate, are relatively small and likely not capable of incorporating more than a few therapeutic protein molecules, whereas the extended bilayers of ionic detergents, such as sodium dodecylsulfate (SDS), may contain substantial numbers of proteins capable of insinuating themselves into the structure.

More likely to be present in therapeutic protein products are denatured protein aggregates that are generated via unfolding of the native conformation of the protein. While aggregates of denatured protein may potently induce antibody, they would not be expected to induce neutralizing antibody because protein conformation was lost on denaturation. However, antibodies directed to linear epitopes within the

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binding site could effectively neutralize if they bound to a linear determinant critical in ligand-receptor binding. Such occurrences appear to be rare. Moreover, through epitope spreading, antibodies that bind to the protein but do not neutralize its activity may facilitate the generation of neutralizing antibodies. However, this is not a rapid and highly efficient process. Thus, for many therapeutic protein products, development of neutralizing antibody is preceded by the existence of a high titered and prolonged binding antibody response. Moreover, other immunologically provocative factors may also be required. In such cases preventing a neutralizing antibody response may be possible by limiting the titer and duration of the primary response, which may limit epitope spread.

Although antibodies that bind but fail to neutralize product are of somewhat lesser concern than neutralizing antibody, in some circumstances such antibodies have been shown to (1) affect the pharmacokinetics of product, necessitating more frequent dosing,[23] (2) mediate anaphylactoid reactions,[24] and (3) potentially facilitate epitope spreading.

Evidence for the Role of Product Aggregates in Induction of Immune Responses to Therapeutic Protein Product: Animal Models

Much of the information framing our understanding of the immunogenicity of protein aggregates and the relative tolerogenicity of soluble protein monomers comes from studies performed in the 1950s and 1960s in which human immunoglobulin (Ig) products were injected into experimental rodents. Such studies found that immune responses to human Ig preparations could be eliminated by techniques that removed high MW material from the preparation.[25] In Gamble's studies in 1966, aggregates generated by heat treatment and added back to deaggregated tolerogenic Ig preparations were shown to induce an immune response that, in a dose-dependent fashion, decreased the time to appearance of antibody and increased the antibody titer.[26]

The use of Ig as a model antigen for studying the effects of aggregates on the immune response was, in retrospect, an interesting one, as these proteins differ from most other proteins in terms of complex immune system interactions. Thus, Ig can modify activation of the immune response to itself through the binding and coligation of multiple receptors on the B-cell surface. While the antigen binding fragment (Fab) of an antibody molecule binds to BCR, the complement fixing fragment, Fc, can simultaneously bind to Fc receptors on the B-cell surface. Coligation of BCR with FcR (FcγRII), the only (and inhibitory) FcR expressed on B cells, limits antibody responses.[27] In contrast, complement bound to the Fc moiety of an antibody molecule can bind to complement receptors (CR2) on the B-cell surface. In this case, simultaneous ligation of BCR and complement receptor (CR2) on the B-cell surface profoundly enhances antibody production.[27] Aggregated IgG and pentameric IgM bound to antigen can directly fix and activate complement. Whether such factors influenced immunogenicity of the aggregated species in these studies in mice is not certain, as the affinity and binding of human Ig with rodent complement, complement receptors, and FcRs is not clear.

Although ordinary protein antigens cannot simultaneously bind to BCR and FcR or CR, immune responses to proteins may be potentiated by the formation of protein:antibody complexes. For example, IgM potently enhances antibody responses to particulate antigen, while IgG enhances responses to soluble proteins. Reciprocally, IgM does not enhance responses to small soluble proteins and IgG suppresses responses to particulate antigens. The boost in antibody response resulting from the binding of IgM to antigen requires complement fixation and activation, whereas enhancement through IgG is mediated by engagement of activating FcR on non-B cell antigen presenting cells, such as macrophages.[27] Thus, IgM bound to large protein aggregates may well enhance the antibody response through direct uptake by complement receptors, or via the simultaneous ligation of BCR by the aggregated protein and CR through complement fragments bound to Fc. IgG binding to smaller soluble aggregates may theoretically also enhance antibody responses via activating FcR.

The ability of protein aggregates to elicit antibody responses to a self-protein was most objectively and thoroughly evaluated by Braun et al, who employed human interferon alpha (huIFN-α) transgenic mice to evaluate the capacity of IFN-α product aggregates to break tolerance.[28] They clearly demonstrated that homogenous aggregates of huIFN-α and composite aggregates of mouse serum albumin and huIFN-α potently induced antibody to huIFN-α, whereas IFN-α monomer failed to do so (within the time frame of the study). Unfortunately, for the preparation containing homogenous huIFN-α aggregates, the characteristics of the aggregate species responsible for the efficiency of response induction—the large amount of dimer in the product versus the "very small amount" of high MW aggregate—were not explored. Also critical in this study was the elucidation of the role of dosing frequency, route of administration, and immunomodulation on generation of antibodies, albeit in response to nonaggregated IFN-α monomer. However, these factors may also be critical in the ability of protein aggregates to trigger immune responses, particularly to antigens to which the immune system is tolerant. Thus, more frequent dosing, a subcutaneous (SC) or intraperitoneal (IP) route of administration rather than an intravenous (IV) route, and administration of concomitant immunomodulators would be expected to facilitate the ability of small amounts of aggregates to enhance the immune response.

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Evidence for the Role of Product Aggregates in Induction of Immune Responses to Therapeutic Protein Product: Clinical Studies

Clinically, evidence that aggregates in therapeutic protein products had profound effects on immunogenicity was apparent from very early studies. In the 1950s and early 1960s intravenous immune globulin (IVIG) preparations contained substantial aggregated material that triggered severe hypersensitivity (anaphylactoid) responses due to fixation and activation of the complement cascade and also potentially to release of histamine.[29,30] Antibody responses to such aggregates appeared to be specific for novel determinants present only on the aggregated species (ie, for higher order structures) or for normally cryptic determinants, which are exposed on aggregates of denatured protein.[26] Of importance, responses were far more prevalent and potent in the antibody-deficient patient population than in those without antibody deficiency, suggesting that antibodies generated to human Ig in nontolerant patients increased the frequency and severity of these reactions.[29] These types of adverse clinical responses were also noted in patients treated with early commercial preparations of human serum albumin (HSA) and pasteurized plasma solutions.[24]

A further example of the potency of large amounts of aggregates on generation of immune responses is that of the clinical experience with human growth hormone (hGH). Originally purified from formalin-fixed pituitary glands, hGH contained substantial amounts of aggregates (50%-70%) that induced immune responses. Although hGH prepared by an improved method contained substantially less aggregated material (10%), antibody responses were nevertheless still substantial. Intriguingly, the level of aggregates appeared to determine not whether there would be an antibody response, but rather the nature of the antibody response. Patients treated with the heavily aggregated product demonstrated persistent antibodies to hGH, whereas those treated with the lesser aggregated product developed a transient antibody response.[31] Despite the frequency of appearance of binding antibody in response to hGH therapy, the development of neutralizing antibody was uncommon. However, neutralizing antibody occurred much more commonly in patients with severe congenital isolated GH deficiency (ie, in protein "knock out" children in whom the lack of hGH protein conferred a lack of immune tolerance to the hormone).

The therapeutic protein IL-2 can be viewed as a good test of the general principles governing immune response generation to large MW aggregates expressing multivalent antigens. Indeed, although the monomeric protein is ~15 kD, the recombinant human product is formulated as an aggregate with an average size of 27 molecules per aggregate.[32] Thus, a considerable amount of the product has a MW >100 kD as well as having >20 ligands per aggregate, conditions that were shown to optimize immunogenicity. Indeed, this product is highly immunogenic, inducing binding antibody responses in 80% to 100% of patients.[33] Of interest, neutralizing antibody to IL-2 largely arises in a group of patients treated with IL-2 in an immunologically provocative fashion (ie, SC administration or concomitant treatment with IFN-α, a known immunomodulator, and extensive treatment over a prolonged time period).[33] Also intriguing is the time course in which the neutralizing response arises, as it is detected several months following detection of binding antibody, implicating epitope spreading as the likely route of development. Moreover, it is not clear whether T-cell help is required for either binding or neutralizing antibody.

In fact, these few examples illustrate a more general principle, which is that for most therapeutic proteins, product neutralizing antibody is much less common than product binding antibody. Neutralizing antibody arises more frequently with the following risk factors: generation of a high titer binding antibody response, sustained treatment, and treatment by a more immunogenic route (ie, SC), concomitant treatment with immune modulators, and genetic deficiency of the factor. There are some very notable exceptions to this rule, one being the case of immune responses to the TPO congener, pegylated megakaryocyte growth and development factor (PEG-MGDF), in which as few as 2 doses were sufficient to induce a vigorous neutralizing antibody response.[9] However, the very low abundance of the endogenous protein counterpart, TPO, in normal physiology[34] predicts that tolerance to this protein would be minimal, a situation somewhat akin to individuals with genetic deficiencies of particular proteins (such as hGH) who are highly prone to formation of neutralizing antibody. The SC route of administration was an additional provocative factor in triggering the TPO neutralizing response, as was the immune competency of the patient population. Moreover, whether immunogenicity was triggered by truncation of the full length TPO, thereby exposing the normally protected active site to the immune system, is not clear. Certainly the pegylation of the truncated TPO would have been expected to confer protection against immune response generation.

Why the immune system commonly makes robust responses to nonneutralizing determinants yet has to be mightily provoked to make responses to neutralizing determinants, suggests that the immune system is more tolerant (or ignorant) of the active site than of other epitopes, which raises several interesting possibilities: the active sites of self-proteins are normally very well shielded from antigen specific T- and/or B-cells[35]; the active sites of proteins lack primary sequence for complexation with self Class II MHC and thus fail to be T-cell immunogens; or the active sites of proteins may be exposed to the immune system in such a way as to induce a

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higher level of tolerance than other portions of the protein (ie, through binding of the active site to a cell surface or internal receptor with resultant altered processing and presentation or via proximity of the active site to disulfide bonds, which may be processed and presented differently than other epitopes).[36] Substantial work needs to be done to investigate such issues.

Measurement of Product Aggregates/High Risk Changes for Aggregate Generation

Protein aggregates in therapeutic protein products are almost exclusively assessed by a size exclusion chromatography (SEC) method. While sensitive, this method has critical flaws, including failure of some high MW species to penetrate the gel. Thus, methods orthogonal to SEC need to be employed to assess product aggregates, particularly in the setting of comparability assessments but potentially also for routine lot release.[37-42] Moreover, in routine testing for product aggregates, an inherent assumption is that very few samples are required to be tested for any given lot, as justified by the demonstrated homogeneity of the bulk fill and the presumed homogeneity of the container closure.

The Food and Drug Administration (FDA) is particularly aware of container closures that are not homogeneous in the content of compounds that might bear on generation of product aggregates. Thus, use of glass syringes as storage vessels has been problematic from the perspective that the tungsten used to create the syringe is left in residual amounts that vary widely across syringes in a given lot. The leaching of tungsten oxides caused reactions with at least 2 protein therapeutic products and caused them to aggregate. Moreover, uncoated plunger stoppers from syringes leached the vulcanizing agent used to cross-link the rubber, and it is speculated that this agent acted as an immune adjuvant in inducing antibody responses to Eprex, causing a surge in PRCA cases in renal failure patients.[43] Finally, vial stoppers leached metals that activated a metalloprotease coeluting with a therapeutic protein, causing product truncation. Thus, the FDA now views changes in container closure to be a high-risk change, necessitating extensive scrutiny for effects on product quality. Furthermore other changes that are likely to affect product aggregation and, hence, immunogenicity consist of changes in formulation, changes in virus and adventitious agent removal, and changes in source material/cell line.

Changes in adventitious agent removal steps are of high risk with respect to altering product aggregation and immunogenicity. Thus, addition of a pasteurization step to manufacture a factor VIII product (63C° for 10 hours) enhanced product immunogenicity. Although increased product aggregation was not investigated, it is very likely that an increase in product aggregates was etiologic in the outcome.[44] Other high-risk changes include changes in formulation and in source materials or cell lines.

Conclusion

In summary, protein product aggregates are potent inducers of immune responses to therapeutic protein products. The extent to which these responses impact on therapy is determined by multiple factors. Manufacturers of therapeutic protein products should ensure that their products contain minimal product aggregates, that they employ several orthogonal methods for assessment of product aggregates, and that manufacturing changes or container closure changes prompt vigorous investigation of changes in levels of product aggregates.

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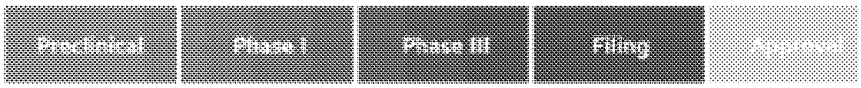
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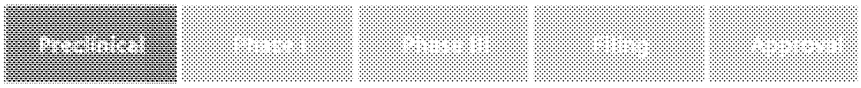


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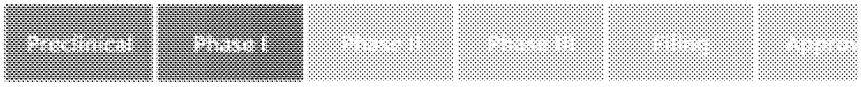
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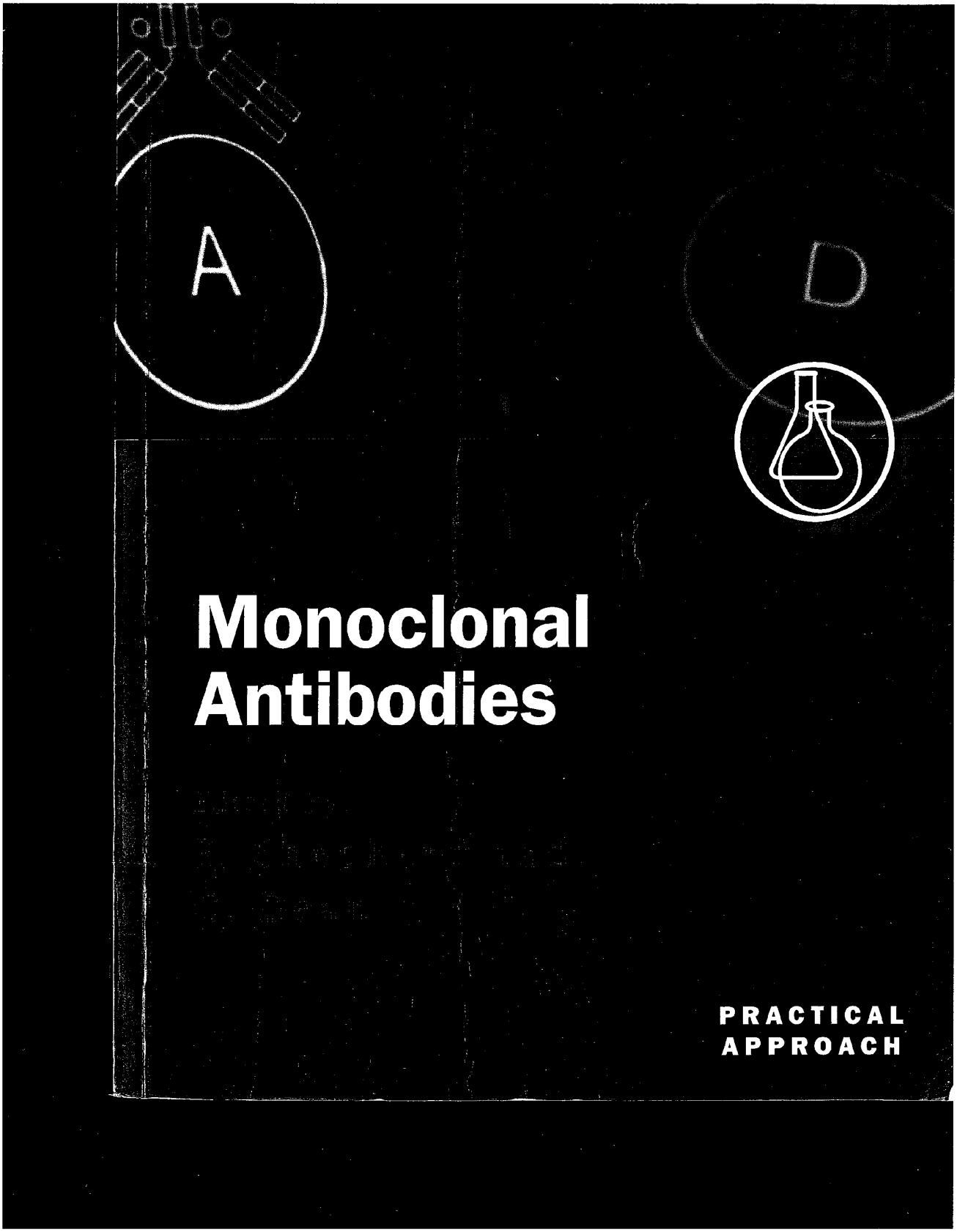
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Monoclonal Antibodies

A Practical Approach

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Appendix

1 The design of the humanized antibody

Humanization by CDR grafting (also called reshaping) is now a well-established procedure for reducing the immunogenicity of monoclonal antibodies (mAbs) from xenogeneic sources (commonly rodent) and for improving their activation of the human immune system; in fact there are many humanized mAbs in clinical trials and a few have been given approval to be used as drugs. Although the mechanics of producing the engineered mAb using the techniques of molecular biology are relatively straightforward, simple grafting of the donor rodent complementarity determining regions (CDRs) into human acceptor frameworks does not always reconstitute the binding affinity and specificity of the original mAb. The design of the engineered mAb is now the critical step in reproducing the function of the original molecule. This design includes various choices: the extents of the CDRs, the human frameworks to use, and the substitution of residues from the rodent mAb into the human framework regions (backmutations). The positions of these backmutations have been identified principally by sequence/structure analysis or by analysis of an homology model of the variable regions 3D structure. Recently, phage libraries have been used to vary the amino acids at chosen positions (1, 2). Similarly, many approaches have been used to choose the most appropriate human frameworks in which to graft the rodent CDRs. Early experiments used a limited subset of well-characterized human mAbs (often where the structure was available), irrespective of the sequence identity to the rodent mAb (the so-called 'fixed frameworks' approach). Some groups use variable regions with high amino acid sequence identity to the rodent variable regions ('homology matching' or 'best-fit'); others use consensus or germline sequences, while still others select fragments of the framework sequences within each light or heavy chain variable region from several different human mAbs. There are also approaches to humanization developed which replace the surface rodent residues with the most common residues found in human mAbs ('resurfacing' or 'veneering') and those which use differing definitions of the extents of the CDRs.

Not surprisingly, some rodent mAbs have proved difficult to humanize using standard protocols (3). The design and engineering of humanized mAbs are still very much areas of research, as much for the light they shed on protein structure and function as well as for the potential therapeutic and diagnostic benefits. For

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this reason, an iterative protocol called the 'Humanization design cycle' (see *Protocol 1*) is described in the accompanying sections. These sections additionally supply data and raise issues of concern to the prospective antibody designers to help them through the bewildering variety of choices. The data is also available on the WWW under either of the two following 'Humanization bY Design' urls:
`http://mathbio.nimr.mrc.ac.uk/jsaldan` or
`http://www.cryst.bbk.ac.uk/~ubcg07s`
with hypertext links to the various resources for database search, sequence analysis, and structural modelling. Search forms for Sequence and Text input are also available under these urls to aid navigation through the list of previously humanized antibodies taken from the literature.

Protocol 1

The humanization design cycle

Equipment

- Any computer of reasonable power (for instance Pentium processor, 32 Mb RAM) which is connected to the WWW through a standard browser (e.g. Netscape) is needed to accomplish the design protocol

Method

1. Examine the source donor amino acid sequences for each chain (see Section 1.1.1).
2. Produce a structural model of the Fv regions (see Section 1.1.2).
3. Consider the human acceptor framework sequences for each chain (see Section 1.1.3).
4. Identify putative backmutations in the chosen frameworks (see Section 1.1.4).
5. Reconsider framework choice and design humanized antibody sequence.
6. Construct humanized and chimeric antibody sequences.
7. Test constructs.
8. Success? If 'No' then return to step 3.

1.1 Issues to consider

1.1.1 The source donor amino acid sequences

In the source donor sequence for each chain (heavy and light) it is important to consider:

(a) The complementarity determining regions. These contain the residues most likely to bind antigen and thus they must be retained in the humanized antibody (unless the precise residues involved in binding are known which is unlikely). CDRs are defined by sequence according to the Kabat scheme or by structure according to the Chothia scheme (see *Table 2*). The advantage of using the latter definitions is that the CDRs are shorter, therefore the humanized antibody will have fewer xenogeneic fragments. However, the

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experience of other groups (4) has shown that using the Kabat definition generally requires fewer iterations in the humanization design cycle. A third scheme for defining CDR extents (see *Table 2*) called the 'contact definition' from Andrew Martin is based on an analysis of antibody/antigen interactions. It is likely to be useful in humanization but is as yet untested.

(b) Canonical residues. These are key residues which determine to a large extent the structural conformation of the CDR loop. They were originally defined by Chothia, but have now been revised by Andrew Martin. His WWW form at url: `http://www.biochem.ucl.ac.uk/~martin/abs/chothia.html` allows input of the variable region sequences and automatic identification of the canonical structure class and important residues. Canonical residues are almost always retained in the humanized antibody if they are different to those in the human frameworks. Numerous examples of the importance of canonical residues can be found in the literature which can be searched for in the 'Humanization bY Design' web sites.

(c) Interchain packing residues. Defined by Novotny and Haber, and Chothia at the interface between the variable heavy and variable light chain domains (see *Table 3*). Generally, unusual packing residues should be retained in the humanized antibody if they differ from those in the human frameworks. Their importance is illustrated in the humanization of mAb 1B4 (5).

(d) Unusual framework residues. These can be located using Andrew Martin's WWW form at url: `http://www.biochem.ucl.ac.uk/~martin/abs/seqtest.html`. For mouse antibodies, it is better to determine the subgroup using the form on the Kabat Database WWW site at url: `http://immuno.bme.nwu.edu/subgroup.html` and identify the residue positions which differ from the consensus. These donor-specific differences may point to somatic mutations which enhance activity. Unusual residues close to the binding site may possibly contact the antigen. However, if they are not important for binding, then it is desirable to remove them because they may create immunogenic neoepitopes in the humanized antibody. Note that sometimes unusual residues in the donor sequence are actually common residues in the human acceptor sequence. Unusual residues in the acceptor frameworks are not desirable because of the possibility of immunogenicity.

(e) Potential *N*- or *O*-glycosylation sites. It is well known that potential *N*-glycosylation sites are specific to a consensus pattern N-X-S/T. These sites can be located in the donor sequence using the Prosite WWW form at url: `http://www.expasy.ch/sprot/scnpsite.html`. Removal of potential *N*-glycosylation sites has not destroyed the binding avidity of a humanized antibody thus far, and in one case actually increased it (6). *O*-glycosylation sites are usually found in helical segments which means that they are not common in the beta-sheet structure of antibodies. They have no consensus pattern, but differences in *O*-linked glycosylation between chimeric and humanized forms of BrE-3 was hypothesized to cause the increased binding of the humanized mAb (7).

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Table 2 CDR loop definitions (numbering according to Kabat *et al.*, ref. 15)

Loop	Kabat	Chothia	Contact
CDR-L1	L24–L34	L26–L32	L30–L36
CDR-L2	L50–L56	L50–L52	L46–L55
CDR-L3	L89–L97	L91–L96	L89–L96
CDR-H1	H31–H35	H26–H32	H30–H35
CDR-H2	H50–H65	H52A–H55	H47–H58
CDR-H3	H95–H102	H95–H101	H93–H101

Table 3 Residues at the VL/VH domain interface[a]

	Kabat number[b]	Mouse[c]	Human[c]
VK	34	H678 N420 A408 Y147 E114	A531 N147 D66
	36	Y1653 F198 L96	Y748 F80
	38	Q1865 H47	Q799 H22
	44(+)	P1767 V132 I40	P839 L5
	46	L1381 R374 P97	L760 V37
	87	Y1457 F448	Y795 F41
	89	Q1170 L206 F144	Q687 M107
	91	W376 S374 G356 Y295 H182	Y404 R115 S105 A84
	96(+)	L537 Y380 W285	L134 Y215 F78 W73 I71
	98(+)	F1724	F654
VH	35	H1001 N636 S402	E184 S527 H340 G167 A143
	37	V2336 I200	V1037 I477 L27
	39	Q2518 K67	Q1539 R16
	45(+)	L2636 P16	L1531 P24
	47	W2518 L64 Y50	W1534 Y21
	91	Y2149 F479	Y1429 F116
	93	A2202 T222 V102	A1346 T90 V71
	95	Y399 G375 S340 D340 R226	D268 G266 R109 E100
	100k(+)	F1285 M450	F540 M109 L33
	103(+)	W1469	W323

[a] The positions of interdomain residues were as defined by Chothia *et al.* (16).

[b] Numbering is according to Kabat *et al.* (15). Residues underlined are in the framework, other residues are in the CDRs. (+) residues are the six that form the core of the VL/VH interface according to Chothia *et al.* (16).

[c] The number following the one-letter amino acid code is the frequency taken from Kabat (November 1997 dataset; see url in the text). The number of sequences examined is identical for each domain and equal to 1997 and 852 for mouse and human sequences, respectively; except for residue 103 in the heavy chain where numbers are 1651 and 369 for mouse and human sequences, respectively.

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1.1.2 Modelling the Fv regions

A carefully built model of the Fv regions of the source antibody and in some cases of the designed humanized antibody, is a common denominator in many of the successful humanization experiments. It is possible to build a model completely automatically using commercial packages or academic servers such as Swiss-Model at url: `http://www.expasy.ch/swissmod/SWISS-MODEL.html`. However, the danger of allowing a computer to make all the decisions is highlighted in the humanization of antibody AT13/5 (8) where the interaction between residues at positions 29 and 78 (Kabat numbering) in the heavy chain was not modelled correctly. The experience of an expert in protein sequence analysis and modelling is always worthwhile.

1.1.3 Choice of human acceptor frameworks

Despite this being the most critical part of the humanization design cycle, there are no hard-and-fast rules for choosing the acceptor frameworks into which to graft the donor CDRs. This is because the benefit of the various choices (in terms of immunogenicity in the patient) has not clearly been proved in the clinic. Therefore, there are only a set of approaches which have arisen from the collective experience of previous humanization experiments.

(a) Fixed frameworks or homology matched. Some groups prefer to use fixed frameworks (usually antibody NEW for the heavy chain and REI for the light chain since their structures are solved) for all their humanized antibodies. Other groups try to use the frameworks that are most similar to their donor sequence (homology matching or best-fit). A subtle comparison of the two methods in terms of the ease of producing a functional humanized antibody appears to favour homology matching (9). Note that it is entirely possible to 'mix and match' framework regions (i.e. choose frameworks 1–4 from different human antibodies according to sequence similarity) although this approach hasn't been tested exhaustively.

(b) Light/heavy chain frameworks from the same/different clone. In general, light and heavy chains from the same antibody are more likely to form a functional binding site than light and heavy chains from different antibodies. However, since the interface between variable domains is so well conserved (see *Table 3*), this is not usually a problem. A comparison of the two approaches once again appears to favour the latter (10).

(c) Human subgroup consensus or individual framework sequences. Being limited to frameworks from particular human antibodies runs the risk of the somatic mutations creating immunogenic epitopes, even though the frameworks are human. An alternative approach is to use the frameworks from human consensus sequences where idiosyncratic somatic mutations will have been 'evened out'. The two approaches have been compared; in one case showing no difference in binding avidities (11) and in the other case showing better binding with individual frameworks (12). Consensus sequences for human and mouse subgroups can be found under urls:

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http://mathbio.nimr.mrc.ac.uk/jsaldan/cons/cons.html; or
http://www.cryst.bbk.ac.uk/~ubcg07s/cons/cons.html.

(d) Frameworks from germline sequences. Consensus sequences are artificial and although having no idiosyncratic residues, may create unnatural sequence motifs which are immunogenic. An alternative is to use human germline sequences which have been compiled in the database VBase at url: http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html.

Having decided on an approach to take in order to choose the human frameworks; which particular human antibody, consensus, or germline sequence should be used? This is simple in the fixed framework approach since the choice is always NEW for the heavy chain and REI for the light (although since modifications to both frameworks are apparent from the literature (13) even there a choice needs to be made!). The choice is usually made by performing a search for the most homologous sequence over the appropriate database. There are many database search programs available, the two most common being *BLAST* and *FASTA* which can be found at the following two urls respectively: http://www.ncbi.nlm.nih.gov/BLAST/ and http://www2.ebi.ac.uk/fasta3/. *BLAST* is often preferable since it allows searching over the Kabat database. In order to search the consensus or germline sequences, they need to be downloaded onto the local computer. Choice of the particular human frameworks for the light and heavy chain variable regions should be made by trying to match the length of the CDRs, the canonical residues and the interface packing residues (as described in Section 1.1.1), as well as trying to find the highest percentage identity between the donor and acceptor sequences. Unusual residues in the acceptor frameworks are not usually tolerated since they may lead to immunogenic epitopes in the resultant humanized antibody. Try to find human frameworks which are similar (in terms of percentage identity) to the source sequences and also require the least number of backmutations (see next section).

1.1.4 Identify putative backmutations

This is the most difficult and unpredictable procedure in the humanization of monoclonal antibodies. It is also the area which throws much light on protein structure and function. A solid body of data for helping to identify strategic alterations (using the sequence and text search forms) is available on the Humanization bY Design web sites under mirrored urls:
http://mathbio.nimr.mrc.ac.uk/jsaldan/selhum.html;
http://www.cryst.bbk.ac.uk/~ubcg07s/selhum.html.

(a) Analysis of donor and acceptor sequences. Putative point backmutations from the human framework residue back to the original source residue will already have been identified from the analysis of the donor and acceptor sequences for canonical residues, interchain packing residues, unusual residues, and glycosylation sites. Experience has shown that it is especially important to retain the source's canonical and interchain packing residues (though unfortunately not in all cases).

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(b) Vernier zone and CDR-H3. Consider residues in the Vernier zone (see *Table 4*) (14) which form a 'platform' on which the CDRs rest and therefore potentially affect their conformation. Due to its extreme variability, special attention should be paid to CDR-H3, analysing the structural model for residues which may potentially affect its conformation.

(c) Proximity to binding site. Using the structural model, analyse residues within 5–6 Å of any CDR residues. These residues are likely to bind to antigen, especially if they are unusual as classified by the analysis of the source sequence.

(d) Glycosylation sites. Consider removal of any potential glycosylation sites, especially if they are on the surface of the structural model, since experience has shown that this can enhance the avidity of the humanized antibody.

Table 4 Residues in the VERNIER zone

Heavy chain	Light chain
2	2
27–30	4
47–49	35–36
67	46–49
69	64
71	66
73	68–69
78	71
93–94	98
103	–

Having decided on the residues to backmutate, return to the question of human acceptor frameworks. It is not unlikely that an overlooked human framework may actually contain the backmutations which are to be retained. If this is the case, then there is no need to introduce residues from the source sequence, thus making the humanized antibody more 'human'.

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PAPERS FROM MEETINGS

After TGN1412: Recent developments in cytokine release assays

An article based on a presentation at the 20th Summerschool in Immunotoxicology, Beaune, France, September 26–28, 2011.

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Abstract

The failure of regulatory science to keep pace with and support the development of new biological medicines was very publically highlighted in March 2006 when the first-in-man Phase I clinical trial of the immunomodulatory CD28-specific monoclonal antibody (mAb) TGN1412 ended in disaster when all six volunteers suffered a life-threatening adverse reaction termed a 'Cytokine Storm'. The poor predictive value of standard pre-clinical safety tests and animal models applied to TGN1412 demonstrated the need for a new generation of immunotoxicity assays and animal models that are both sensitive and predictive of clinical outcome in man. The non-predictive result obtained from pre-clinical safety testing in cynomolgus macaques has now been attributed to a lack of CD28 expression on $CD4^+$ effector memory T-cells that therefore cannot be stimulated by TGN1412. In contrast, high levels of CD28 are expressed on human $CD4^+$ effector memory T-cells, the source of most TGN1412-stimulated pro-inflammatory cytokines. Standard *in vitro* safety tests with human cells were also non-predictive as they did not replicate *in vivo* presentation of TGN1412. It was subsequently shown that, if an immobilized therapeutic mAb-based assay or endothelial cell co-culture assay was used to evaluate TGN1412, then these would have predicted a pro-inflammatory response in man. New *in vitro* assays based on these approaches are now being applied to emerging therapeutics to hopefully prevent a repeat of the TGN1412 incident. It has emerged that the mechanism of pro-inflammatory cytokine release stimulated by TGN1412 is different to that of other therapeutic mAbs, such that standard pro-inflammatory markers such as TNFα and IL-8 are not discriminatory. Rather, IL-2 release and lymphoproliferation are optimal readouts of a TGN1412-like pro-inflammatory response.

Keywords: TGN1412, cytokine release assays, therapeutic monoclonal antibody

Introduction

In March 2006, the Phase I clinical trial of TGN1412, a therapeutic CD28-specific monoclonal antibody (mAb), ended in a disaster that brought into question the safety of new biological medicines. TGN1412 was a new class of immunomodulatory therapeutic intended for the treatment of rheumatoid arthritis, leukemia and multiple sclerosis, but instead it caused near fatal reactions in all six healthy volunteers. Within 2 h of receiving a single intravenous dose of 0.1 mg/kg, all six volunteers had a systemic inflammatory response characterized by a rapid induction of pro-inflammatory cytokines, later termed a 'cytokine storm' (Suntharalingham et al., 2006). Within 16 h, all six volunteers became critically ill with acute lung injury, multiple organ failure, and disseminated intravascular coagulation that would result in amputation of fingers and toes for the worst affected. All six patients were transferred to an intensive care unit where they received intensive organ support and high-dose immunosuppressive therapy, but two developed prolonged cardiovascular shock and acute respiratory distress. Fortunately, all six patients survived but have

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been warned that they have a greatly increased chance of developing cancers and immune system disorders in later life. The company that developed TGN1412, TeGenero AG, filed for insolvency 4 months later.

The adverse drug response to TGN1412 had been unanticipated since it had passed a standard battery of pre-clinical safety tests without raising any alarms and been approved by both UK and German regulatory agencies for first-in-man Phase I clinical trials. Initially a contaminant was suspected of causing the adverse drug responses, but subsequent analysis showed that the trial material was not contaminated and had been correctly formulated (Duff, 2006; Stebbings et al., 2007). When standard pre-clinical safety tests were independently repeated, using recovered clinical trial material, no pro-inflammatory cytokine release could be detected either *in vitro* with human white blood cells or when infused into cynomolgus macaques (Duff, 2006; Stebbings et al., 2007). The conclusion was that an unexpected biological effect of TGN1412 in man had caused an adverse drug response that was not predicted by pre-clinical safety testing (Duff, 2006; Stebbings et al., 2009). This posed a serious dilemma for regulators asked to grant licences for trials of new biological medicines where the potential to cause a 'cytokine storm' in man could not be confidently assessed. Concomitantly, fears of another TGN1412 incident made it difficult for research-based pharmaceutical companies to attract new investment in therapeutic mAbs, postponing the implementation of new and potentially life-saving drugs.

Why did pre-clinical safety testing in cynomolgus macaques not predict human responses?

Cynomolgus macaques were chosen for pre-clinical safety testing of TGN1412 because they share 100% sequence homology for the extracellular loop of CD28 with man, demonstrated equivalent binding affinity for TGN1412, and did not reveal differences in patterns of tissue staining (Hanke et al., 2006). However, cynomolgus macaques were the wrong species for pre-clinical safety testing of TGN1412 because the $CD4^+$ effector memory sub-set of this species does not express CD28 and, therefore, cannot be stimulated by anti-CD28 agonists (Eastwood et al., 2010; Figure 1). In contrast the $CD4^+$ effector memory T-cells of man express high levels of CD28 so can, therefore, be stimulated by TGN1412 (Sallusto et al., 1999; Eastwood et al., 2010). The evolutionary reason for this subtle difference in CD28 expression on human $CD4^+$ effector memory T-cells is not understood, but likely enhances antigen responsiveness by providing co-stimulatory signals. Human and cynomolgus macaque $CD8^+$ effector memory T-cells do not express CD28, thus explaining the $CD4^+$ T-cell bias of the TGN1412 response in man (Pitcher et al., 2002; Moniuszko et al., 2004; Sallusto et al., 2004; Figure 1). Only a comparative analysis of target cell subsets would

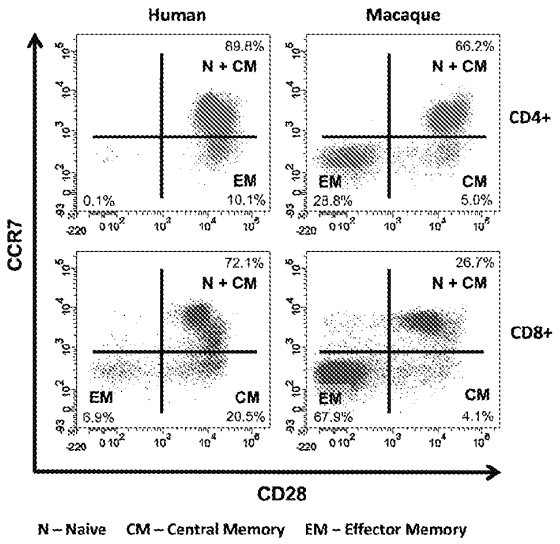


Figure 1. Comparative immunophenotyping of human and cynomolgus macaque $CD4^+$ T-cells. Analysis gated on $CD4^+$ T-cells shown in top panels and on $CD8^+$ T cells shown in bottom panels. Human and cynomolgus macaque naive (N) and central memory (CM) $CD4^+$ and $CD8^+$ T-cells are $CCR7^+CD28^+$. Human $CD4^+$ effector memory (EM) T-cells are $CCR7^-CD28^+$ in contrast to cynomolgus macaque $CD4^+$ EM T-cells, which are $CCR7^-CD28^-$. Cynomolgus macaque $CD4^+CCR7^-CD28^+$ T-cells are a transitional sub-set with CM properties. Both human and cynomolgus macaque $CD8^+$ N and CM T-cells express CD28 compared to $CD8^+$ EM T-cells that lack CD28 expression. (See colour version of this figure online at www.informahealthcare.com/imt)

have identified the species difference in $CD4^+$ effector memory T-cells prior to pre-clinical safety testing.

Although cynomolgus macaque naive and central memory T-cells express CD28, these subsets were not stimulated *in vitro* by TGN1412 unless exogenous IL-2 was added to cultures, producing a weak proliferative response compared to human lymphocytes (Stebbings et al., 2007). It is possible that initial release of cytokines, including IL-2, by activated $CD4^+$ effector memory T-cells in concert with TGN1412 similarly drove proliferation and then differentiation of naive and central memory $CD4^+$ T-cells in man, resulting in a second reactive phase. This may correspond with the time line of events after infusion of TGN1412 where a transient recovery after the first corticosteroid dose was followed by a rapid deterioration and more severe adverse response including multi-organ failure (Suntharalingham et al., 2006). The driving force for the second reactive phase was most likely IL-2, highlighting the importance of measuring release of this cytokine and associated lymphoproliferation during pre-clinical safety testing of new therapeutics.

Why were pre-clinical *in vitro* safety tests with human cells non-predictive?

TGN1412 is an immunomodulatory drug that targets the surface marker CD28 in order to exploit co-stimulatory

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signals required for T-cell activation (Jenkins et al., 1991; Tacke et al., 1997; Lühder et al., 2003). In rat models of experimental autoimmune encephalomyelitis and adjuvant arthritis, treatment with anti-CD28 mAbs orthologous to TGN1412 stimulated the release of anti-inflammatory cytokines and the expansion of regulatory T-cells believed to counter autoimmune responses (Rodríguez-Palmero et al., 1999; Lin and Hünig, 2003; Beyersdorf et al., 2005). Since TGN1412 stimulated massive pro-inflammatory cytokine release in man it was postulated that pre-clinical *in vitro* safety tests with human lymphocytes did not replicate *in vivo* presentation due to the biology of CD28 (Stebbings et al., 2007). The natural ligands for CD28, i.e., CD80 and CD86, are present on antigen-presenting cells that interact with T-cells through localized engagement at the point of cell-to-cell contact (Dustin et al., 1998). This promotes formation of an immunological synapse that consists of a central cluster of T-cell receptors surrounded by a ring of adhesion molecules that sequesters kinases required for T-cell activation from across the inner cell membrane (Dustin et al., 1998; Monks et al., 1998; Wang et al., 2004). Conversely, engagement of CD28 outside of the immunological synapse or disruption of its formation impedes T-cell activation (Sanchez-Lockhart and Miller, 2006; Thoulouze et al., 2006). Standard *in vitro* safety tests employ aqueous phase presentation of mAb to human lymphocytes where binding occurs over the whole cell surface that does not replicate localized cell receptor engagement.

In order to mimic delivery of a localized signal TGN1412 was immobilized onto the surface of tissue culture plates so that human T-cells could only interact with the mAb at one interface (Stebbings et al., 2007). The result of this simple change in presentation was dramatic; massive lymphoproliferation and pro-inflammatory cytokine release that mirrored trial volunteer responses to TGN1412 (Stebbings et al., 2007; Figure 2). The possibility that this response was an artefact was ruled out by the observation that lymphocytes from cynomolgus macaques were not stimulated by immobilized TGN1412 at any dose (Stebbings et al., 2007). The species-specific response of human lymphocytes to TGN1412 *in vitro* mirrored the *in vivo* adverse drug response in man but not cynomolgus macaques. Moreover, the calculation of a safe starting dose in man based upon pre-clinical safety testing of TGN1412 in cynomolgus macaques was flawed. A no-observable adverse effect level (NOAEL) of 50 mg/kg in cynomolgus macaques was used to justify a low safe starting dose of 0.1 mg/kg in man (TeGenero, 2005). In reality, this dose correlated with 45–80% receptor occupancy in man and a near maximum immunostimulatory dose *in vitro* (Stebbings et al., 2007; Waibler et al., 2008). A NOAEL approach is no longer preferred; a minimum anticipated biological effect level (MABEL) is now recommended for calculation of safe starting doses of therapeutics in man (Duff, 2006; CHMP, 2007).

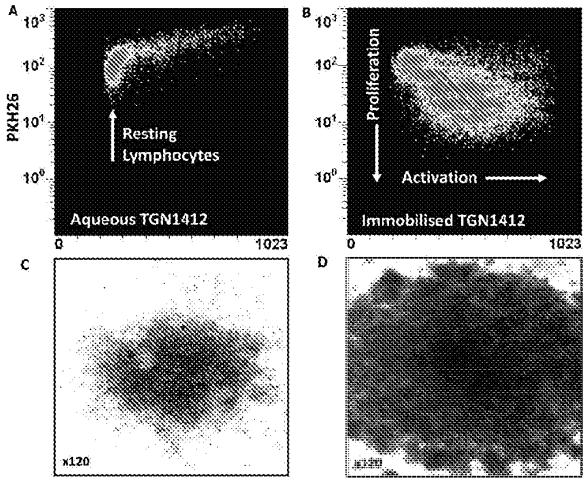


Figure 2. Proliferation of PKH26-stained human PBMC incubated 3 days with (a) 1 μg/well aqueous TGN1412 vs (b) 1 μg/well immobilized TGN1412. Lymphocyte proliferation appears as bands of cells with decreasing PKH26 fluorescence as dye is divided equally between daughter cells and increased forward scatter (FSC) corresponding to increased size due to blasting. No proliferation is observed with aqueous TGN1412 (a) compared to a strong proliferative response with immobilized TGN1412 (b). Low power light microscope images of 2×10^5 human PBMC in tissue culture after 3 days culture with (c) 1 μg/well aqueous TGN1412 vs (d) 1 μg/well immobilized TGN1412. (See colour version of this figure online at www.informahealthcare.com/imt)

Predictivity of newly-proposed cytokine release assays

Initial *in vitro* studies of pro-inflammatory cytokine release from human lymphocytes stimulated with immobilized TGN1412 measured high levels of tumor necrosis factor (TNF)-α and interleukin (IL)-8 (Stebbings et al., 2007). Yet, when later compared with immobilized Campath-1H (an anti-CD52 mAb used for the treatment of B-cell chronic lymphocytic leukemia) and Rituximab, (an anti-CD20 mAb used to treat hematological neoplasm such as leukemia and lymphomas), two therapeutics known to induce cytokine release syndrome in some patients (Moreau et al., 1996; Winkler et al., 1999), levels of TNFα (3000–5000 pg/ml) and IL-8 (50,000–100,000 pg/ml) release after 24 h stimulation were not significantly different to TGN1412 (Eastwood et al., 2010; Figure 3). After 72 h, TGN1412 does stimulate significantly ($p < 0.05$) more TNFα than Rituximab, but not Campath-1H (Figure 3). This result does not correlate well with the severity of the response to TGN1412 compared to Campath-1H and Rituximab, so TNFα and IL-8 release are poorly predictive of the severity of response (Eastwood et al., 2010).

Subsequently, both interferon (IFN)-γ and IL-2 were found to be better predictive markers of a TGN1412-like cytokine release using immobilized mAbs (Eastwood et al., 2010). All three of these therapeutic mAbs stimulate IFNγ release *in vitro* when immobilized, but levels are significantly ($p < 0.05$) higher with TGN1412 at 48 and 72 h compared to Rituximab and Campath-1H,

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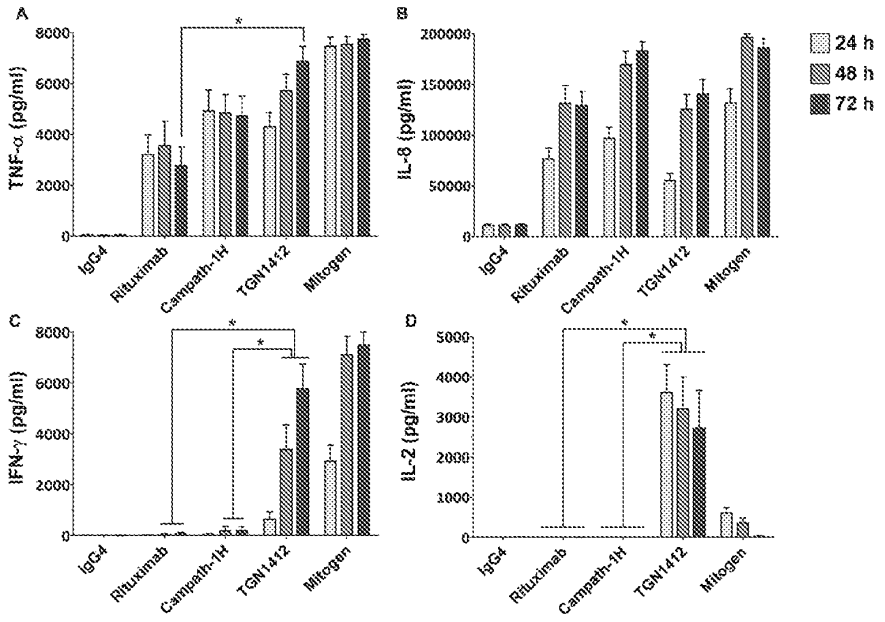


Figure 3. Cytokine release from human PBMCs stimulated *in vitro* for 24, 48, and 72 h with 1 μg/well immobilized isotype-matched control ($IgG_4\kappa$), Rituximab (IgG_1), Campath-1H (IgG_1), and TGN1412 ($IgG_4\kappa$). The lectin PHA at 10 μg/ml was used as mitogen control. Cytokine release into culture supernatants was measured by ELISA. (a) TNFα, (b) IL-8, (c) IFNγ, and (d) IL-2 release are expressed in pg/ml. Donors responses shown ($n = 9$). * Denotes $p < 0.05$ (one-way analysis of variance with bonferroni correction).

correlating with the severity of the response (Figure 3). However, the best predictive marker for TGN1412 is IL-2, with high levels released after 24, 48, and 72 h (4000–5000 pg/ml) compared to no response with Campath-1H and Rituximab (Figure 3). The only other therapeutic mAb found to stimulate IL-2 release is Muromonab-CD3, an anti-CD3 mAb used to treat allograft rejection, but whose administration is often complicated by serious adverse reactions related to cytokine release (Suthanthiran et al., 1989). In renal allograft patients, not pre-treated with immunosuppressant, serum levels of IL-2 peak around 400 pg/ml 4 h after Muromonab-CD3 administration (Chatenoud et al., 1990). In contrast, TGN1412 stimulated release of >5000 pg/ml IL-2 in trial volunteer sera after 4 h (Suntharalingam et al., 2006), consistent with the greater severity of response compared to Muromonab-CD3. Thus, a level of T-cell activation beyond that seen with Muromonab-CD3 is increasingly likely to cause a TGN1412-like response.

Whilst it is tempting to suggest that levels of IL-2 release up to those caused by Muromonab-CD3 may be acceptable it should be remembered that this response is self-limiting, cytokine producing cells are depleted by treatment. For immunomodulatory mAbs, lower levels of IL-2 release will have the potential to cause a severe response. The ability of IL-2 release to amplify pro-inflammatory T-cell cytokine responses by promoting lymphoproliferation suggests that this was the driving force with respect to the adverse effects caused by TGN1412. It was established that TGN1412 stimulates $CD4^+$ T-cells to undergo lymphoproliferation and release pro-inflammatory cytokines (Stebbings et al., 2007). In contrast, Campath-1H and Rituximab stimulate cytokine release from natural killer (NK) and $CD8^+$, but not $CD4^+$, T-cells (Wing et al., 1996; Eastwood et al., 2010: Figure 4). Muromonab-CD3 was found to represent a third pattern of cytokine release stimulating subsets of $CD4^+$ and $CD8^+$ T-cells and NK cells (Figure 4). Since IL-2 is produced by mainly $CD4^+$ T-cells, not NK cells; this observation explained why immobilized Campath-1H and Rituximab stimulation did not induce release of this cytokine. Stimulation of more $CD4^+$ T-cells by TGN1412 than Muromonab may also explain quantitative differences in IFNγ and IL-2 release (Figure 4).

TGN1412 stimulates IFNγ release from $CD4^+$ T-cells, whereas Campath-1H and Rituximab stimulate release of this cytokine from NK and $CD8^+$ T-cells, showing that there are at least two different mechanisms for inducing IFNγ release (Figure 4). However, a cytokine ELISA will not discriminate between cellular sources giving the impression that these pro-inflammatory responses are the same if equivalent amounts of cytokines are released. Differences between Campath-1H and Rituximab cytokine release are also evident in the proportions of $CD8^+$ T-cells and $CD8^{+/-}$ NK cells stimulated implying activation of different subsets. Understanding the cellular sources of these cytokines is important for assessing safety of new therapeutics, since each mediates a different pro-inflammatory mechanism with different clinical outcomes. The mechanism of TGN1412 cytokine release (a $CD4^+$ T-cell-restricted response) is different to that reported for other therapeutic mAbs and is mediated by binding to antigen

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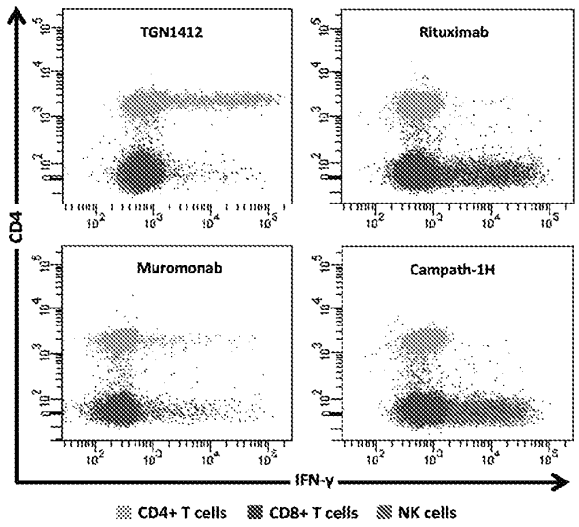
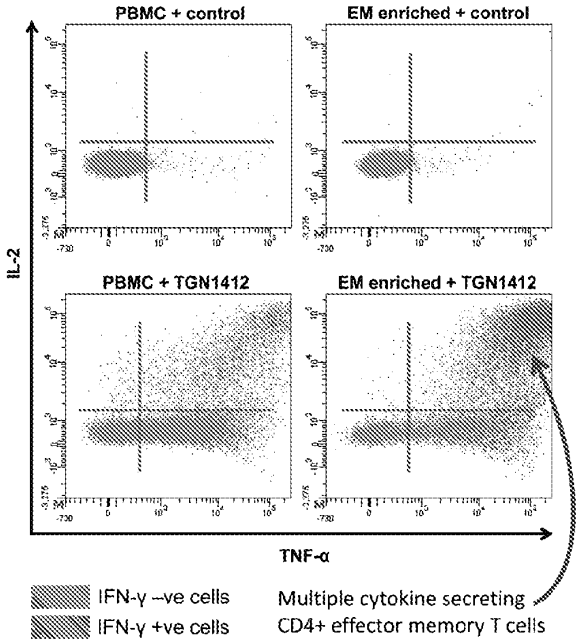


Figure 4. Intracellular IFN-γ staining of human PBMC stimulated for 24 h with 1 μg/well of immobilized TGN1412, Rituximab, Muromonab, and Campath-1H. $CD4^+$ T cells, $CD8^+$ TT cells and NK cells are identified using the key above. TGN1412 stimulates IFN-γ secretion from $CD4^+$ T-cells, Rituximab, and Campath-1H stimulate IFNγ secretion from NK cells and $CD8^+$ T-cells and Muromonab stimulate IFN-γ secretion from $CD4^+$ and $CD8^+$ T-cells and NK cells. (See colour version of this figure online at www.informahealthcare.com/imt)



	PBMC + control	EM enriched + control	PBMC + TGN1412	EM enriched + TGN1412
IL-2 –, TNF-α –, IFN-γ –	98.9%	99.1%	34.0%	14.2%
IL-2 +, TNF-α –, IFN-γ –	0.0%	0.0%	1.3%	0.1%
IL-2 –, TNF-α +, IFN-γ –	1.0%	0.8%	43.8%	27.5%
IL-2 –, TNF-α –, IFN-γ +	0.0%	0.0%	0.0%	0.0%
IL-2 +, TNF-α +, IFN-γ –	0.1%	0.1%	18.7%	42.5%
IL-2 +, TNF-α –, IFN-γ +	0.0%	0.0%	0.0%	0.0%
IL-2 –, TNF-α +, IFN-γ +	0.0%	0.0%	0.4%	2.1%
IL-2 +, TNF-α +, IFN-γ +	0.0%	0.0%	1.8%	13.6%

Figure 5. Intracellular TNFα, IL-2, and IFNγ staining of human PBMCs and effector memory T-cell (EM) enriched PBMC following stimulation with either 1 μg/well immobilized IgG_4 isotype matched control mAb or TGN1412. Analysis shown is gated on $CD3^+CD4^+$ T-cells. Cells in the right hand quadrants are positive for TNFα, cells in the upper quadrants are positive for IL-2, cells positive and negative for IFN-γ are identified using the key above. Cells in the upper right corner in red are positive for TNFα, IL-2, and IFNγ. The frequency of cytokine positive cells is expressed as a percentage of $CD3^+CD4^+$ T-cells. Quadrant statistics for cytokine positive $CD3^+CD4^+$ T-cells are shown in the table below. (See colour version of this figure online at www.informahealthcare.com/imt)

via the complementary determining regions (Findlay et al., 2011a). In contrast, the F_c component of therapeutic mAbs mediates NK cell cytokine release. A therapeutic mAb of the IgG_1 isotype, such as Campath-1H and Rituximab, with high affinity for F_c receptors, will likely stimulate NK cell cytokine release, whereas an IgG_4 mAb such as TGN1412 with low F_c receptor affinity will likely not (Bruhns et al., 2009). Whilst IL-2 release is an important marker of a TGN1412-like cytokine response, development of a robust assay for future trials of therapeutic mAbs would have to include cytokines that are expressed by all target cell types, such as TNFα or IFNγ.

Reconciling differences between *in vivo* and *in vitro* cytokine release

High concentrations (> 5000 pg/ml) of the cytokines TNFα, IL-2, and IFNγ were measured in the plasma of trial volunteers within 4 h of TGN1412 infusion (Suntharalingam et al., 2006). In comparison, the kinetics of cytokine release *in vitro* using immobilized TGN1412 was much slower, particularly in the case of IFNγ where an incubation time of 48–72 h was required to achieve parity (Eastwood et al., 2010; Figure 3). The search for an explanation of this led to the identification of $CD4^+$ effector memory T-cells being the source of TGN1412-stimulated IFNγ release (Eastwood et al., 2010; Figure 5).

$CD4^+$ effector memory T-cells are known to have the capacity for rapid release of considerable levels of cytokines, with TNFα, IL-2, and IFNγ being major components that are consistent with the pro-inflammatory response to TGN1412 (Sallusto et al., 1999; Chung, 2008). In the body, high concentrations of $CD4^+$ effector memory T-cells are found in the lungs and gastrointestinal mucosa (Campbell et al., 2001; Picker, 2006). Moreover, up to 90% of all the lymphocytes in the body are contained within the lungs and gastrointestinal mucosa, compared to only 2–5% in the peripheral blood (Cerf-Bensussan and Guy-Grand, 1991; Smit-McBride et al., 1998). It is, therefore, likely that infusion of TGN1412 into trial volunteers caused high concentrations of cytokines to be rapidly released into the blood by large numbers of $CD4^+$ effector memory T-cells within the mucosal tissues. In contrast, peripheral blood lymphocytes contain low numbers of $CD4^+$ effector memory T-cells, so their use for

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in vitro assays is likely to under-estimate TGN1412-like cytokine responses such as IFNγ release, with the caveat that the method of presentation *in vivo* is different.

Delay in release of high levels of IFNγ *in vitro* by peripheral blood mononuclear cells (PBMC) was most likely due to the time required for naive and central memory $CD4^+$ T-cells to undergo proliferation and differentiation into effector memory $CD4^+$ T-cells with the capacity to secrete this cytokine (Geginat et al., 2001). For ethical and practical reasons, it is not feasible to use lymphocytes harvested from healthy human tissues in order to perform *in vitro* safety tests that replicate mucosal and lymphoid responses. Therefore, it is important to understand the differences between peripheral blood, lymphoid, and mucosal tissue lymphocytes when interpreting results of *in vitro* safety tests. Alternatively, enrichment of $CD4^+$ effector memory T-cells from peripheral blood lymphocytes for *in vitro* assays will better replicate *in vivo* cytokine responses to TGN1412 in terms of both kinetics and cytokine secretion (Eastwood et al., 2010; Figure 5), but is limited by low cell yields.

Assay refinements and co-culture alternatives

Levels of cytokine release with immobilized mAbs treated are hugely dependent upon assay conditions such as antibody coating method, serum concentration, cell concentration, and whether dilute whole blood or isolated lymphocytes are used. For example, low serum concentrations (e.g., 2%) are quickly exhausted by stimulated lymphocytes, blunting cytokine release. Originally air-drying, but not wet-coating, of TGN1412 onto polystyrene tissue culture plates was necessary to elicit a response (Stebbings et al., 2007). It is now known that superior responses can be obtained with polypropylene tissue culture plates and that both wet coating and air drying methods can be employed (Eastwood et al., 2010; Findlay et al., 2010). This was originally thought to be a property of the plastic used, but non-tissue culture treated polystyrene plates are equally effective and can be wet-coated with mAb. The process of modifying tissue culture plates to make it easier for adherent cells to grow appears to reduce the amount of mAb that can be bound (Findlay et al., 2010). The density of immobilized mAb appears critical for cytokine release, suggesting a threshold below which no response is seen. This is reminiscent of 'trigger pharmacology'. Conversely, a prozone effect is observed with high concentrations of mAb and air drying, probably due to leaching of mAb into aqueous phase where it can inhibit responses to immobilized TGN1412 (Findlay et al., 2010).

Another approach to immobilize therapeutic mAbs for presentation to lymphocytes in cytokine release assays is to employ polystyrene beads coated with protein A or anti-human IgG to act as artificial antigen-presenting cells (Walker et al., 2011). This method greatly increases the surface area available for interaction with lymphocytes, allowing responses to lower concentrations of therapeutic mAbs. Regardless of the approach employed, immobilization of TGN1412 onto plastic is an effective *in vitro* method for replicating TGN1412 cytokine release in man, but does not mimic *in vivo* presentation.

Attempts to model *in vivo* presentation of TGN1412 employed a co-culture of human endothelial cell monolayers with donor lymphocytes have been shown to result in pro-inflammatory cytokine release, particularly IL-6, IL-8, and TNFα (Stebbings et al., 2007; Findlay et al., 2011b; Dhir et al., 2012). It has subsequently been reported that the cytokine profile of this model does not fully match that of the trial volunteers or assays with immobilized TGN1412, as only low levels of the cytokines IL-2 and IFNγ are produced (Findlay et al., 2011b). If endothelial cell interactions are only part of the story then other mechanisms are likely to account for *in vivo* presentation, for example F_c-capture, but since TGN1412 is of the IgG_4 sub-class, this would then be restricted to the $F_c\gamma RI$ receptor (Bruhns et al., 2009), possibly involving a monocyte component (Sandilands et al., 2010). Alternatively, pre-culture of human lymphocytes at high density, to promote tissue-like conditions, has recently been reported to increase the sensitivity of circulating T-cell responses to TGN1412 in aqueous phase (Romer et al., 2011). Other methods being investigated include 3D cell and skin biopsy cultures to attempt to replicate tissue responses.

Conclusions

The mechanism of cytokine release caused by TGN1412 is different to that caused by other therapeutic mAbs because it involved a different arm of the immune system, the $CD4^+$ effector memory T-cells. Unlike other therapeutic mAbs, aqueous presentation of TGN1412 *in vitro* is not immunostimulatory and gives a false negative result that, combined with use of the wrong species for pre-clinical safety testing, led to the erroneous prediction that it was safe to test in man. Although immobilization has since been demonstrated to render TGN1412 immuno-stimulatory *in vitro* it should be noted that this is an artificial means of stimulation unlikely to replicate *in vivo* presentation, but nevertheless it is the only method that induces substantial cytokine release similar to that experienced by trial volunteers. Stimulation of different pro-inflammatory pathways produces different cytokine profiles and clinical outcomes so it is important to measure the relevant responses in order to discriminate between them. In the case of TGN1412, measurement of IL-2 and lymphoproliferation provide the most unambiguous biomarkers. The likelihood that another therapeutic mAb or immunomodulatory agent is capable of stimulating a TGN1412 like 'cytokine storm' can now be linked to the ability to activate $CD4^+$ effector memory T-cells. However, activation of other arms of the immune system also has the potential to cause a 'cytokine storm'.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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PLoS one

Exchanging Murine and Human Immunoglobulin Constant Chains Affects the Kinetics and Thermodynamics of Antigen Binding and Chimeric Antibody Autoreactivity

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Mouse-human chimeric antibodies composed of murine variable (V) and human (C) chains are useful therapeutic reagents. Consequently, we investigated whether heterologous C-regions from mice and humans affected specificity and affinity, and determined the contribution of C_H glycosylation to antigen binding. The interaction of a 12-mer peptide mimetic with monoclonal antibody (mAb) 18B7 to *Cryptococcus neoformans* glucuronoxylomannan, and its chimeric (ch) and deglycosylated forms were studied by surface plasmon resonance. The equilibrium and rate association constants for the chAb were higher than for mAb 18B7. V region affinity was not affected by C_H region glycosylation whereas heterologous C region of the same isotype altered the Ab binding affinity and the specificity for self-antigens. Structural models displayed local differences that implied changes on the connectivity of residues. These findings suggest that V region conformational changes can be dictated by the C_H domains through an allosteric effect involving networks of highly connected amino acids.

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INTRODUCTION

Monoclonal antibodies have found a wide range of applications *in vitro* for immunochemical characterization and quantization of antigens (Ags) as well as many different therapeutic applications, for the treatment of microbial, autoimmune and malignant diseases. Although several murine mAb are in clinical use, their use is limited because treated patients develop human anti-murine antibodies (HAMA) that may reduce the effectiveness of a treatment due to the formation of immune complexes. Ideally, human Abs would be used in therapy but these remain difficult to produce. One alternative is the construction and production of mouse-human chAbs. These molecules have human C regions to provide effector functions and mouse V regions that bind antigen. Since the C region constitutes most of the mass of the immunoglobulin (Ig), chAbs are largely human in composition and significantly less immunogenic. However, a central assumption in the construction and use of mouse-human chAbs is that they retain the affinity and specificity of the parental murine mAb. Although this assumption is supported by an overwhelming amount of data showing that V regions interact with Ag, there is now considerable data that the C region can affect V region structure therefore affecting Ab affinity and specificity [1–6]. In this regard, the different C_H domains can impose diverse structural constrains to the interaction of Ab with Ags, especially multivalent Ags such as polysaccharide. This raises the question of whether similar effects can follow the construction of mouse-human chimeric antibodies where heterologous C regions manifesting differences in sequence are exchanged to create a less immunogenic molecule.

The murine mAb 18B7 is being developed as an adjunctive passive immunotherapy treatment of cryptococcal meningitis in patients with AIDS [7]. Infusion of mAb 18B7 into patients induced HAMA responses, despite their immunosuppressed status [7]. Since cryptococcosis is a chronic disease, there is interest in generating therapeutic Ab reagents that are non-immunogenic and suitable for multiple infusions. Given that the murine mAb 18B7 has undergone clinical testing and that has been extensively studied in the laboratory, one approach was to generate chAbs as potential therapeutic reagents, with the assumption that expression of mAb 18B7 V regions in combination with human C regions would maintain the specificity and affinity of the murine mAb. That assumption was challenged by the observation that a set of mouse-human chAbs derived from mAb 18B7 [8] to the capsular polysaccharide of *C. neoformans* glucuronoxylomannan (GXM), manifested subtle differences in their binding characteristics [1]. Furthermore, there is extensive anecdotal and unpublished evidence that many attempts to generate humanized mAbs based on mouse V regions have failed to produce useful Abs because of loss of specificity or affinity. However, the available data are only suggestive since one can always blame differences in avidity resulting from different hinge region geometry for differences in the binding of chAbs relative to the parental murine mAb.

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In this study, we used surface plasmon resonance (SPR) to investigate the thermodynamic and kinetic properties of binding of the GXM-binding mAb 18B7, its deglycosylated form (18B7dg) and its mouse-human chAb counterpart. These Abs have identical V regions but differ in their C domains. Most of the Ig glycosylation sites are found in the C_H domains but glycosylation seems to have little if any effect on Ab binding to Ag [9,10]. Comparison of the binding kinetics and thermodynamics between the glycosylated and deglycosylated murine mAb 18B7 forms and the chAb 18B7 revealed differences in binding affinity attributed to the heterologous C_H region, however no contribution from the carbohydrate motif was observed. The results have important implications for the design and use of heterologous V and C chains in therapeutic Abs.

RESULTS

Effect of glycosylation and heterologous constant region on the kinetic and equilibrium binding constants

To study the role of glycosylation we compared mAb 18B7, chAb 18B7, and 18B7dg binding to the GXM mimetic peptide P1. Analysis of these mAbs by SPR revealed that the association rate constants for the encounter step (k_{+1}) for mAb 18B7 and 18B7dg were similar, but different from chAb 18B7 (**Figure 1A**). The equilibrium dissociation constants (K_D) for mAb 18B7, 18B7dg and chAb 18B7 are 2.33×10^{-3}, 1.07×10^{-3} and 5.41×10^{-4} M at 25°C, respectively. Van't Hoff plots of the interaction of these Abs with peptide P1 revealed that their affinity decreased with temperature (**Figure 2A**). In addition, the equilibrium association constants for the binding of mAb 18B7 and its deglycosylated form to P1 manifested similar affinity constants and their binding characteristics were more influenced by the change in temperature than that observed for chAb 18B7 (**Figure 2A**). It is also noticeable that chAb 18B7 had a higher overall equilibrium affinity constant (K_A) than the parental mAb 18B7. In addition, for all these complexes, the encounter equilibrium association constant K_{a1} showed a pattern that is almost identical to the equilibrium association constant (**Figure 2B**), while the docking equilibrium association constant K_{a2} was relatively unaffected by changes in temperature, showing similar trends between Ab-peptide complexes (**Figure 2C**).

Effect of glycosylation and heterologous constant region on the thermodynamic parameters of Ab binding

Analysis of the Gibbs free energy of binding (ΔG) profiles of these mAb-peptide complexes showed that for mAb 18B7 and its deglycosylated form, the energy of binding increases with temperature, followed by a decrease at temperatures above 30°C (303 K), whereas the energy of binding for chAb 18B7-P1 complex was relatively insensitive to temperature changes (**Figure 3A**). A similar pattern was observed for the encounter energy of binding ($\Delta G1$) (**Figure 3B**) for the complexes formed by mAb 18B7 and 18B7dg and chAb 18B7 relative to ΔG. The docking energy of binding ($\Delta G2$) increased slightly at low temperatures, but remained otherwise relatively insensitive to changes in temperature, and manifested similar patterns for all Ab-peptide complexes (**Figure 3C**). From these data, we can establish that most of the free energy changes come from the encounter step. Furthermore, ΔS for mAb 18B7 and its deglycosylated form decreased as the temperature increased (**Figure 3D**), but showed a sharp increase above 30°C (303 K). In contrast, ΔS for the chAb 18B7-P1 complex decreased only slightly with increasing temperature (**Figure 3D**). Also, differences in ΔG ($\Delta\Delta G$) of mAbs 18B7dg and chAb 18B7 relative to mAb 18B7 indicate the interaction formed by chAb 18B7 is energetically more favorable than for mAb 18B7dg (**Figure 4A**), with contributions to the differences in energy of binding coming from the encounter and docking steps (**Figure 4B and 4C**). Interestingly, for the chAb 18B7-P1 and 18B7dg-P1 complexes, most of the contributions to activation energy of the transition state $\Delta\Delta G^{\neq}$ (**Figure 5A**) are from the encounter step $\Delta\Delta G1^{\neq}$ (**Figure 5B**). Hence, deglycosylation leads to differences in the energies of binding with respect to the glycosylated form. These differences are more accentuated for the encounter activation energy of chAb 18B7, indicating that the transition state for this complex is energetically more favorable than for the mAb 18B7dg-P1 complex at temperatures ranging from 20°C to 35°C (293 K to 308 K). In summary, kinetic and thermodynamic calculations indicate that deglycosylation does not affect the affinity of binding, but a heterologous associated C region will, as shown above.

Molecular modeling analysis

At a first glance, the backbone conformation of mAb 18B7 and chAb 18B7 models differed significantly (**Figure 6A**). However, a detailed analysis showed that these changes were due to different Ab elbow angles, i.e. angle between variable (V_L and V_H) and C domains (C_L and C_{H1}). If any of the two domains, V or C, for any of the chains, H and L, is kept fixed during structural superposition, a good structural agreement can be observed (L chain: **Figure 6B and 6C** keeping V_L and C_L fixed during structural superposition; H chain: **Figure 6D and 6E** superposing domain V_H and C_{H1} respectively).

Analysis of the connectivity of the Fab for murine mAb 18B7 and chAb 18B7 showed that these differences were located mainly at the interface between the C_H and V_H domains (**Figure 7A and 7B**). Highly connected residues (i.e. Zscore$\geq$1.5) were assembled forming a network that spans from the C_{H1} domain to the V_H domain. This network can provide a logical explanation for a long-range allosteric effect that involves residue-to-residue communication. It is worth noting that Phe^{103} (**Figure 7B**) is located in one of the CDRs loop and the network of highly connected residues extends to the C_{H1} domain. **Figure 7C** shows the sequence alignment and the connectivity analysis; chAb 18B7 and mAb 18B7 display a differential connectivity pattern. This difference in connectivity pattern could explain the differential affinity of mAb 18B7 and chAb 18B7 toward an Ag. The analysis also suggests that these conformational variations are a consequence of species class-specific constraints.

Polyreactive patterns of Abs 18B7

The polyreactive properties of mAb 18B7, 18B7dg and chAb 18B7 were measured by a panel of self-antigens consisting of actin, tubulin, thyroglobulin, and single-stranded DNA (**Figure 8A–D**). Polyreactivity was evident among these mAbs, showing differences in binding reactivity within the Ags used for the study. It is noticeable that chAb 18B7 has an overall lower binding reactivity for the self-Ags used in this study, in accordance with differences in specificity for this Ab with respect to mAb 18B7. In addition, we have shown that cross-reactivity does not only occur with closely related molecules because the studied cross-reactants are not related to the capsular polysaccharide GXM.

DISCUSSION

In previous studies, we established that a family of V-region identical murine IgG1, IgG2a, IgG2b, and IgG3 manifested diverse binding affinity and specificity that were attributed to C region effects on the V region. This effect was attributed to amino

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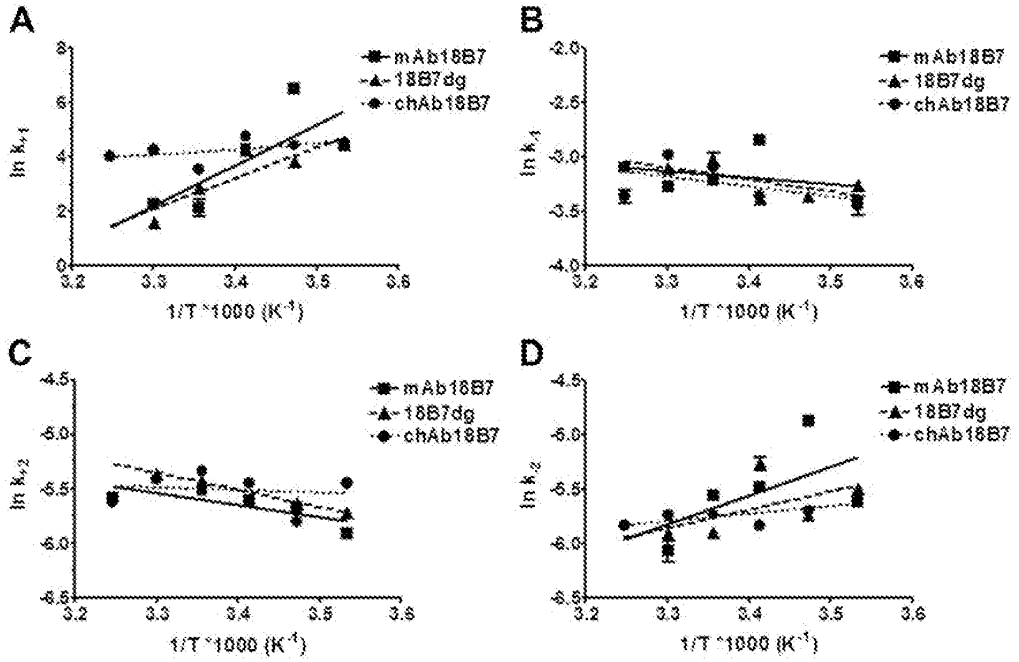


Figure 1. Analysis of the association and dissociation rates constants by SPR. Van't Hoff plots of the rate constants *A*, k_{+1}, *B*, k_{-1}, *C*, k_{+2} and *D*, k_{-2} for mAb 18B7, 18B7dg, and chAb 18B7 binding to peptide P1. All data points were obtained from the BIAevaluation 4.1 software using a two-state model. Lines represent the best fit to a linear regression using a 95% confidence interval. Errors bars were calculated using propagation of error for SE of each assay.
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acid changes in C_{H1} that caused structural differences in the attached V region that translated into variations in antigen binding [11]. An implication of those results was that chAbs might also manifest differences in Ag binding as a consequence of amino acid sequence dissimilarities between heterologous C regions. Consistent with this notion a prior study comparing the binding of mAb 18B7 derived chAbs revealed differences in Ag binding but the results were only strongly suggestive, since avidity contributions resulting from Ab geometry and flexibility could have influenced the types of Ab-Ag complexes formed [1]. To rigorously establish whether heterologous C regions affected V region interactions with Ag it was necessary to use an experimental system whereby each V region interacted with only one epitope to avoid avidity contributions combined with a sensitive measure for detecting binding differences. To investigate the characteristics of binding we used SPR, a method that can detect the interaction of mAb immobilized on a sensor chip with a small antigen such as a peptide, thus avoiding avidity contributions and allow examination of the kinetics and thermodynamics of Ab-Ag complex formation.

A characteristic of all IgGs is glycosylation in the C_{H2} domain at Asn^{297} [12], which is necessary for structure recognition and stability [13], and several effector functions, such as complement binding and activation, and interactions with Fc receptors [14–18]. Glycosylation can affect the conformation of some Ab molecules by increasing or decreasing the stability of amino acid residues in the Ag binding site [19,20]. Furthermore, numerous studies have shown that C_{H2} domain associated oligosaccharides play an essential role in IgG1-mediated Ab effector function [14,15,21–23]. However, few studies have addressed the issue of how differences in the structure of the C_H domains influence Ab-Ag binding [15].

Given the differences observed in binding affinities for the Fab molecules observed in our previous work [11], we could not rule out contributions of the C_{H2} domain to the Ab-Ag interaction. We have approached this issue by removing glycan chains using endoglycosidase digestion with a method known to be very effective in deglycosylating this mAb [24]. mAb 18B7 has a conventional IgG glycosylation pattern with a single *N*-linked glycosylation at Asn^{263} residue [24]. Circular dichroism spectral analysis of parental mAb 18B7 and 18B7dg revealed no major change in the secondary structure as a result of *N*-glycan removal, and their binding patterns to *C. neoformans* are essentially identical [24]. Native and deglycosylated mAb 18B7 binding to peptide P1 manifested similar binding kinetics and thermodynamics, suggesting that structural changes imparted upon C_{H2} domain by glycosylation do not extend into the V region and had little or no effect on the binding site of the murine IgG1 used for this study. This result is consistent with, and complementary of, the findings observed with anti-GXM mAbs 3E5, in that most of the isotype-related contributions to V-region binding affinity are a result of conformational changes on the C_{H1} domain, rather than C_{H2} [11].

In contrast to the similar interactions measured for the native and deglycosylated murine mAb 18B7, we found significant kinetic and thermodynamic differences in the comparison between mAb 18B7 and chAb 18B7. The V region of mAb 18B7 manifested a higher binding affinity when attached to a human γ1 chain. chAb 18B7 had a greater affinity, as determined by the equilibrium association constant than the parental murine mAb and different kinetics of binding, suggestive of differences in their fine specificity. These results imply that the nature of the C region electrostatic and hydrophobic interactions with the attached V region modulates the affinity and specificity of binding to Ag among Abs with an identical variable region. Consistent with these

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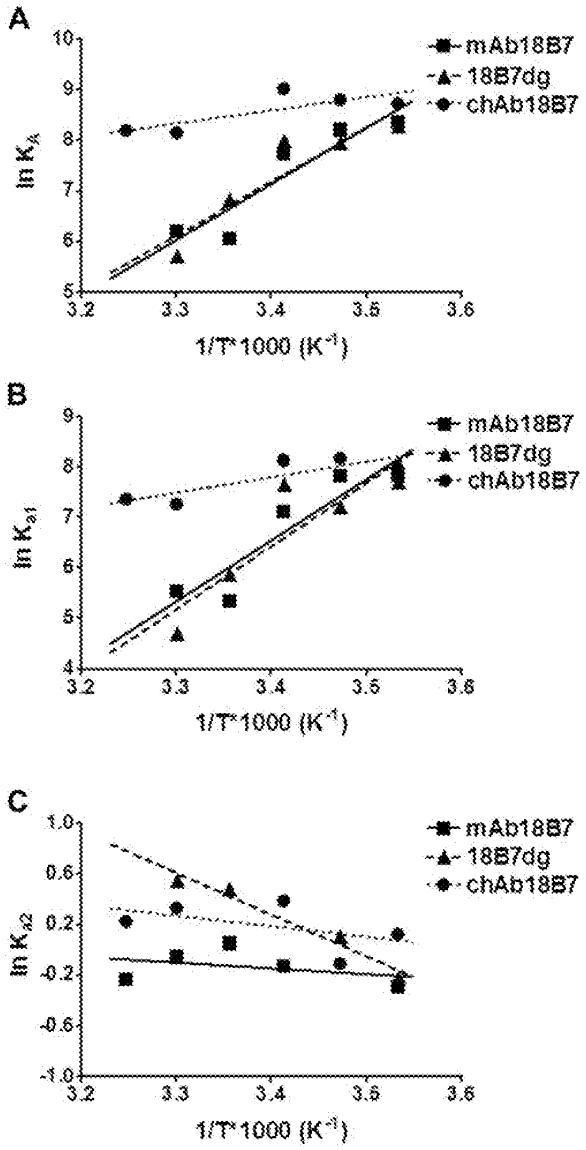


Figure 2. Equilibrium affinity constants for the binding of mAb 18B7, 18B7dg, and chAb 18B7 to peptide P1. Van't Hoff plots of the *A*, equilibrium association constant (K_A), *B*, encounter equilibrium association constant (K_{a1}) and *C*, docking equilibrium association constant (K_{a2}) for mAbs 18B7 binding to peptide P1. All equilibrium constants were calculated from the rate constant obtained from the fittings to a two-state model. Lines represent the best fit to a linear regression using a 95% confidence interval. Errors bars were calculated using propagation of error for SE of each assay.
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findings, others have reported that the immunogenicity of an Ab can be influenced by regions adjacent to the V domains [25,26].

The stability of Ab-Ag complexes is regulated by the same forces as in other biological structures, namely electrostatic interactions and the hydrophobic effect [27,28]. The structural modeling revealed that m18B7 and ch18B7 are structurally very similar, predicting only changes in the Ab elbow angles. Prior studies have speculated that the elbow angles might change in response of ligand binding [29], but no convincing experimental data has been found to support that hypothesis [30]. We favor the view that conformational differences imposed by the C_{H1} domain on the Ag-binding site could affect binding affinity and specificity. These conformational differences are probably a result of small changes in the electrostatic interactions between amino acid residues rather than by 'gross' conformational changes caused by hinge flexibility or by structural constrains imposed by the oligosaccharide moieties [31,32].

Identifying functional residues is a complex issue. Activity can be modulated by residues that are distant from the binding site and these interactions within and between functional sites are crucial for protein activity. The study of closeness values in protein structure has proved useful in characterizing functional sites [33]. Such studies are also useful for describing the interaction networks within protein structures that are connected to active site residues, as those found in enzymes where effectors bind to residues distant from the active sites. Central residues have high closeness values and they are assumed to transmit and efficiently integrate in formation to the rest of the protein [33]. Our earlier studies [11] suggested that highly connected residues may be involved in the differences in fine specificity in four murine mAbs expressing different C_H regions and identical V regions. As observed in the homology models, an electrostatic network between the tyrosines residues interacting at the interface of the V region and the C_{H1} domain of murine mAb 18B7 could be more unfavorable for the net electrostatic forces of binding. These interaction networks formed at the C_{H1} domain appear to be directly responsible for the differences in affinity and specificity between these mAbs.

The differences observed in the specificity of these mAbs also affected the characteristics of binding to a panel of self-antigens, indicating that polyreactivity is not only preserved after Ig class switch [34], but it also depends on the contribution of structural changes caused by the C_H domains. This finding implies that polyreactivity is influenced by the surrounding C_H structures, possibly making the Ab-combining site more plastic, thus allowing these Abs to recognize a variety of Ags. Consequently, the structural heterogeneity conferred by the different C_H regions may result in the production of polyreactive Ab by changing charge and/or hydrophobicity of the V region. This implies that mouse-human chAb construction may yield Igs with binding characteristics that are different from the parental murine mAb, including the possibility for unexpected self-reactivity.

In summary C_H region glycosylation does not affect Ag binding, but exchanging murine and human C_H domains can have profound effects on affinity and specificity. Although the generalizability of the observations with mAb 18b7 and chAb 18B7 to other chimeric Abs is unknown, our findings suggest caution with assuming that simply replacing the C region domain maintains the specificity and affinity of the V region. Given that different V region genes encode for proteins with differences in protein sequence, Abs encoded by different V region genes may vary in their susceptibility to C region structural effects. Furthermore, we anticipate that these effects might also be expected to occur in chAbs composed of human V and mouse C regions [35]. Substituting the mouse C regions with human C regions in chAbs was a major advance in reducing the immunogenicity of mouse mAbs but our results show that this approach can have unpredictable effects on the thermodynamics of Ag-Ab complexes and the self-reactivity of chAbs. We do not know if the new reactivities exhibited by chAb 18B7 relative to the parent mAb 18B7 are clinically relevant, but the fact that polyreactivity occurs is the need for additional caution when examining the potential for cross-reaction with host tissues. Since most of the Abs currently used in drug therapy are of the IgG1

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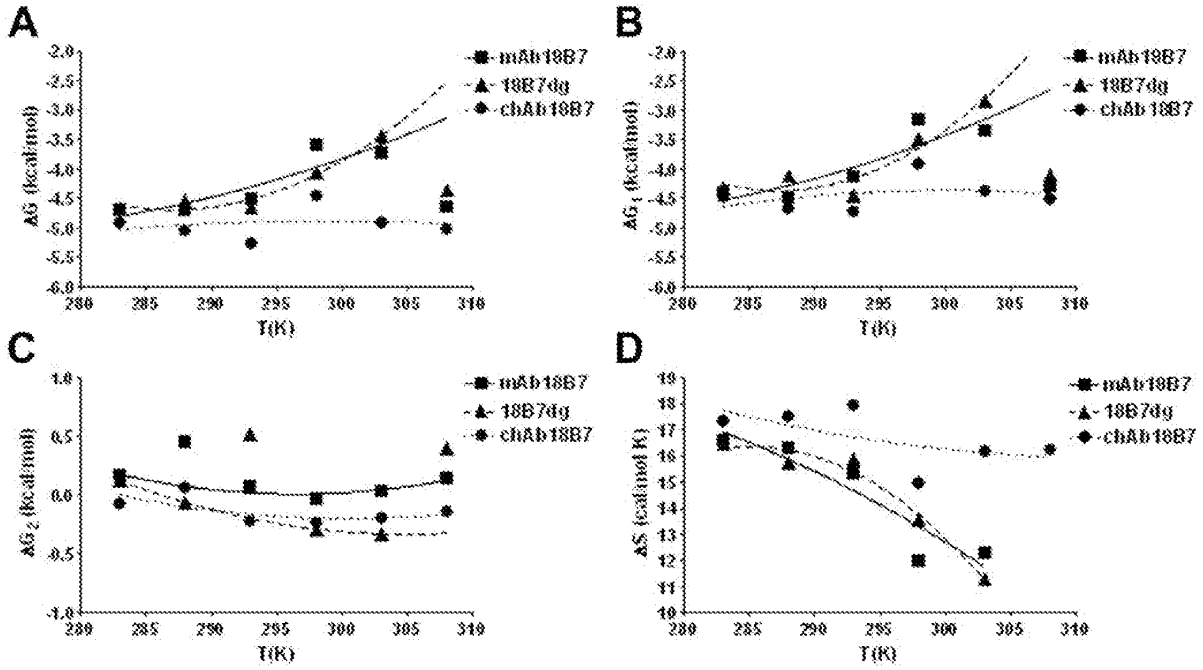


Figure 3. Thermodynamics of binding of mAbs 18B7, 18B7dg and chAb 18B7 to a peptide mimetic of GXM. Behavior of *A*, free energy of binding (ΔG), *B*, encounter free energy of binding (ΔG_1), *C*, docking free energy of binding (ΔG_2) and *D*, entropy of binding (ΔS) as a function of temperature for the mAb 18B7, 18B7dg and chAb 18B7 binding to peptide P1. Free energies were calculated from the formula $\Delta G = -RT \ln K_A$, $\Delta G = -RT \ln K_{a1}$, $\Delta G = -RT \ln K_{a2}$ and ΔS from the formula $\Delta G = \Delta H - T\Delta S$.
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isotype it is important to understand how substituting a $\gamma 1$ C_H chain affects murine V region binding affinity and specificity. An improved understanding of the mechanisms underlying this effect may allow the design of less immunogenic Abs for therapeutic use that maintain greater Ag-binding fidelity and lower polyreactivity.

METHODS

MAbs and peptides

GXM-binding mAb 18B7 was made from a mouse immunized with a GXM-Tetanus Toxoid conjugate vaccine [8]. Mouse-human chAb 18B7 (IgG1) was generated by cloning and transfection as described [36]. mAb 18B7 was purified by protein G affinity chromatography (Pierce) from hybridoma culture supernatants, and dialyzed against at least two changes of PBS. Mouse-human chAb 18B7 was purified by protein L agarose beads (Sigma). mAb 18B7 was deglycosylated as described [24]. Briefly, 337 μg of mAb 18B7 were incubated at 37°C for 24 h with 100 mU of PNGaseF (Glyco). Then, the digestion mixture was dialyzed against at least two PBS changes. All Abs were analyzed by polyacrylamide gel electrophoresis to verify their integrity and correct molecular weight. Ab concentration was determined by ELISA and Bradford measurements. Peptide mimetic of GXM P1 (SPNQHTPPWMLK) [3] was synthesized and biotinylated by the Laboratory for Macromolecular Analysis at the Albert Einstein College of Medicine.

Immobilization of mAb 18B7, 18B7dg and chAb 18B7

The BIAcore 3000 system and the research grade sensor chip CM5 (BIAcore) were used. mAbs were immobilized in the surface of the CM5 chip through primary amino groups using reactive esters. First, the carboxylated matrix was activated with 70 μl of a 1:1 mixture of N-ethyl-N′(dimethylaminoporpyl)-carbodiimide (EDC) (Pierce) and N-hydroxysuccinimide (NHS) (Pierce). Then, mAbs 18B7 and 18B7dg were injected at 25 μg/ml in 10 mM 2-(N-morpholino) ethanesulphonic acid (MES) (Sigma-Aldrich), pH 6.0. chAb 18B7 was injected at 25 μg/ml in 10 mM sodium acetate (Sigma-Aldrich), pH 5.42. After mAb injection, remaining NHS-ester groups were blocked by injection of 70 μl of 1M ethanolamine (Sigma-Aldrich), pH 8.5.

Kinetic Measurements

To analyze the kinetic and thermodynamic parameters of the Abs utilized in this study we used the peptide mimetic of GXM P1 [3]. The interaction of the mAbs-peptide complexes was analyzed at 10, 15, 20, 25, 30, and 35°C. Peptide P1 was tested at concentrations ranging from 1.56 μM to 200 μM at high flow rate (100 μl/min) in 0.2 mM K_2HPO_4/KH_2PO_4 pH 6.5, 150 mM KCl and 0.01% Tween 20. At the end of the dissociation phase the surface was regenerated by injecting 50 μl of 50 mM ethanolamine in 50% ethylene glycol (Sigma-Aldrich) and 50 μl of 10 mM HCl. An unmodified dextran surface was used as reference. Kinetic data was analyzed with the BIAevaluation 4.1 software. Data for each group was analyzed globally using a two-state model to obtain the forward (k_{+1}, k_{+2}) and reverse (k_{-1}, k_{-2}) rate constants. The rate constants for each global fit were used to calculate the association equilibrium constant, based on the following equations:

$$K_{a_1} = k_{+1}/k_{-1} \quad \text{and} \quad K_{a_2} = k_{+2}/k_{-2} \tag{1}$$

$$K_A = K_{a_1}(1 + K_{a_2}) \tag{2}$$

The Gibbs free energy changes ΔG were calculated from the

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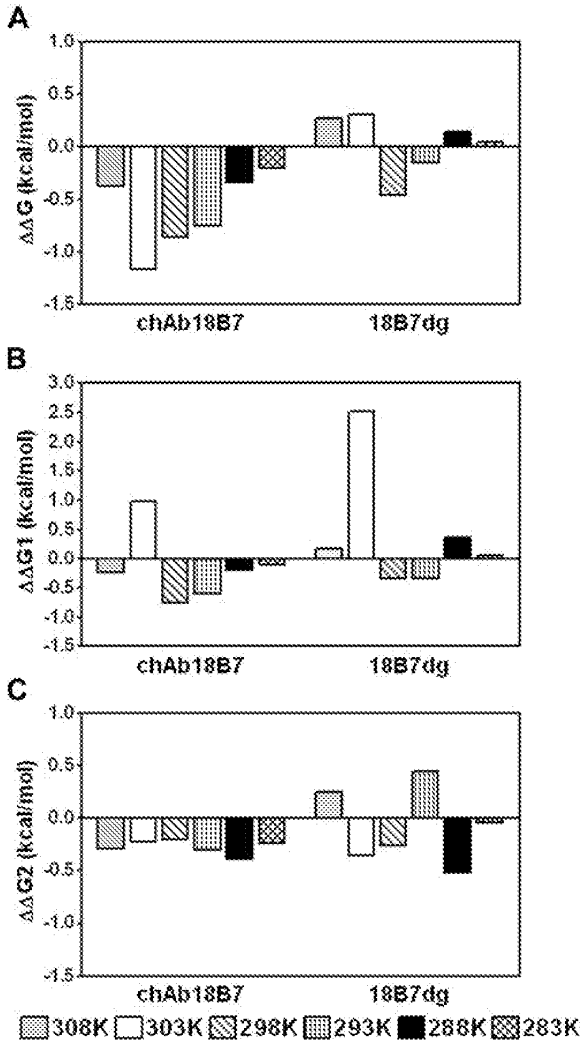


Figure 4. Effect of temperature on the differences in free energy of binding ($\Delta\Delta G$) of chAb 18B7 and 18B7dg complexes. Each panel corresponds to the ΔG of chAb 18B7-P1 and 18B7dg-P1 complex formation, relative to the ΔG of parental mAb 18B7, at different temperatures. ***A***, overall difference of the free energy of binding ($\Delta\Delta G$) and overall difference of the free energy of binding of ***B***, encounter and ***C***, docking steps ($\Delta\Delta G1$ and $\Delta\Delta G2$) for the mAbs 18B7dg and chAb 18B7 at 35°C, 30°C, 25°C, 20°C, 15°C, 10°C. Values of $\Delta\Delta G$ were calculated as $\Delta G_{ch/dgAb} - \Delta G_{mAb18B7}$.
doi:10.1371/journal.pone.0001310.g004

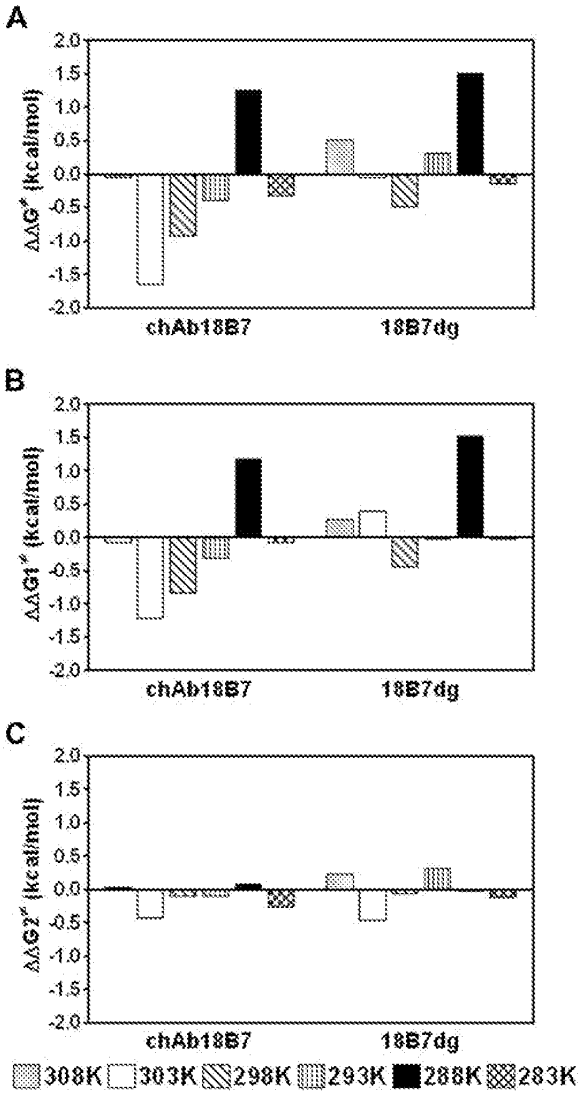


Figure 5. Differences in free activation energy of binding ($\Delta\Delta G^{\ddagger}$) of the transition states of chAb 18B7 and 18B7dg for the binding to peptide P1. Each panel corresponds to the ΔG of chAb 18B7-P1 and 18B7dg-P1 complex formation, relative to the ΔG of parental mAb 18B7. ***A***, free activation energy of binding ($\Delta\Delta G^{\ddagger}$), ***B***, encounter activation free energies ($\Delta\Delta G1^{\ddagger}$) and ***C***, docking activation free energies ($\Delta\Delta G2^{\ddagger}$), as a function of temperature. Values of $\Delta\Delta G$ were calculated as $\Delta G_{ch/dgAb} - \Delta G_{mAb18B7}$. In all cases, the activation energies for the encounter and docking steps ($\Delta G1^{\ddagger}$ and $\Delta G2^{\ddagger}$) were calculated from k_{+1} and k_{+2}, respectively, according to the transition state theory: $\Delta G^{\ddagger} = -RT \ln K^{\ddagger}$, $\Delta\Delta G^{\ddagger} = -RT \ln K^{\ddagger}_{ch/dg\ Ab}/K^{\ddagger}_{mAb18B7}$, and $K^{\ddagger} = k_B\ T\ k_a/\hbar$, where k_a is the forward rate constant, k_B is Boltzmanns constant and $\hbar$ is Plank's constant.
doi:10.1371/journal.pone.0001310.g005

equilibrium constants:

$$\Delta G = -RT \ln K_A \tag{3}$$

$$\Delta G1 = -RT \ln K_{a_1} \quad \text{and} \quad \Delta G2 = -RT \ln K_{a_2} \tag{4}$$

$$\Delta G = \Delta H - T\,\Delta S \tag{5}$$

These equations can be rearranged to yield the relation:

$$\ln K_A = -\Delta H/RT + \Delta S/R \tag{6}$$

By measuring K_A as a function of temperature, one can plot $\ln K_D$ versus $1/T$ (eq. 6). Ideally, this plot, known as van't Hoff plot, should yield a straight line with a slope of $-\Delta H/R$ and an intercept $\Delta S/R$. Enthalpy changes (ΔH) were calculated from the slope of the van't Hoff plot. Entropy changes (ΔS) were calculated from the equation 5, by substituting the values of ΔG (eq. 3) and assuming a constant ΔH with temperature. Equilibrium constants were calculated without considerations of the errors of the rate constants.

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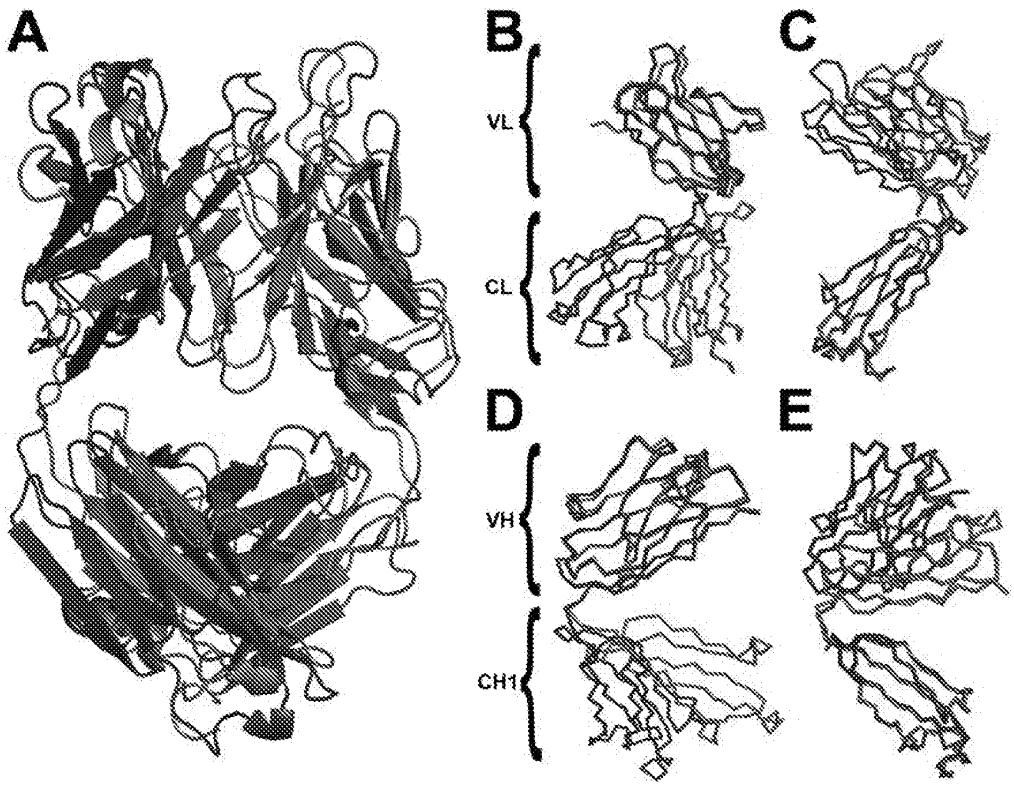


Figure 6. Structural models for chAb18B7 (red) and murine mAb18B7 (blue). ***A,*** Global structural superposition of the models; light chain depicted in gray. ***B,*** Structural superposition of light chain keeping fixed V_L and ***C,*** C_L domain. Structural superposition of ***D,*** heavy chain keeping fixed V_H and ***E,*** C_{H1} domain. Figures 6 and 7 were generated using PyMOL (http://pymol.sourceforge.net).
doi:10.1371/journal.pone.0001310.g006

Molecular modeling

mAb 18B7 and ch18B7 were modeled using the sequence for V_H and V_L from mAb 18B7 obtained from the protein GeneBank[37] with accession numbers AJ309266 and AJ309267, respectively. Each sequence was used to scan the Protein DataBank (PDB) [38] using PSI-BLAST [39] with default parameters. PSI-BLAST outputs were filtered using BlastProfiler [40] to select templates with the highest sequence coverage and sequence identity. Selected templates were manually inspected to choose those ones with the highest crystallographic quality and sequence coverage. The models were built with M4T [41] combining two experimental structures as templates for each sequence. A common structure for all sequences (PDB code 2h1p), was used as template for V and C regions of both L and H chain; and a specific template for the C_{H1} domain depending on the Ab species (PDB codes of 1sbs and 1pz5 C_{H1} domain, for human and mouse respectively). The 2h1p structure corresponds to the experimental three-dimensional structure of anti-GXM mAb 2H1 with peptide mimetic of GXM PA1. The average sequence identities between target sequences and templates were larger than 90%, assuring high quality models [42,43]. The quality of the models was assessed using PROSA-II [44] and PROCHECK [45].

Connectivity analysis of residues

Structural models of mAb 18B7 and chAb 18B7, were transformed into interaction graphs. The nodes of the graphs are the residues and the edges between nodes are any type of inter-atomic interactions, namely covalent (peptide bond) and non-covalent interactions (hydrogen bonds, polar interactions, and hydrophobic interactions). Inter-atomic interactions were described using the CSU program [46]. For each node (or residue) of the graph a *closeness centrality* Z-score was calculated as described in Amitai G. *et al* [33]. The *closeness centrality* is a measure of connectivity; a high closeness centrality value indicates a high number of interactions with the rest of nodes (or residues).

ELISA

mAb 18B7, 18B7dg and chAb 18B7 were screened for their polyreactive properties against a panel of antigens by ELISA. Polystyrene plates were coated overnight at 4°C with actin (Sigma), tubulin (ICN) and thyroglobulin (Sigma) at 5 μg/ml in bicarbonate buffer pH 9.6, single stranded (ss) and DNA (Sigma) at 100 μg/ml in sodium citrate buffer, pH 6.0. Plates were blocked with 1% BSA in TBS for 2 h at 4°C. Abs were added at 40 μg/ml in 1% BSA/TBS and serially diluted. Plates were incubated overnight at 4°C. Ab binding was determined with alkaline-phosphatase conjugated goat anti-mouse H+L (Southern Biotech) for the murine Abs and alkaline-phosphatase conjugated goat anti-human IgG (Caltag) for chAb 18B7. The plates were developed with *p*-nitrophenyl phosphate substrate by measuring absorbance at 405 nm.

Statistics

Standard deviations (SD) for the ELISA were calculated using Microsoft Excel functions for SD. The error bars on the van't Hoff plots correspond to the error propagation (ep) of the natural log

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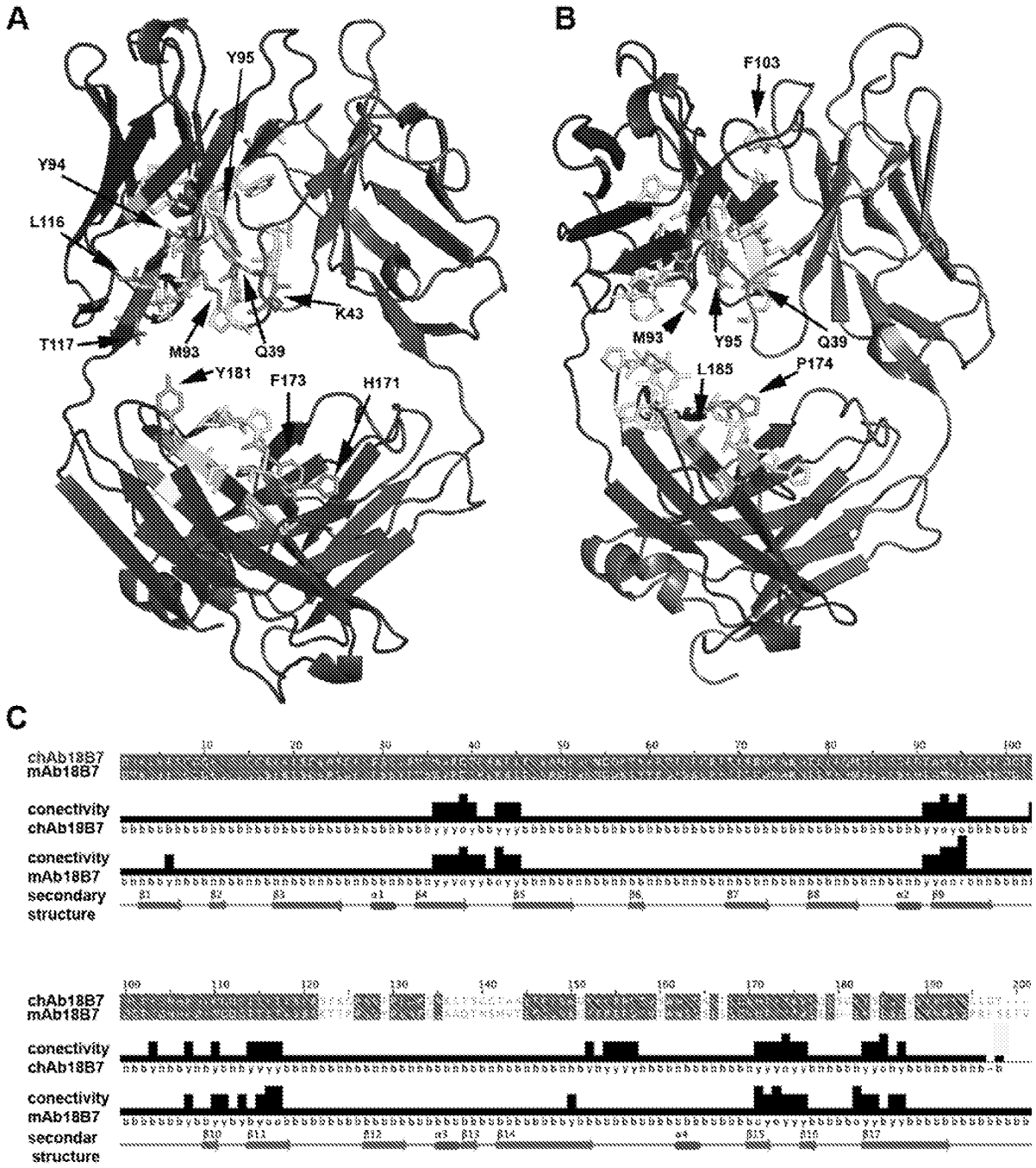


Figure 7. Connectivity analysis of residues in mAb 18B7 and ch18B7 models of C_{H1} domain. Cartoon representation of ***A***, murine mAb 18B7 V_H-C_{H1} and ***B***, chAb 18B7. Areas of high connectivity are indicted by red and orange (red Zscore>2.0; orange: 1.5≤Zscore<2.0); medium connectivity residues are indicated in yellow (1.0≤Zscores<1.5), whereas low connectivity is indicated in blue (Zscore<1.0). Light chain is shown in gray. Side chains of amino acids with high connectivity values (Zscore≥1.5) are indicated by arrow. ***C***, Residues masked in blue in the sequence alignment of H chain are equal in both Abs (domain V_H: 1–120; domain C_{H1}: 121–200). Connectivity values for each residues are shown in the alignment (in form of histogram and color coding, being b, y, o, and r: blue, yellow, orange, and red respectively); secondary structure elements are also shown.
doi:10.1371/journal.pone.0001310.g007

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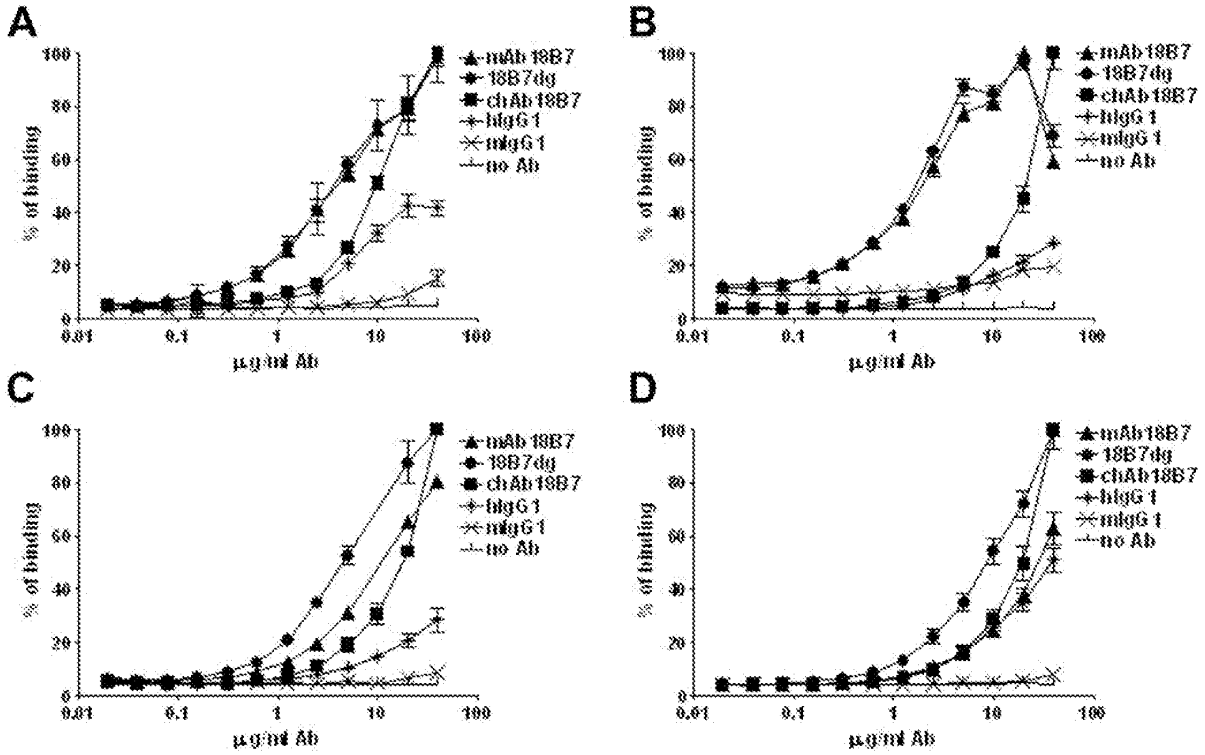


Figure 8. Reactivity patterns of mAb 18B7, 18B7dg and chAb 18B7 against polyreactive antigens. Panels correspond to the binding profiles of these Abs to, *A*, tubulin, *B*, ssDNA, *C*, thyroglobulin and *D*, actin. Human (hIgG1) and mouse (mIgG1) IgG1 were used as control. Data represent mean±SD of three measurements.
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(ep = SE_k/k) of the standard error (SE) obtained with the BIAevaluation software for each rate constant individually.

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Author Contributions

Conceived and designed the experiments: MT. Performed the experiments: MT. Analyzed the data: MT. Wrote the paper: MT. Other: Conceived the experiments, Reviewed the paper: AC Conceived and design molecular modeling analysis, Reviewed the paper: AF Performed molecular modeling analysis, Reviewed the paper: NF.

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The Immunoglobulin Heavy Chain Constant Region Affects Kinetic and Thermodynamic Parameters of Antibody Variable Region Interactions with Antigen*[S]

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A central dogma in immunology is that antibody specificity is a function of the variable (V) region. However serological analysis of IgG_1, IgG_{2a}, and IgG_{2b} switch variants of murine monoclonal antibody (mAb) 3E5 IgG_3 with identical V domains revealed apparent specificity differences for *Cryptococcus neoformans* glucuronoxylomannan (GXM). Kinetic and thermodynamic binding properties of mAbs 3E5 to a 12-mer peptide mimetic of GXM revealed differences in the affinity of these mAbs for a monovalent ligand, a result that implied that the constant (C) region affects the secondary structure of the antigen binding site, thus accounting for variations in specificity. Structural models of mAbs 3E5 suggested that isotype-related differences in binding resulted from amino acid sequence polymorphisms in the C region. This study implies that isotype switching is another mechanism for generating diversity in antigen binding and that isotype restriction of certain antibody responses may reflect structural constraints imposed by C region on V region binding. Furthermore, isotype affected the polyreactivity of V region identical antibodies, implying a role for C region in determining self-reactivity.

Antibodies are heterodimeric proteins composed of two heavy (H)[2] and two light (L) chains. Each H and L chain contains a V domain that when combined defines the antigen (Ag) binding site. In the mammalian genome, there are five major classes of heavy constant (C_H) domains that determine Ab isotype. These are usually assumed to have effector functions without directly affecting the Ag binding affinity and/or specificity. However, the C_H domains are known to contribute to the apparent affinity through avidity effects that result from the polyvalent nature of certain immunoglobulins (Ig) classes. Hence, the classical view of Ig function is that the V domains are solely responsible for Ab affinity and specificity, whereas the C region is responsible for the biological properties such as complement activation, Fc receptor binding, avidity, and serum half-life (1). This concept posits that when B cells switch from one C_H region to another, the avidity and effector functions of an Ab change without altering the specificity for the antigen (2). However, V region-identical Abs have been reported to manifest differences in the magnitude of binding to Ag, fine specificity, and idiotypic (Id) recognition (3–7), thus challenging this central immunological dogma.

A critical condition for Ab-Ag binding is the formation of a specific complex between the Ab and the Ag. Understanding the interaction of these two biological macromolecules requires detailed knowledge of the structure and functional characteristics of the complex. The structure of the Ab-Ag complex can be described using x-ray crystallography and computer-generated structural models. The functional activity can be described by the kinetic rate constants ($k_{on/off}$), equilibrium constants (K), and thermodynamic parameters of the binding complex. The equilibrium constant is related to the Gibbs free energy of binding (ΔG) by the relationship $\Delta G = -RT\ln K_A = \Delta H - T\Delta S$. A net negative change in ΔG indicates successful binding interactions, and the magnitude of this change directly determines the binding affinity. ΔG involves and is modulated by two other thermodynamic parameters, the enthalpy change (ΔH) and the entropy change (ΔS) (8, 9). Enthalpy describes heat changes resulting from molecular interactions at the binding site involving electrostatic, hydrophobic, hydrogen bonds, and van der Waals intermolecular interactions, whereas entropy changes reflect the net conformational, stereochemical, and structural perturbations that occur within the interacting entities or in the surrounding solvent molecules (8, 9).

Our previous studies using a family of mouse-human chimeric (ch) Abs and two families of murine IgG isotype switches demonstrated that isotype contributes to Ab fine specificity differences for multivalent Ags by showing differences in binding and Id recognition for these mAbs to their Ags (7, 10). Those studies relied entirely on serological methods to investigate binding differences. To formally prove the notion that the C_H region affects binding to a monovalent ligand, we have used surface plasmon resonance (SPR) in a Biacore system. SPR allows measurements of the formation and dissociation of com-

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[2] The abbreviations used are: H, heavy chain; L, light chain; GXM, glucuronoxylomannan; Ab, antibody; mAb, monoclonal antibody; Ag, antigen; C, constant region; V, variable region; ch, chimeric; SPR, surface plasmon resonance; Fab, antigen-binding fragment; Id, idiotype/idiotypic; K, equilibrium constant; k, kinetic rate constant; ΔG, Gibbs free energy; ΔH, enthalpy; ΔS, entropy; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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plexes, in real time and in a label-free environment. Using SPR analysis we determined the kinetic and thermodynamic constants for the interaction of V region-identical IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 with a monovalent Ag in the form of peptide mimetic. The binding kinetics are best described by a two-state bimolecular association model consistent with an Ab and Ag encounter step followed by a docking step, which may include conformational rearrangements. Consequently, the results presented here indicate kinetic and thermodynamic variations that imply diverse Ab-Ag interactions within IgG molecules expressing different C_H regions but identical V regions.

EXPERIMENTAL PROCEDURES

mAbs and Peptides—GXM-binding mAb 3E5 (IgG_3) was produced by a hybridoma isolated from a mouse immunized with a GXM-tetanus toxoid conjugate vaccine (11). The IgG_1, IgG_{2a}, and IgG_{2b} switch variants of 3E5 IgG_3 were generated *in vitro* by sib selection (12). Members of the mAb 3E5 family share identical V_H and V_L sequences (7). All murinem mAbs were purified by protein A or G affinity chromatography (Pierce) from hybridoma culture supernatants and dialyzed against PBS. mAb concentration was determined by ELISA and Bradford measurements. All Abs were tested by PAGE to verify their integrity and correct molecular weight. mAbs 3E5 were analyzed by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry at the Laboratory for Macromolecular Analysis, Albert Einstein College of Medicine, to confirm the absence of mAb aggregates (7, 13). Peptide mimetic P1 (SPNQHTPPWMLK) (7) was synthesized and biotinylated by the Laboratory for Macromolecular Analysis, Albert Einstein College of Medicine.

Generation of Fabs—Fabs were generated from mAbs 3E5 IgG_{2a} and 3E5 IgG_3. mAb 3E5 IgG_{2a} was dialyzed against 20 mM Na_2PO_4, 10 mM EDTA, pH 7.0, before enzymatic digestion. Papain digestion was carried out overnight at room temperature in 20 mM Na_2PO_4, 10 mM EDTA, pH 7.0, and 0.8 mM dithiothreitol with an *E*:S ratio of 1:20. mAb 3E5 IgG_3 was dialyzed against 20 mM Tris-NaCl pH 7.4, before enzymatic digestion. Papain digestion was carried out overnight at room temperature in 20 mM Tris-NaCl, pH 7.4, and 0.8 mM dithiothreitol with an *E*:S ratio of 1:20. After digestion, Fab molecules from both mAbs were purified by protein A affinity chromatography (Pierce) and dialyzed against at least two changes of PBS.

GXM-3E5 mAbs Binding ELISA at Different pHs—Polystyrene plates were coated with a 5 μg/ml solution of GXM in PBS, incubated 1.5 h at 37 °C, and blocked overnight at 4 °C with 1% BSA in TBS. mAbs 3E5 were added at 50 μg/ml in 0.2 M phosphate buffer at pH ranging from 4.4 to 10.4 and serially diluted. The plates were incubated for 1.5 h at 37 °C. mAb binding was determined with alkaline-phosphatase-conjugated goat anti-mouse κ (Southern Biotech) and *p*-nitrophenyl phosphate substrate by measuring absorbance at 405 nm.

GXM-3E5 mAbs Binding ELISA in Ethanol—Polystyrene plates were coated with a 5 μg/ml solution of GXM in PBS, incubated 1.5 h at 37 °C, and blocked with 1% BSA in TBS. mAb was added at 50 μg/ml in 5% ethanol, 1% BSA. Serial dilutions were made, and plates were incubated for 1.5 h at 37 °C. mAb binding was determined as above.

Polyreactivity ELISA—mAbs 3E5 were screened for polyreactivity against a panel of antigens by ELISA. Polystyrene plates were coated overnight at 4 °C with actin (Sigma), tubulin (ICN), and thyroglobulin (Sigma) at 5 μg/ml in bicarbonate buffer, pH 9.6, and with double-stranded DNA (Sigma) and single-stranded DNA (Sigma) at 100 μg/ml in sodium citrate buffer, pH 6.0. Plates were blocked with 1% BSA in TBS for 2 h at 4 °C. mAbs were added at 50 μg/ml in 1% BSA/TBS and serially diluted. Plates were incubated overnight at 4 °C. mAb binding was determined with alkaline-phosphatase-conjugated goat anti-mouse κ (Southern Biotech) and *p*-nitrophenyl phosphate substrate by measuring absorbance at 405 nm.

Immobilization of 3E5 mAbs onto CM5 Chips—The Biacore 3000 system and research grade sensor chip CM5 (Biacore) were used. mAbs and Fabs were immobilized on the surface of a CM5 chip through primary amino groups using reactive esters. The carboxylated matrix was first activated with 70 μl of a 1:1 mixture of *N*-ethyl-*N*′(dimethylaminoporpyl)-carbodiimide (Pierce) and *N*-hydroxysuccinimide (Pierce). mAbs 3E5 were injected at 25 μg/ml in 10 mM MES (Sigma-Aldrich), pH 6.0. Fab 3E5 IgG_{2a} and Fab 3E5 IgG_3 were injected at 100 and 50 μg/ml, respectively, in 10 mM sodium acetate (Sigma-Aldrich), pH 4.99. After mAb injection, remaining *N*-hydroxysuccinimide ester groups were blocked by an injection of 70 μl of 1 M ethanolamine (Sigma-Aldrich), pH 8.5.

Biacore Binding Assays—Peptide mimetic of GXM P1 was used to analyze the kinetic and thermodynamic parameters of the mAbs utilized in this study. This peptide was recovered from a peptide phage display library screened with 3E5 IgG_3, and it has been previously shown to bind all mAb 3E5 isotypes (7). The interaction of the mAb-peptide complexes was analyzed at 10, 15, 20, 25, 30, and 35 °C. Peptide P1 was tested at concentrations ranging from 1.56 to 200 μM at high flow rate (100 μl/min) in 0.2 mM K_2HPO_4/KH_2PO_4, pH 6.5, 150 mM KCl, and 0.05% P20. At the end of the dissociation phase, the surface was regenerated by injecting 50 μl of 50 mM ethanolamine in 50% ethylene glycol (Sigma-Aldrich) and 50 μl of 10 mM HCl. Nonderivatized flow cells served as a reference surface. The continuous flow ensured that no changes in analyte concentration occurred during the measurements. The availability of a constant free analyte concentration is particularly important for the determination of kinetic parameters. By using one of the flow channels as an in-line reference, a control is obtained on the same sample aliquot, eliminating the variations that occur with separate reference and sample measurements. Kinetic data were interpreted with BIAevaluation 4.1 software. Data for each group were analyzed globally using a two-state model to obtain the individual forward (k_{+1}, k_{+2}) and reverse (k_{-1}, k_{-2}) rate constants. The rate constants for each global fit were used to calculate the equilibrium constant (K_A).

$$K_{a1} = k_1/k_{-1} \quad \text{(Eq. 1)}$$

$$K_{a2} = k_2/k_{-2} \quad \text{(Eq. 2)}$$

$$K_A = K_{a1}(1 + K_{a2}) \quad \text{(Eq. 3)}$$

The Gibbs free energy changes (ΔG) were calculated from the equilibrium constants,

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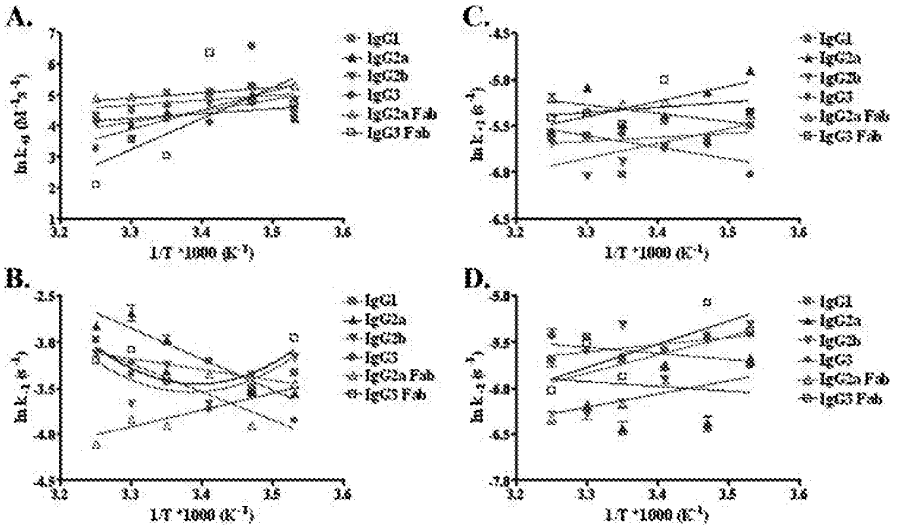


FIGURE 1. **Analysis of the association and dissociation rates constants by SPR.** Van't Hoff plots of the rate constants k_{+1} (*A*), k_{-1} (*B*), k_{+2} (*C*), and k_{-2} (*D*) for mAbs 3E5 binding to peptide P1. All data points were obtained from the BIAevaluation 4.1 software using a two-state model. *Lines* represent the best fit to a linear regression using a 95% confidence interval. *Errors bars* were calculated using propagation of error for S.E. of each assay.

$$\Delta G_1 = -RT\ln K_{a1} \quad \text{(Eq. 4)}$$

$$\Delta G_2 = -RT\ln K_{a2} \quad \text{(Eq. 5)}$$

$$\Delta G = -RT\ln K_A = \Delta G_1 + \Delta G_2 \quad \text{(Eq. 6)}$$

$$\Delta G = \Delta H - T\Delta S \quad \text{(Eq. 7)}$$

By rearranging the equations above, we can rewrite the relationship into

$$\ln K_A = -\Delta H/RT + \Delta S/R \quad \text{(Eq. 8)}$$

By measuring K_A as a function of temperature, one can draw a plot of $\ln K_D$ ($1/K_A$) *versus* $1/T$. Ideally, this plot, known as a van't Hoff plot, should yield a straight line with a slope of $-\Delta H/R$ and an intercept $\Delta S/R$. Enthalpy changes (ΔH) were calculated from the slope of the van't Hoff plot. Entropy changes (ΔS) were calculated from the equation $\Delta G = \Delta H - T\Delta S$ by substituting the values of ΔG (from $\Delta G = -RT\ln K_A$) and assuming ΔH is constant with temperature. Equilibrium affinity constants were calculated without considering the errors of the rate constants.

Molecular Modeling—The variable domains of the heavy and light chains of mAbs 3E5 IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 sequences are identical. Thus, only one set of sequences corresponding to the V_H and V_L sequences of 3E5 IgG_1, with accession numbers AAT81538 and AAT81540, were downloaded from GenBank™ (14). Each sequence was used to scan the Protein Data Bank (15) using PSI-BLAST (16) with default parameters. PSI-BLAST outputs were filtered using BlastProfiler (17) to select templates with the highest sequence coverage and high sequence identity. Selected templates were inspected manually to choose those with the highest crystallographic quality. The models were built with MODELLER (18, 19), combining two experimental structures as templates for each sequence. A common structure for all sequences (Protein Data Bank code 2h1p), was used as template for V and C regions of both the L and H chains and a specific template for the C_{H1} domain depending on the Ab isotype (Protein Data Bank codes 1sbs, 1jgu, 2cgr, and 1pz5 for IgG_1, IgG_{2a}, IgG_{3b}, and IgG_3 C_{H1} domain, respectively). The 2h1p structure corresponds to the experimental three-dimensional structure of anti-GXM mAb 2H1 with peptide mimetic of GXM PA1. The average sequence identity between target sequences and templates was greater than 90%, assuring high quality models (19). Five models were generated for each mAb, and the one with lowest energy (according to MODELLER energy function) was selected and further analyzed with PROSA-II (20) and PROCHECK (21) to determine the quality of the models.

Identifying "Highly Connected" Regions—Structural models were submitted to the SARIG (structural analysis of residue interaction graphs) server (22). The SARIG server converts protein structures into residue interaction graphs (RIGs) in which amino acid residues serve as the nodes of the graph and their interatomic contacts with each other are the graph edges. SARIG analyzes the so-called closeness of the residue(s). Closeness is a property that measures how close each node (or residue) is to all other nodes in the network. The distance is defined as the length of the shortest path between the two nodes. Closeness values are expressed in Zscores and thus are standardized by the standard deviation from the mean value calculated for each protein structure. It was shown that functionally important residues typically have high closeness values. Residues with high closeness values, *i.e.* highly connected ones, may interact directly or through intermediates (allostery) with other residues of the protein.

RESULTS

One-step Versus Two-step Kinetic Models—We chose the two-state model based on the proposal that these mAbs may have changes in their Fab structure or Ig surface properties, which could be a result of binding to Ag (7). The two-step model fitted better than one-step model to our experimental data (Fig. 1S in supplemental data, and data not shown) and it has been shown to be a more precise representation of Ab-Ag interactions (23, 24). The simulated curves generated with the two-state model were almost identical to the experimental curves (data not shown). In addition, we found that the Langmuir one-step binding model was not applicable because of the higher apparent affinity obtained with intact IgG. Thus, binding kinetics indicates that mAbs 3E5-P1 complexes are best described by a two-state model,

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$$A + B \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AB^* \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} AB$$

REACTION 1

This model allows the estimation of two forward rates constants (k_{+1}, k_{+2}) and two reverse rate constants (k_{-1}, k_{-2}). The rate constants k_{+2} and k_{-2} are not true on- and off-rate constants but rather are the rates of monomolecular complex conversion between the docked and undocked form. The rate constants k_{+2} and k_{-2} modulate the observed on- and off-rates when they become rate-limiting, *i.e.* when $k_{+2} < k_{+1}$ or $k_{-2} < k_{-1}$ (25). The fitting of sensorgram data to a two-state model (supplemental data, Fig. 1S) is significantly better than to a one-step model based on low and evenly distributed residuals, low χ^2 (<1), and visual inspection of the binding curves. This fitting is not attributable to heterogeneity of the IgG sample or to nonspecific interactions, because all mAbs were obtained from hybridoma culture supernatant, affinity-purified, and tested for GXM binding by ELISA. PAGE and mass spectrophotometry were used to verify mAb integrity and to discard the possibility that some of our results were due to the presence of mAb aggregates.

Effect of Antibody Subclass on the Kinetic and Equilibrium Rate Constants—SPR analysis revealed differences between the binding kinetics of the mAbs 3E5-P1 complexes that were also observed throughout the temperature range studied. Examination of the dissociation equilibrium constants and individual rate constants indicates that all four Abs and the two Fabs exhibited different kinetic and thermodynamic properties on binding. These changes were also observed with variation in temperature. Furthermore, the magnitude of the temperature effect was characteristic for each Ab-peptide complex (Figs. 1 and 2). Rate and equilibrium constants also revealed that the variation in temperature had different impacts on the encounter and docking steps.

Slightly different results were observed for the effect of temperature on the association and dissociation rates of mAb 3E5-P1 complex formation with the different isotypes. For all complexes, the encounter forward rate constant (k_{+1}) decreased at higher temperatures (Fig. 2*A*). Fig. 1*C* shows that for 3E5 IgG_1-P1, IgG2a-P1, and IgG_{2b}-P1 complexes, the docking association rate constant (k_{+2}) decreased at high temperatures, whereas for 3E5 IgG_3, Fab IgG_{2a}, and Fab IgG_3 the magnitude of this parameter increased. The curves for the encounter reverse rate constant (k_{-1}) increased at higher temperatures for 3E5 IgG_1-P1, IgG_{2a}-P1, and IgG_3-P1 complexes, whereas for Fab 3E5 IgG_{2a}-P1 complex a decrease of k_{-1} was observed. For 3E5 IgG2b-P1 and Fab 3E5 IgG_3-P1 complexes, the data most closely fit a curvilinear regression, and therefore these interactions appeared to be more complex (Fig. 1*B*). The plots for docking reverse rate constant (k_{-2}) showed a decrease with a temperature increase for 3E5 IgG_{2a}-P1, IgG_{2b}-P1, and Fab IgG_3-P1 complexes, whereas k_{-2} increased with temperature for the remaining complexes (Fig. 1*D*). However, quantitatively the individual rate constants showed differential response to temperature, with variations on the amount of these decreases between mAbs 3E5-P1 complexes.

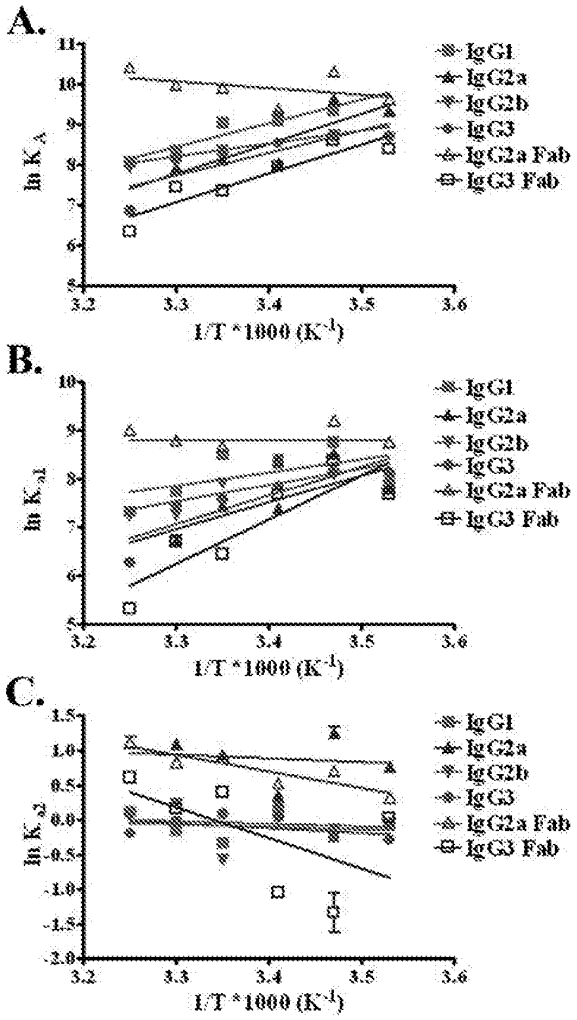


FIGURE 2. **Equilibrium affinities for the binding of mAbs 3E5 to peptide P1.** Van't Hoff plots of the equilibrium association rate constant (K_A) (*A*), encounter equilibrium association rate constant (K_{a1}) (*B*), and docking equilibrium association rate constant (K_{a2}) (*C*) for mAbs 3E5 binding to peptide P1. All equilibrium constants were calculated from the rate constant obtained from the fittings to a two-state model. *Lines* represent the best fit to a linear regression using a 95% confidence interval. *Errors bars* were calculated using propagation of error for S.E. of each assay.

The differences in the rate constants for these complexes accounted for the variations observed in the equilibrium rate constants. The affinity of mAbs 3E5 for peptide P1 calculated from the van't Hoff plots decreased at higher temperatures, except for Fab 3E5 IgG_{2a}, in which affinity for the peptide increased (Fig. 2*A*). The equilibrium constants for the encounter steps (K_{a1}) for these complexes decreased as temperature increased, except for the 3E5 Fab IgG_{2a}-P1 complex, where K_{a1} increased (Fig. 2*B*). For the IgG_{2a}-P1 complex, the decrease of k_{-1} (Fig. 1*C*) as a function of temperature may contribute to the temperature increase of K_{a1}. Also, it is noticeable that the van't Hoff plots for the encounter association constant K_{a1} were approximately parallel to those for the overall affinity constants K_A (Fig. 2*A*). In contrast, the docking equilibrium constants (K_{a2}) were relatively insensitive to changes in temperature for

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all complexes (Fig. 2*C*), with the exception of the Fab 3E5 IgG_{2a}-P1 complex where this parameter decreased at lower temperatures. The K_{a2} of the Fab 3E5 IgG_3 also decreased initially at lower temperatures but increased again at temperatures below 15 °C (288 K) (Fig. 2*C*). The decrease of overall affinity with increasing temperatures reflects a primary temperature-related reduction in the encounter equilibrium constants of the mAbs 3E5-P1 complexes except for the Fab 3E5 IgG_{2a}-P1 complex. For this complex, the increase in overall affinity reflects a temperature-related increase in the encounter equilibrium constant.

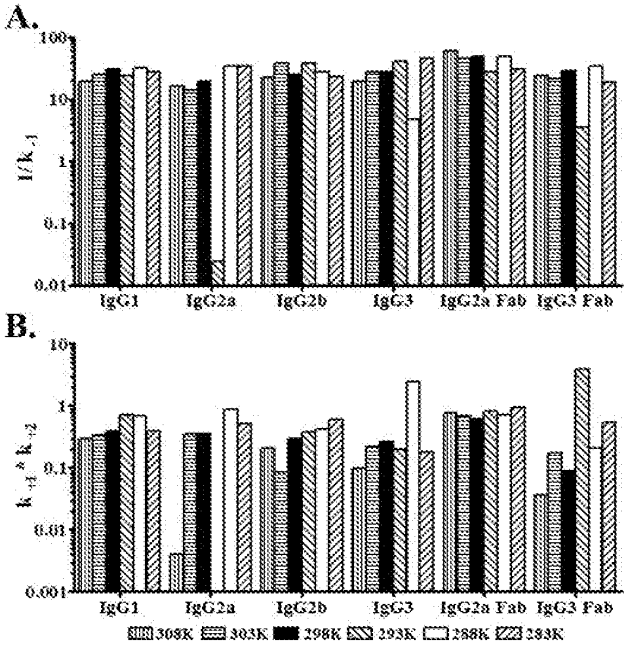


FIGURE 3. **Effect of temperature on the stability and steering of mAbs 3E5 for the binding to peptide P1.** The tendency of the encounter complex to evolve into the final docked complex is proportional to $k_{+1}k_{+2}/k_{-1}$, where $1/k_{-1}$ relates to the stabilization of the docked complex (*A*) and $k_{+1}{}^{*}k_{+2}$ relates to the contribution of electrostatic forces to molecular steering (*B*). Each *bar* represents a different temperature value in degrees Kelvin.

The k_{+2}/k_{-1} ratio is a sensitive indicator of favorable docking (23, 26). Throughout the range of temperatures studied, this ratio is <1 (supplemental data, Table 2S) suggesting that all mAb 3E5-P1 complexes are more likely to remain at the encounter step than to dock. In addition, $k_{+1} > k_{+2}$ for all mAbs 3E5 binding to P1 at the entire range of temperature, thus increasing the time required for the complexes to reach equilibrium and making the docking step rate-limiting. Because of the slower docking and faster dissociation of the encounter complex, the apparent association rate constant for the mAbs 3E5-P1 interactions appeared to be much slower than it is. The reaction rates can be modified through the action of long range electrostatic forces, which can maneuver a protein complex into a different configuration, a phenomenon known as electrostatic steering (27, 28). The stability of the encounter complex is proportional to $1/k_{-1}$ (Fig. 3*A*), whereas the relative overall impact on electrostatic steering from the changes in rate constants due to the associated C_H regions is related to the product of $k_{+1}k_{+2}$ (Fig. 3*B*). The overall stability for mAbs 3E5-P1 complexes are similar (Fig. 3*A*), thus suggesting that the proportion of the complexes within the steering region is comparable for each isotype. In addition, there is an overall increase in the electrostatic steering of Fab 3E5 IgG_{2a} relative to the intact molecule (Fig. 3*B*), and thus this complex will form an encounter complex faster. In contrast, there was an overall decrease in the electrostatic steering for the Fab 3E5 IgG_3 relative to the parental mAb (Fig. 3*B*), and thus this complex will form an encounter complex more slowly than 3E5 IgG_3.

Antibody Subclass Has Different Effects on the Thermodynamic Parameters—The variation in the relative responses reflects differences in the encounter and docking rate constants for the different mAb 3E5-P1 complexes. ΔG decreases with temperature for Fab 3E5 IgG_{2a}-P1 complex while increasing for the remaining complexes (Fig. 4*A*). When the encounter and docking energies of binding, ΔG_1 and ΔG_2, respectively, are considered individually, a similar trend of ΔG_1 (Fig. 4*B*) with respect to ΔG is noticeable (Fig. 4*A*). In contrast, the binding energies of docking (ΔG_2) are relative constant throughout the temperature range examined (Fig. 4*C*), with a slight decrease at high temperatures for Fab 3E5 IgG_{2a}-P1 and Fab 3E5 IgG_3-P1 complexes. From these data, we can establish that for all of these interactions most of the free energy changes come from the encounter step (ΔG_1). Also, the differences in ΔG ($\Delta\Delta G$) for the mAbs 3E5-P1 interactions with respect to

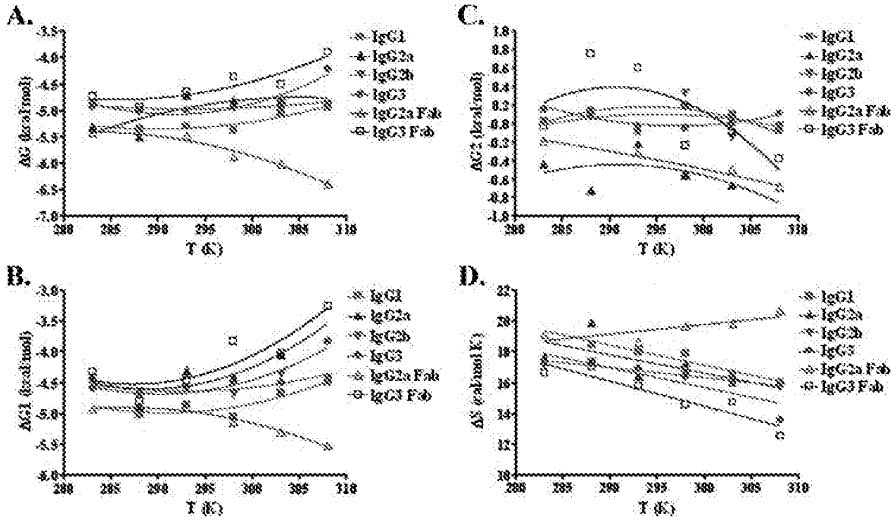


FIGURE 4. **Thermodynamics of binding of mAbs 3E5 to a peptide mimetic of GXM.** Behavior of free energy of binding (ΔG) (*A*), encounter free energy of binding (ΔG_1) (*B*), docking free energy of binding (ΔG_2) (*C*), and entropy of binding (ΔS) (*D*) as a function of temperature for the mAb 3E5 binding to peptide P1. Free energies were calculated from the formula $\Delta G = -RT\ln K_A$, $\Delta G = -RT\ln K_{a1}$, $\Delta G = -RT\ln K_{a2}$, and ΔS is from the formula $\Delta G = \Delta H - T\Delta S$.

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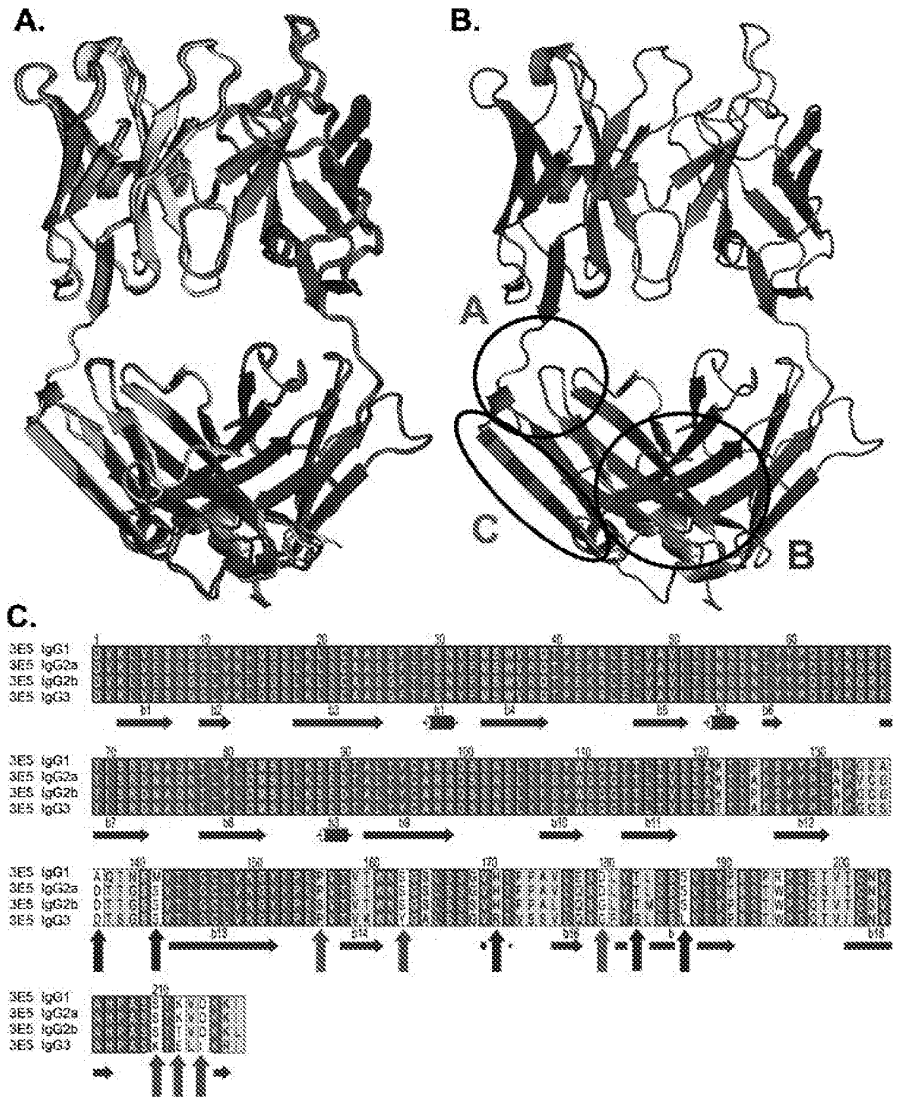


FIGURE 5. **Homology models and the corresponding sequences of 3E5 mAbs V-C_{H1} domains.** *A*, structural superimposition of mAbs 3E5 V_H-C_{H1} is shown in *color*, whereas the associated light chain is *gray*. *B*, homology-based model of 3E5 IgG switch variants shows three regions of structural differences within the C_{H1} domain (regions A, B, and C are indicated by *circles*). *C*, amino acid sequences for the V_H-C_{H1} region of the 3E5 IgG switch variants; *arrows* indicate structural differences in their amino acid sequences (*red*, *blue*, and *green arrows* for regions A, B, and C, respectively). This figure was generated using PyMOL.

the parental 3E5 IgG_3 increased with higher temperatures (supplemental data, Fig. 2S). Furthermore, if we compare the difference of energy of the encounter and docking steps of 3E5 IgG_{2a} with the parental 3E5 IgG_3, there is an overall increase in the binding energies at all temperatures for the docking step ($\Delta\Delta G_2 < -0.5$ kcal/mol) (supplemental data, Fig. 2S), indicating that the associated C_H region of an Ab can affect the energy of binding. This favoring in energy includes its Fab counterpart, Fab 3E5 IgG_{2a}, in which both $\Delta\Delta G_1$ and $\Delta\Delta G_2$ have become more favorable than the paternal 3E5 IgG_3. Free energy loss is observed for the Fab 3E5 IgG_3 ($\Delta\Delta G > 0.5$ kcal/mol) with respect to the parental 3E5 IgG_3, with most of this loss coming from the encounter step ($\Delta\Delta G1$) (supplemental data, Fig. 2S). The binding energy differences for the transition states for the mAbs 3E5-P1 complexes revealed that the γ_1, γ_{2a}, and γ_{2b} C_H regions have more favorable activation energy for the transition state than that of γ_3 C_H domains, with the exception of Fab 3E5 IgG_3 where the binding energy ($\Delta\Delta G > 0.5$ kcal/mol) and the activation energy of the transition state ($\Delta\Delta G^{\ddagger} > 0.5$ kcal/mol) increased relative to the parental mAb (supplemental data, Fig. 3S).

The changes in the entropy of association (ΔS) (Fig. 4*D*) for the mAbs 3E5 to P1 follow a pattern similar to that of ΔG (Fig. 4*A*). We observed that for all mAbs 3E5 complexes, except Fab 3E5 IgG_{2a}, ΔS decreased at higher temperatures with Fab 3E5 IgG_{2a} being the highest. For all mAbs, except Fab 3E5 IgG_{2a}, a favorable ΔS was complemented by a favorable ΔH (supplemental data, Table 1S). For the Fab 3E5 IgG_{2a} an unfavorable ΔH was compensated by a favorable ΔS (supplemental data, Table 1S).

Molecular Modeling—In the absence of experimental structures, comparative protein structure modeling can be used to generate three-dimensional models for Abs and to put in a structural context the experimental observations and changes in biophysical properties. The three-dimensional structures of the Fab domains from the different 3E5 IgG subclasses have been modeled based on the x-ray structure of the highly homologous anti-GXM mAb 2H1 (Fig. 5*A*). At such a high level of sequence identity (>90%) comparative modeling can deliver a three-dimensional model with competitive accuracy to the resolution of a medium x-ray crystallographic solution (19).

Three regions of structural differences (identified as regions A, B, and C) were observed within the C_{H1} domain of 3E5 IgG isotypes (Figs. 5*B* and 6, *A–C*). Residues in these regions may form interchain and/or solvent electrostatic or hydrophobic interactions that differed between isotypes. Of particular importance are Asp^{180} and Thr^{183}, in regions A and B, respectively, because of their location at regions that have highly connected interactions. Furthermore, these two amino acids are not only important for their ability to form hydrogen bonds but also because they are located within an area of high electrostatic interactions between amino acids that could affect the structure of the Ag binding site (Fig.

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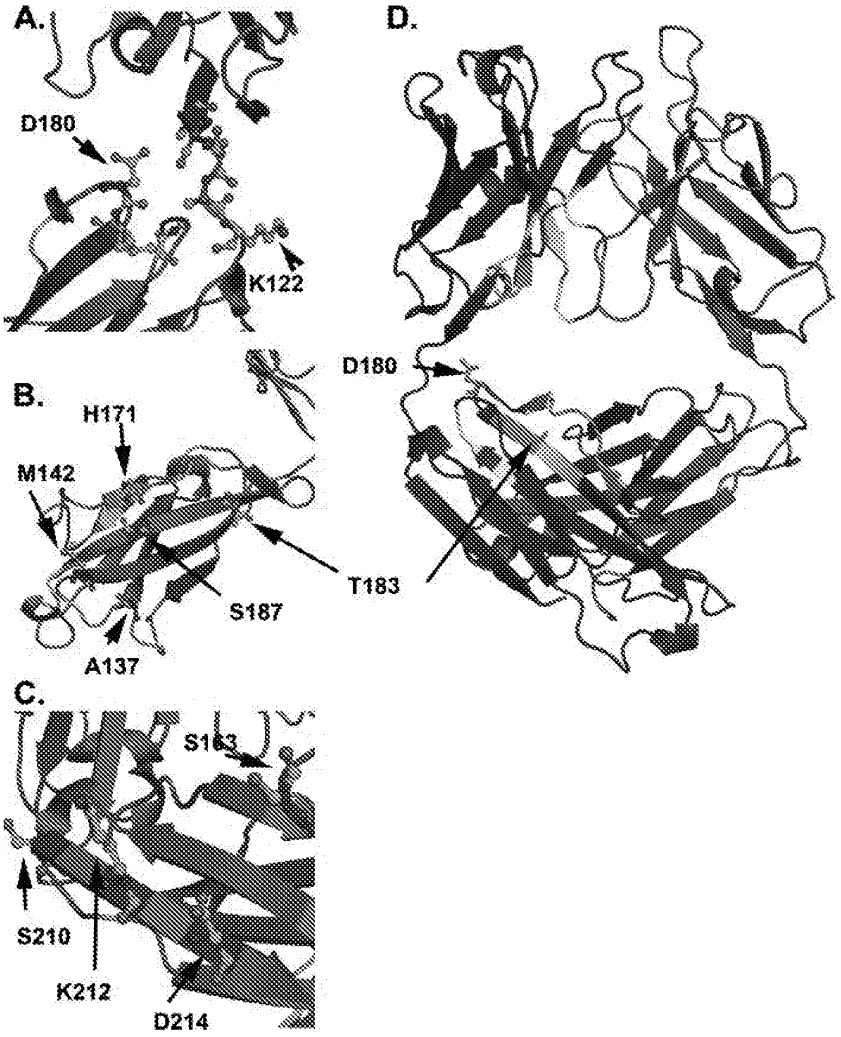


FIGURE 6. **Homology modeling analysis of mAb 3E5 C_{H1} domain.** Magnified views of regions A, B, and C are showed in *panels A, B,* and *C,* respectively. Side chains of amino acids with structural differences between C_{H1} domains of different IgG isotypes are indicated by *arrows*. *D,* amino acid connectivity analysis of mAb 3E5 IgG_1. Areas of high connectivity are indicted by *red* and *orange* (*red,* Zscore > 2.0; *orange,* 1.5 < Zscore < 2.0). Medium connectivity residues are indicated in *yellow* (1.0 < Zscores < 1.5), and low connectivity is indicated in *blue* (Zscore < 1.0). Light chain is shown in *gray*. Amino acids Asp^{180} and Thr^{183} are located in the *orange area* and are indicated by *arrows*. This figure was generated using PyMOL.

6*D*). It is notable that the SARIG calculations do not show the Ag binding site as a highly connected one. Hence, the few differences observed in the homology models of different mAbs in the C_{H1} domain, may have rather significant impact in the Ag binding site.

Ethanol and pH Affect Binding Patterns of mAb 3E5—Because molecular modeling predicted that there were no significant changes in conformation of the V region of these mAbs, we considered whether the observed residue changes in the C_H regions and the consequent perturbations in the solvent environment would translate into changes in affinity for Ag. The premise for these studies was to obtain supporting evidence for the concept that sequence differences in the C_{H1} domain of IgG isotypes could result in structural changes that propagated into the V region and altered binding properties. For that purpose we tested the effect of pH and ethanol in the binding of the mAbs 3E5 to GXM. We examined the areas of greatest variability in the various C_H regions and hypothesized that changing the solvent and/or pH would perturb local interactions and result in differences in Ag binding for the various 3E5 IgG subclasses. These residues could possibly interact with water molecules or form hydrophobic or electrostatic interactions.

We used pH to perturb the charged interactions predicted by molecular modeling in the C_{H1} domain. We observed that mAbs 3E5 were able to bind to GXM at different pHs at room temperature. However, for 3E5 IgG_1 there was a decrease in binding relative to the other isotypes as the pH increased (Fig. 7, *A* and *B*). The pH changes may have a stronger influence in the electrostatic and hydrophobic interactions in the Ab combining site and/or its surroundings, thus resulting in pH-mediated conformational changes. This finding also correlates with the higher ΔG_1 for 3E5 IgG_1 with respect to the other isotypes, suggesting that this mAb forms more electrostatic and hydrophobic interactions at the binding site that could be easily disturbed by changes in pH. It is noteworthy that there was little or no difference between the binding curves of the four IgG subclasses at the pH used for Biacore analysis.

The binding of 3E5 IgG subclasses to GXM in ethanol (Fig. 7*C*) was different from that observed under buffer conditions (7). There was an increase in the relative binding affinity, where $IgG_{2b} > IgG_{2a} > IgG_3 > IgG_1$ to GXM (Fig. 7*C*). Ethanol changes the polarity and dielectric constant of the environment and decreases protein solubility. The change toward GXM binding was expected to come from changes caused by the removal of solvent molecules from the Ag binding site and its surroundings. This could cause polar groups that were exposed to become buried inside the protein consequently changing the IgG electrostatic and hydrophobic surface properties of IgGs. Since these mAbs have identical V regions, the contribution to these effects must originate from the differences of side chains in the C_H domains. These results correlate with the binding entropies, where 3E5 IgG_1 has the most favorable ΔS (Fig. 4*D*), likely because of changes in the hydrophobic interactions.

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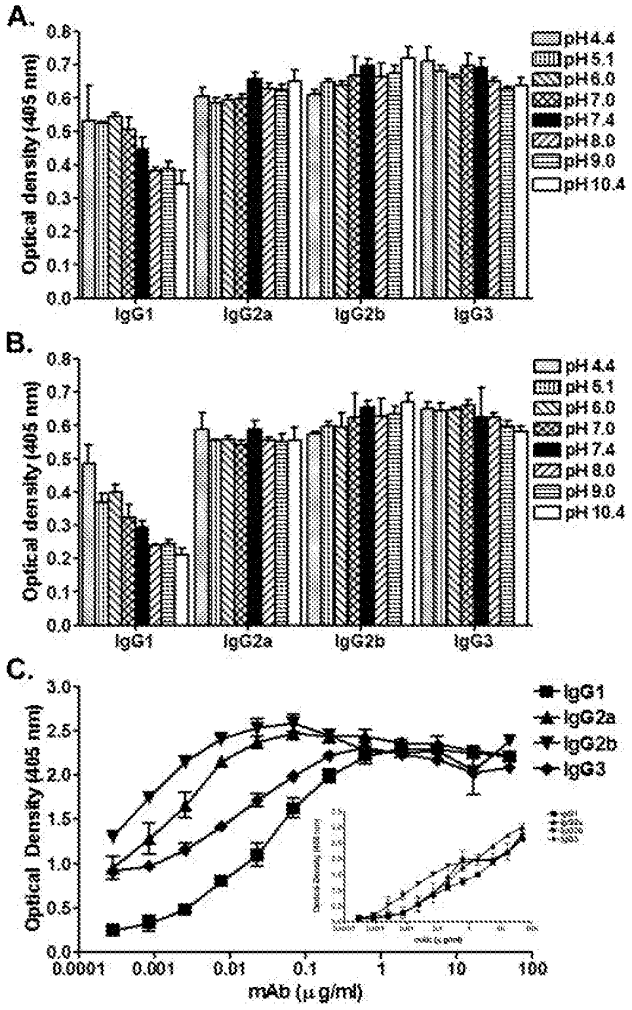


FIGURE 7. **Binding profiles of the mAbs 3E5 to GXM as a function of pH at two different concentrations by ELISA.** *Panels A* and *B* correspond to 0.6 and 1.9 μg/ml mAbs 3E5, respectively. Each *bar* represents a different pH of the binding buffer. *C*, binding profiles of the mAbs 3E5 in 5% ethanol to bound GXM on an ELISA plate. The *inset* shows the binding of 3E5 mAbs in TBS-1% BSA buffer conditions to bound GXM. Data represent the mean ± S.D. of three measurements.

mAbs 3E5 Exhibit Polyreactive Characteristics—The polyreactive properties of the 3E5 IgG switch variants were measured by a panel of antigens consisting of actin, tubulin, thyroglobulin, single-stranded DNA, and double-stranded DNA (Fig. 8). Polyreactivity was evident among these mAbs, showing differences in binding reactivity with the Ags used for the study. All mAbs show some degree of binding to thyroglobulin (Fig. 8*A*), whereas only IgG_3 shows binding reactivity for tubulin (Fig. 8*C*). In addition, the studied cross-reactants are not related to the capsular polysaccharide, GXM, thus showing that cross-reactivity does not only occur with closely related molecules.

DISCUSSION

For isotype-switched variant Abs with identical V regions to have different thermodynamic parameters in their interaction with a peptide, one must conclude that the molecular interactions between the peptide and the Ab paratope differ depending on the constant region. Different molecular interactions in turn imply different forms of complexes and thus differences in fine specificity. Consequently, mAbs with identical V regions differing in isotype can manifest variations in their fine specificity and functional affinity as a consequence of their associated C_H region.

The binding of mAbs 3E5 to a peptide mimetic of GXM are interactions of relatively low binding affinity, but the ability of the four IgG subclasses to discriminate among peptides indicates high specificity for these mAbs. Interestingly, these mAbs not only can bind to GXM-derived oligosaccharides and peptides that are mimetics of GXM with high specificity but they also bind to unrelated antigens, making this IgG set polyreactive or cross-reactive. Furthermore, the polyreactivity of a given V region is a function of its associated C_H region. Affinity was also influenced by C_H region, as Fab 3E5 IgG_3 had the lowest binding affinity within this set of mAbs. In contrast, Fab 3E5 IgG_{2a} had a higher affinity than the intact 3E5 IgG_{2a} or the parental 3E5 IgG_3. These observations suggest that structural differences in the C_{H1} domain are responsible for the differences in affinity constants and, consequently, for variations in Ag binding. The mechanism for this effect could involve transforming the structure of the binding site into a more kinetically competent form in mAbs with higher binding affinities (29). In this regard, it has been shown that segmental flexibility is controlled by the C_{H1} domain and the hinge regions of Abs (30, 31). However, binding differences due to the hinge do not explain the higher binding affinity obtained with Fab 3E5 IgG_{2a}. In the case of Fab 3E5 IgG_3, this molecule could be less flexible or have fewer interactions with the solvent, thus reducing the hydrophobic interactions and increasing the "hydrophobic effect," resulting in increased entropy.

The small structural variations predicted by the homology models of the Fab of mAbs 3E5 support our thesis that differences in fine specificity result from minor changes in the neighboring amino acid residues of the C_{H1} region. Consistent with this notion, homology modeling revealed that the Fab structures of mAbs 3E5 were very similar, and structural differences were expected to be below the level of x-ray crystallography resolution. This supports the use of a thermodynamic solution to approach differences in Ab-Ag complex formation as a function of isotype. The homology models identified three regions in the C_{H1} domain that potentially caused structural differences in the mAbs 3E5. The differences in C_{H1} domains could develop in structural isomers that could alter the association rates at which the Ab-Ag complex is formed. Thus, fine specificity differences might arise from even the minor variations in the side chains of the C_H domains, probably by structuring the Ab binding site into a more kinetically competent form. In addition, it is known that flexible side chains of polar and charged residues, such as Asp, Lys, Asn, Arg, and Ser, play an important role in binding (32). The homology models revealed that the three regions demonstrating structural differences involved at least one or more of these amino acids that could participate

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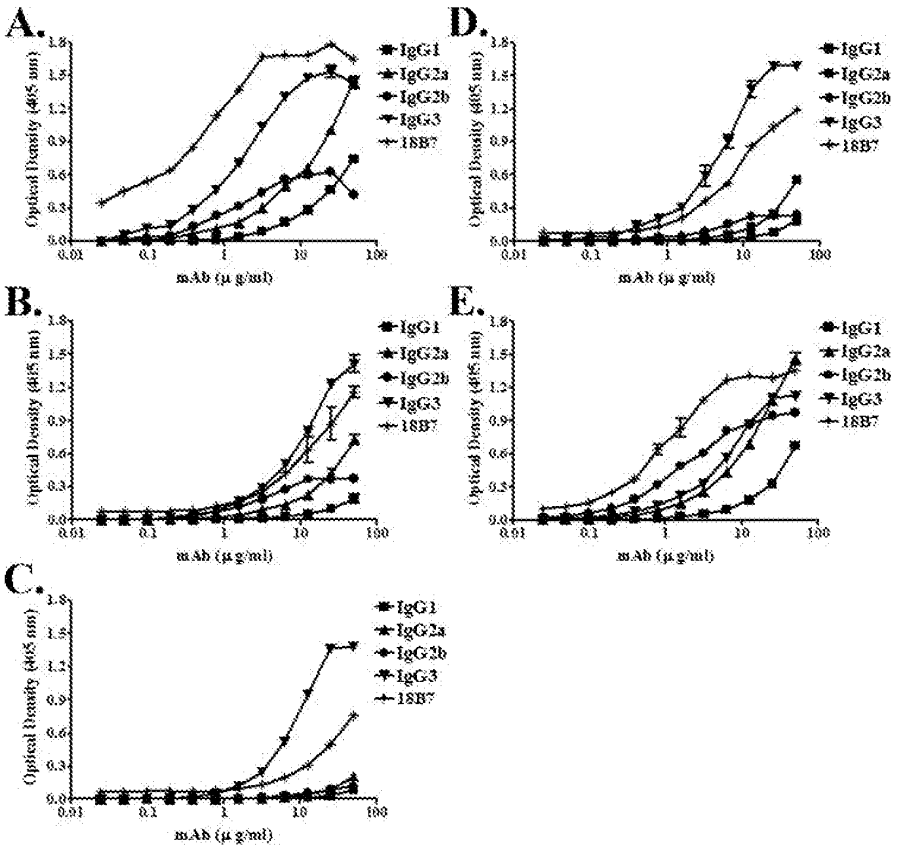


FIGURE 8. **Reactivity patterns of mAbs 3E5 against polyreactive antigens.** *Panels* corresponds to the binding profiles of 3E5 IgG switch variants to: *A*, thyroglobulin; *B*, double-stranded DNA; *C*, tubulin; *D*, actin; *E*, single-stranded DNA. Anti-GXM mAb 18B7 (IgG_1) was added as control. Data represent the mean ± S.D. of three measurements.

in the formation of electrostatic and hydrophobic interactions. Among the three regions, Asp^{180} and Thr^{183} in regions A and B, respectively, present in the C_{H1} domain of IgG_1 and IgG_{2a} isotypes, are of particular interest because of their ability to form hydrogen bonds. Furthermore, analysis of residues connectivity revealed that these amino acids are within a highly connected region. Highly connected areas were related to functionally important regions of proteins where a network of long range interactions between amino acids modulate direct or allosteric communications between proteins (22, 33, 34). Thus, the alterations in fine specificity and affinity observed for these mAbs could be a result of long range electrostatic interactions resulting from differences in the microenvironment of the C_H domains that affect the Ab combining site. Many studies support the idea that a limited number of V region differences (35–37) and non-contact residues can significantly change Ab conformation and function (36, 38). In addition, there is evidence that amino acid changes in the V_H region may induce different functional roles in the context of an altered H-chain microenvironment (36, 39) even if these residues are not within the binding site or do not directly necessary contribute to the total Ag binding (36, 39). Because these Abs share the same V region, changes in paratope conformation may well be attributed to changes in the microenvironment of the binding site due to the associated C_H.

Environmental variations such as in pH and ionic strength can lead to the redistribution of conformational states. Desolvation also plays a key role in determining the effect of electrostatics by counteracting the favorable electrostatics interactions formed between the Ag and Ab (40). The reactivity of mAbs 3E5 was not affected by small amounts of ethanol as a solvent. However, the affinity of binding in the alcoholic solutions increased relative to that observed in buffer without ethanol. This suggests that in the presence of ethanol more favorable hydrophobic interactions are formed between the Ab-Ag binding site and the solvent. Furthermore, by decreasing the pH, we observed a significant reduction in the binding of mAb 3E5 IgG_1 to GXM relative to the other isotypes. For this isotype, pH may have a greater influence on the alternation between a compact and a more open conformation, resulting in a "conformational switch" that affects Ag binding specificity. This also suggests that there are more interactions of polar and charged amino acids with the solvent occurring for 3E5 IgG_1 than for the other isotypes. Differences in the pH dependence of binding for the different isotypes raises the intriguing possibility of isotype-related differences in binding affinity and specificity for Ab-Ag complexes in acidic compartments such as phagosomes.

The ability of the immune system to produce Abs with different specificities through class switch recombination may be advantageous to the host in providing an additional source of diversity in the Ab response. Thus, the immune response could be enhanced by expressing a different isotype. This notion is supported by immunization studies with oxazolin, where upon antigen binding isotype-specific patterns arise, a feature observed only in autoimmune diseases (41). Amplification of the Ab immune response through isotype switching could conceivably allow for the formation of certain V region combinations that are self-reactive, which are not eliminated through the negative selection processes because of their low affinities. Thus, isotype switching could be a way to generate antibodies with wider specificity that still conserve their ability to bind to the original Ag and provide a way to manage the continuous genetic variation of pathogens, as well as facilitating the rapid recruitment of protective Abs against pathogens such as *Cryptococcus neoformans*, *Streptococcus pneumoniae*, and influenza virus.

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The plasticity of the paratope site of an anti-carbohydrate Ab could allow for a convergence of specificities of Abs recognizing a common subset of antigenic determinants (9). Thus, rather than having to generate a new Ab with a different specificity, through a change of isotype combined with plasticity of the binding site, a "fit" to the Ag can be induced by isotype switching. This can be of particular importance for multivalent Ags, such as GXM, in which Abs with the same V region and different C_H region may recognize slightly different epitopes according to microenvironment variations induced by the associated C_H chain. This in turn would increase the heterogeneity of the Ab combining site and may be important for the development of Ab cross-reactivity and for Id networks; consequently, it could play a major role in immunoregulation of the humoral response. Reitan and Hannestad (42, 43) demonstrated that the anti-Id response can also be induced by monomeric IgM, IgA, and IgE molecules and even $F(ab')_2$ fragments, although the Fc regions of IgGs seem to have a dominant effect and induce long lasting unresponsiveness to the associated V regions. As idiotypic interactions dispense of antigens, they might operate in the selection of preimmune repertoires and therefore in the establishment of natural tolerance.

For the modeled structures, we assumed that the peptide-bound structures for the Fab of these mAbs were very similar, with only slight alterations in a few side chains. This supported the notion of Ab multispecificity due to the formation/change of hydrogen bonds or/and salt bridges rather than by hydrophobic interactions. Polyspecificity is also a characteristic observed in auto-Abs (44). Polyreactive antigen binding B cells, which can bind to and process endogenous host antigens from peripheral tissues and are found in the thymus, may contribute to immunological tolerance. If this turns out to be the case, then the B cell repertoire would have a dual function: high affinity Abs to protect against foreign invaders and low affinity polyreactive Ag-binding B cells to protect against autoimmune diseases by contributing to immunological tolerance. The recruitment of B cells expressing low affinity, polyreactive Abs may also reinforce self-tolerance in a constantly renewing of B lymphocytes (45).

Our study suggests another way of generating immune diversity. As such, the immunological memory could be affected to determine the course of the secondary response by the generation of Abs with identical V regions but different isotype, giving origin to higher or lower memory response depending on isotype. Given that isotype could affect specificity, Ag binding to the B cell receptor could directly trigger clonal expansion of isotype-restricted Ag-specific B cells. Consequently, the well known isotype restriction of anti-polysaccharide and anti-viral responses may reflect isotype-related effects on Ab specificity for an Ag.

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Variable-Region-Identical Antibodies Differing in Isotype Demonstrate Differences in Fine Specificity and Idiotype[1]

Marcela Torres,[2,3]* Rena May,[2†] Matthew D. Scharff,*† and Arturo Casadevall[3]*‡

A central tenet of the current understanding of the relationship between Ab structure and function is that the variable region domain is solely responsible for Ag specificity. However, this view was recently challenged by the observation that families of mouse-human chimeric Abs with identical V regions demonstrate differences in fine specificity and by reports of changes in Ab Id structure with isotype switching. Here we revisited this question by evaluating the reactivity of two families of murine IgG switch variants that differed in V region usage for *Cryptococcus neoformans* glucuronoxylomannan, glucuronoxylomannan peptide mimetics, and anti-Id mAbs. The results reveal isotype-related differences in fine specificities and Id for two mAb isotype switched families, thus establishing the validity of this observation with sets of homologous Abs. The results suggest that the C region affects V region protein conformation, leading to differences in fine specificity and Id. The finding that isotype can affect fine specificity has major implications for current concepts of the generation of secondary responses, idiotypic network regulation, and isotype function. Given that isotype class switching and Ig gene somatic hypermutation share molecular mechanisms, these observations unify these processes in the sense that both can alter specificity and affinity. *The Journal of Immunology,* **2005, 174: 2132–2142.**

The current understanding in immunology is that the interactions between V region amino acids and the cognate Ag define Ab specificity. This concept originates from the observation in the 1960s that Ab diversity arises from VDJ recombination, somatic mutations in the CDR regions of the V gene, and class switch recombination (1, 2). A number of studies over the past three decades, including x-ray crystal diffraction studies (3), have validated this principle. However, several recent observations strongly suggest that for certain Ag-Ab combinations, the C region can affect the specificity of the interactions between the Ab-binding site and Ag (4, 5). Cooper and colleagues (5) identified a family of mouse hybridoma switch variants that bound the GlcNac residues of the cell wall polysaccharide of group A streptococci. These Abs had identical V region sequences, yet the magnitude of binding to GlcNAc differed for each isotype (5). Although some aspects of this phenomenon probably reflected changes in avidity, an explanation based on changes in specificity was not formally excluded. Another observation suggesting that the C region could affect some aspects of the V region structure was the report that immunogenic Id-Abs acquired tolerogenicity by switching to IgG1, IgG2a, IgG2b, IgG3, and pIgA (6). These Abs regained their immunogenicity when the Fc portion of IgG1 was removed and switched to IgE or monomeric IgA. That finding was interpreted as suggesting that V regions expressed with IgG or pIgA negatively control the immune response to shared Ids (7). Additional support for the concept that C region can affect V region structure came from studies with the cell line SAMM 368, which produces IgG2b and IgA Abs with specificity for the same Ag, without sharing idiotypic determinants (8). Furthermore, certain mouse idiotypic determinants have been associated with the serum IgA fraction, suggesting that some Ids are isotype restricted (9). Although Id isotype restriction could represent the preferential associations of certain V regions with certain C regions, it could also imply that certain isotypes affect V region conformation (9).

We have recently reported a family of mouse-human chimeric Abs that bind to the *Cryptococcus neoformans* glucuronoxylomannan (GXM)[4] polysaccharide. Despite having identical V regions, the IgM and IgG3 chimeras had altered fine specificity compared with the IgG1, IgG2, IgG4, and IgA chimeras as measured by several serological assays (10). Idiotypic studies using mAbs revealed differences in reactivity with chimeric Abs expressing the same V region with different C regions. These results were interpreted as indicative of fine specificity changes originating from differences in isotype, possibly as a result of differences in the folding and exposure of antigenic epitopes of the V region (10). Because chimeric Ab synthesis involves joining of V and C region sequences from two species we could not exclude the possibility that this unusual finding was a result of creating an artificial molecule. Given the finding that the C region can affect V region fine specificity and Id might have major implications for several current immunological concepts, and that none of the observations suggesting this view are conclusive, we have investigated this problem further by comparing the fine specificity of two families of murine IgG switch variants, 3E5 and 4H3, that bind to *C. neoformans* GXM polysaccharide.

mAbs 3E5 and 4H3 both bind GXM, but each differ in specificity and V region usage (11). mAb 3E5 IgG3 (11, 12), which was generated from a B cell isolated from a mouse immunized with GXM-tetanus toxoid, uses V_H7183 and V_K 5.1 gene elements and

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[4] Abbreviations used in this paper: GXM, glucuronoxylomannan; IF, immunofluorescence; KLH, keyhole limpet hemocyanin; KLH-g, gluteraldehyde-treated KLH; KLH-p, periodate-treated KLH.

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binds to an epitope found in all serotypes of GXM (13). The murine Ab response to GXM is highly restricted because the majority of mAbs to GXM that were isolated either from infected mice or from mice immunized with GXM-tetanus toxoid (11) are encoded by V_H7183. Administration of mAbs 3E5 IgG1, IgG2a, and IgG2b diminished the fungal burden and prolonged the life of lethally infected mice, whereas 3E5 IgG3 was not protective (14). The difference between 3E5 IgG3 and the other 3E5 mAbs was believed to have been FcR mediated (15), because their V regions were sequenced and shown to be identical (11) and the efficacy of the different isotypes was modified in mice genetically defective for some FcR (16). mAb 4H3 IgG3, which was generated from a B cell isolated from a mouse infected with *C. neoformans*, uses VH441 and Vλ1 gene elements (11) and binds a different epitope of GXM than that recognized by mAb 3E5. None of the 4H3 isotype variants are protective in mice (15), and some even enhance infection (16).

Our results demonstrate difference in binding and Id recognition for V-region-identical IgGs that are consistent with the notion that isotype contributes to Ab fine specificity for certain Ags.

Materials and Methods

Murine mAbs

GXM-binding mAbs 3E5, 4H3, and 12A1 have been previously described (17, 18). The IgG1, IgG2a, and IgG2b switch variants of mAbs 3E5 were generated in vitro by sib selection (19). The IgG1 and IgG2b switch variants of mAb 4H3 were isolated in vitro by sib selection (12). The V regions of the 3E5 and 4H3 family have been sequenced and shown to be identical (11, 12). mAbs 3E5 and 12A1 are encoded by V_H 7183 and V_K 5.1(17), and based on the use of the same variable gene elements and shared Id (20), these Abs have been classified as members of class II mAbs to GXM. mAb 4H3 is encoded by V_H 441 and Vλ2 (11). mAb 6A10 is an anti-idiotypic Ab that binds the combining site of class II mAbs, as has been previously described (21). As a negative control mouse mAb MOPC 21 (mIgG1) (Sigma-Aldrich) was used. All IgG1 mAbs were purified from hybridoma supernatants using protein G columns (Pierce) following the manufacturer's instructions. All non-IgG1 mAbs were purified using protein A (Pierce) following the manufacturer's instructions. Next, the purified mAbs were dialyzed against PBS and concentration was determined by ELISA using isotype matched standards. The 3E5 family of switch variant immunoglobulins was analyzed by MALDI-TOF mass spectrometry at the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine (Bronx, NY), to evaluate for the presence of aggregates. The MALDI-TOF mass spectrometry analysis revealed no evidence of IgG aggregation.

PCR amplification of Ig H and L chains

RNA was isolated from hybridoma cells using TRIzol reagent (Invitrogen Life Technologies). cDNA was generated using SensiScript RT kit from Qiagen. For mAbs 3E5 IgG1 and IgG2a, DNA containing the V_H was amplified using specific sense and antisense primers as follows: 5′-GGGGCCAGTGGATACAC and 5′-GAAGTGAAGCTGGTGGAGTC. The cDNA template was then amplified using the PCR Core Kit (Qiagen) with 3.0 mM Mg^{2+} and 0.5 μM of each primer under the following conditions: initial denaturation at 94°C for 3 min, 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a final 10-min extension at 72°C. For mAb 3E5 IgG2b the following primers were used: 5′-V_HUni, 5′-TGAGGTGCAGCTGGAGGAGTC and 3′-msCγ, 5′-AGAC CGATGGGGCTGTTGTTTTGGC (22). The cDNA template was then amplified using the PCR Core Kit (Qiagen) with 0.5 μM of each primer under the following conditions: initial denaturation at 94°C for 3 min, 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 40 cycles, followed by a final 10-min extension at 72°C. DNA containing the V_L of 3E5 IgG1, IgG2a, and IgG3 mAbs was amplified using the sense and antisense primers V_k5.1, 5′-GATGTTGTGATGACCCAA and V_K, 5′-TGGATGGTGG GAAGATG (12). The cDNA template was then amplified using the PCR Core Kit (Qiagen) with 3.0 mM Mg^{2+} and 0.5 μM of each primer under the following conditions: initial denaturation at 94°C for 3 min, 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a final 10-min extension at 72°C. For 4H3 IgG1, IgG2b, and IgG3 mAbs, DNA containing the V_H was amplified using the sense and antisense primers as follows: 4H3V_H, 5′-AAGTAGCCTTTGACAAGGCA and 4H3V_H3′, 5′-GAGGTGAAGCTTCTCGAGTCT (11). The cDNA template was then amplified using the PCR Core Kit (Qiagen) with 3.5 mM Mg^{2+} and 0.5 μM of each primer under the following conditions: initial denaturation at 94°C for 3 min, 94°C for 30 s, 46°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a final 10-min extension at 72°C. The cDNA encoding the V_L domains of 4H3 IgG1, IgG2b, and IgG3 hybridomas Ig was generated by PCR with specific sense and antisense primers as follows: 5′-CCCAGCCCATAC TAAGAGTTAT and 5′-CTCAATGATTCTATGTTGTGCC. The cDNA template was then amplified using the PCR Core Kit (Qiagen) with 3.5 mM Mg^{2+} and 0.5 μM of each primer under the following conditions: initial denaturation at 94°C for 3 min, 94°C for 30 s, 46°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a final 10-min extension at 72°C. Purified PCR products by QIAQuick Gel Extraction kit (Qiagen) were then sequenced bidirectionally by automated gel sequencing (DNA Sequencing Facility, Albert Einstein College of Medicine).

Antigens

GXM was purified from *Cryptococcus neoformans* culture by cetyltrimethylammonium bromide precipitation, as described elsewhere (23). The serotype D *C. neoformans* strain 24067 from the American Type Culture Collection was used for all experiments. The GXM solution was tested for detectable protein contaminants, after which it was lyophilized and resuspended in sterile saline to a 5 mg/ml solution. *O*-Acetyl groups were removed from GXM prepared from *C. neoformans* serotype D, American Type Culture Collection strain 24067 by treatment with NH_4OH (pH 11.25–11.50) for 24 h at room temperature. De-*O*-acetylated GXM was recovered by lyophilization and resuspended in sterile saline (24). Keyhole limpet hemocyanin (KLH) (3 mg; Pierce) was dissolved in PBS, and then treated with gluteraldehyde (KLH-g), as described (25). The reaction was incubated overnight at room temperature on an end-over-end rocker and then dialyzed (Slide-A-Lyzer MWCO 10,000; Pierce) at 4°C against sterile PBS for 3 days with daily changes. Periodate-treated KLH (KLH-p) was generated by treating KLH with 0.2 M sodium periodate (Sigma-Aldrich) in 0.01 M sodium acetate buffer (pH 4.5) overnight at 4°C (26). The reaction was stopped by the addition of an equal volume of 0.05 M sodium borohydride (Sigma-Aldrich) for 30 min at room temperature. KLH-p was then dialyzed against sterile PBS for 48 h with daily changes as described (27).

Binding ELISAs

Ab binding to GXM, de-*O*-acetylated GXM, KLH-g, and KLH-p was measured by ELISA, as described (15). Briefly, the ELISA plates were coated with 50 μl of a solution of 10 μg/ml Ag. The plates were incubated for 1.5 h at 37°C, and then overnight at 4°C with 1% BSA/TBS. Serial dilutions of Ab were incubated on the plates and detected with phosphatase-conjugated goat anti-mouse L chain (κ or λ) (Southern Biotechnology). The plates were then developed by adding substrate and determining OD at 405 nm.

Ab competition and anti-Id ELISAs

Competition ELISAs were done as described previously (12). Briefly, polystyrene plates were coated with 50 μl of a solution of 10 μg/ml GXM in PBS followed by blocking with 1% BSA in TBS. Then, 3E5 mAbs were added and serially diluted followed immediately by the addition of mAb 12A1 (IgM) (final concentration, 2 μg/ml). Plates were incubated overnight at 4°C, and binding of mAbs was determined with alkaline-phosphatase conjugated goat anti-mouse IgM. The plates were then developed by adding substrate and determining OD at 405 nm. Reactivity of 3E5 mAbs with the murine anti-idiotypic mAb 6A10 (IgG1) was also measured by ELISA. The anti-idiotypic mAb 6A10 binds to the Ag-combining site of class II mAbs (21). Plates were coated with mAbs 3E5 serially diluted in PBS, and then blocked with 1% BSA in TBS. The plates were then incubated with biotinylated 6A10 mAb, and developed with Vectastain ABC kit (Vector Laboratories). mAb 6A10 was biotinylated using the EZ-Link NHS-PEO solid phase biotinylation kit from Pierce, following the manufacturer's instructions. As a control for the amount of 3E5 IgG subclass bound to the plate, the same ELISA was done using an alkaline phosphatase-conjugated anti-mouse κ Ab (Southern Biotechnology) instead of the biotinylated 6A10 mAb. This ELISA revealed differences in the binding of the various isotypes to the polystyrene matrix. To normalize the data for differences in binding, we measured the relative amount of each 3E5 IgG subclass mAbs bound to the plate using anti-mouse κ Ab. The IgG subclass with the lowest concentration in the plate was assigned a value of one, and the concentration of the other IgG subclasses was normalized relative to this number.

Peptide ELISA

Polystyrene plates were coated with a 1 μg/ml solution of streptavidin (Sigma-Aldrich) in PBS followed by blocking with 1% BSA in TBS. Biotinylated peptide was then added at 2 μg/ml. The peptides PA1

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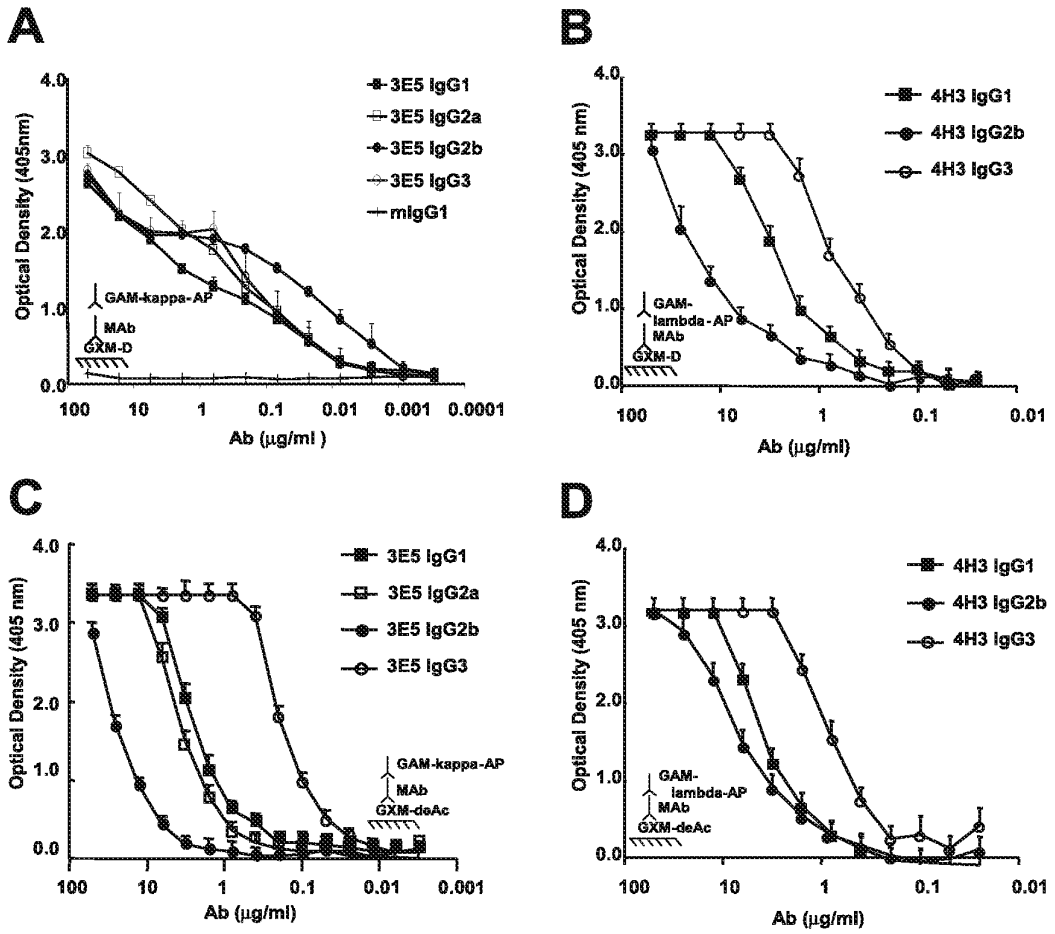


FIGURE 1. Binding of 3E5 and 4H3 families of isotype switch variants to GXM by ELISA. Reactivity of 3E5 switch variants to GXM-D (*A*) and to de-*O*-acetylated GXM-D (*C*) immobilized on polystyrene plates. Binding of the 4H3 family of switch variants to GXM-D (*B*) and to de-*O*-acetylated GXM-D (*D*) immobilized on polystyrene plates. Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

(LQYTPSWMLV), P601E (SGSGDSYSWMYEA), and P206.1 (FGGET FTPDWMMEVAIDNE) are mimetics of GXM (28–30). The peptides were biotinylated and synthesized by the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine. mAb 3E5 was added in serial dilutions and specific binding was detected using phosphatase conjugated goat anti-mouse κ Ab (Southern Biotechnology). The plates were then developed by adding substrate and determining OD at 405 nm.

Indirect immunofluorescence

A suspension of 10^6 *C. neoformans* 24067 cells per millilter was incubated for 30 min at 37°C with 2% BSA (ICN Biomedicals) and 0.5% goat serum (Sigma-Aldrich). mAbs 3E5 or 4H3 or control mAbs were then added at 20 µg/ml in 2% BSA and 0.5% goat serum, and incubated for 1 h at 37°C. Binding of primary mAb was detected by adding FITC-labeled goat anti-mouse IgG (H + L; Southern Biotechnology) at 10 µg/ml. To distinguish the cell wall of *C. neoformans* from its capsule, 25 µg/ml Fluostain I (Sigma-Aldrich) was added with the secondary Ab. Samples were then incubated at 37°C for 30 min. Prior studies have established that the immunofluoresence (IF) pattern is the same regardless of whether one uses FITC-labeled primary or secondary Ab (31). The cells were collected by centrifugation and washed with PBS between the applications of reagent. Then, 30 µl of mounting medium (0.1 M *n*-propyl gallate–50% glycerol in PBS; Sigma-Aldrich) was added. Finally, 5–10 µl of this suspension was added to Poly-Prep slides (Sigma-Aldrich) and coverslips were placed. The slides were viewed with an Olympus AX70 microscope equipped with FITC and DAPI filters. Images were recorded by a Retiga 1300 digital CCD camera using the QCapture 2 software for real time image preview and capture (QIMAGING).

Biopanning to select peptides that are mimetics for 3E5 mAbs

A Ph.D.-12 Phage Display Peptide Library was obtained from New England Biolabs with a concentration of 1.5×10^{13} PFU/ml and a complexity of 2.7×10^9 transformants. The biopanning protocol followed the manufacturer's instruction and involved incubating the library on a 6-well plate (BD Biosciences) coated with the affinity-purified target mAb (25 µg/ml in carbonate buffer, pH 8.7), washing away the unbound phage and eluting the bound phage. The eluted phage was then amplified and taken through additional cycles of biopaning and amplification to enrich for the phages with the highest affinity. After three rounds of screening, three individual clones were selected, amplified, and characterized by a capture ELISA with the target anti-GXM mAb 3E5 IgG3, as coating Ab. The library was absorbed against plastic-binding phages by incubating the amplified phages onto a 6-well plate and recovering the unbound phages. The individual clones were selected by luminescence screening assay using the Super Signal West Pico Chemiluminescent Substrate from Pierce.

Selection for phage-expressing peptides that bind to 3E5 mAbs

A capture ELISA was used to confirm positive phage clones as described by the instructions of the peptide library kit with minor modifications. Briefly, the supernatant of the amplified culture of each clone was used as a testing sample. Polystyrene plates were coated with $\sim 1 \times 10^{11}$ PFU/ml phage (UV inactivated) followed by blocking with 1% BSA in TBS. mAbs were added in serial dilutions starting at 50 µg/ml in 1% BSA in TBS. Plates were incubated for 1 h at 37°C and specific binding was detected using phosphatase conjugated goat anti-mouse κ Ab (Southern Biotechnology). Plates were washed with TBS-0.5% Tween 20 between steps. The binding

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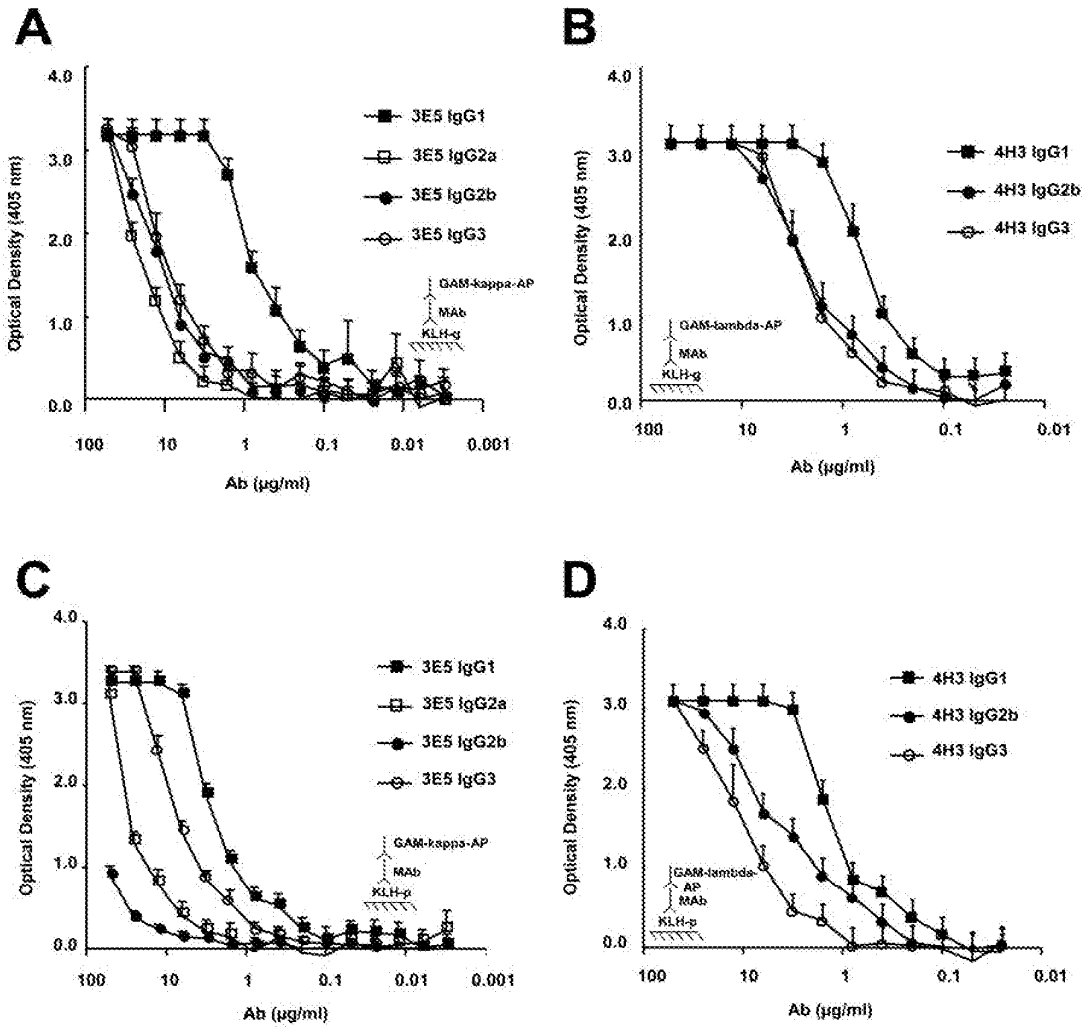


FIGURE 2. Binding of 3E5 and 4H3 families of isotype switch variants to KLH by ELISA. Binding of 3E5 isotype switch variants to KLH-g (*A*) and KLH-p (*C*) immobilized on polystyrene plates. Binding of 4H3 isotype switch variants binding to KLH-g (*B*) and KLH-p (*D*) immobilized on polystyrene plates. Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

reactivity between 3E5 mAbs and the selected phage clones was evaluated by OD at 405 nm after addition of substrate. DNA from selected clones was isolated using the QIAprep spin M13 kit (Qiagen) and then sequenced (DNA Sequencing Facility, Albert Einstein College of Medicine). Based on the sequence of the inserts of two phage, peptides were synthesized and biotinylated by the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine. These biotinylated peptides were tested for their reactivity with the 3E5 switch variants by a peptide ELISA as described above.

GXM competition ELISA with 3E5-derived peptides

Polystyrene plates were coated with a 1 μg/ml solution of GXM in PBS followed by blocking with 1% BSA in TBS. Peptides were added in serial dilutions starting at a concentration of 20 μg/ml. An irrelevant peptide was used as a negative control (data not shown). 3E5 mAbs were added at a constant concentration of 10 μg/ml. Plates were incubated for 1 h at 37°C and specific binding was detected using phosphatase conjugated goat anti-mouse κ Ab (Southern Biotechnology). The plates were then developed by adding substrate and determining OD at 405 nm.

Results

V region sequences

The 3E5 IgG1 and IgG3 V_H and V_L nucleotide sequences have been previously reported based on direct mRNA sequencing (12). The 3E5 V_H and V_L sequences reported here are identical to the ones reported previously. These 3E5 family V region sequences are deposited in GenBank under accession numbers AY674872, AY674873, AY674874, AY674875, AY674876, AY674877, and AY679102. The 4H3 family V region sequences are deposited in GenBank under accession numbers AY679096, AY679097, AY679098, AY679099, AY679100, and AY679101. These 4H3 V_H and V_L region sequences reported are also identical.

Reactivity of mAbs 3E5 and 4H3 switch variants for GXM

GXM binding of the different isotype switch variants from the mAb 3E5 and 4H3 families revealed different binding curves for some of the isotypes (Fig. 1). Because all of the switch variants have identical V_H and V_L regions, the binding curves were expected to be similar, if not identical. Although there were differences in mAb 3E5 IgG subclass binding to GXM (Fig. 1A), these were relatively small and could potentially be explained by differences in avidity as has been suggested previously (32). However, significantly larger differences in GXM binding were detected with the 4H3 switch variants, with the relative order of reactivity being

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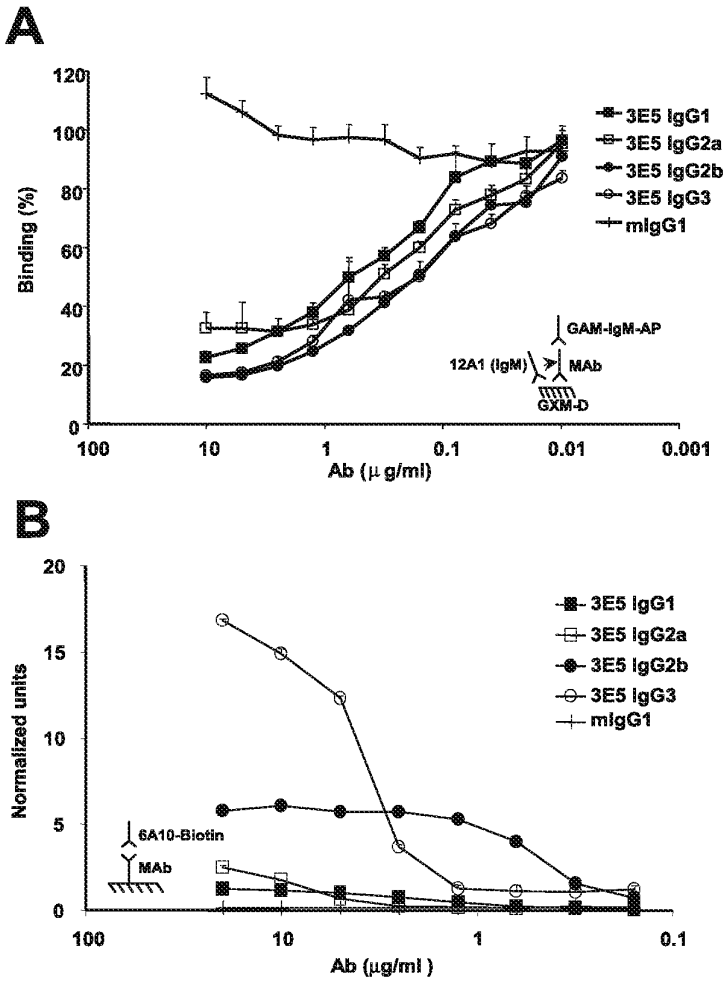


FIGURE 3. *A*, Competition binding assay between 3E5 mAbs to bound-GXM in competition with mAb12A1 (IgM). Reactivity of 3E5 mAbs with the biotin-labeled anti-idiotypic mAb 6A10 (*B*) by ELISA, after normalization of concentration for isotype-related differences in binding to the polystyrene microtiter plate. Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

IgG3 > IgG1 > IgG2b (Fig. 1*B*). This finding suggested that the C region can affect the fine specificity of distinct V regions, albeit in different ways. However, we are unable to rule out the possibility that these results are due to avidity differences of the Abs. Hence, we investigated reactivity with chemically de-*O*-acetylated GXM. Most protective Abs lose reactivity with GXM when the polysaccharide is chemically de-*O*-acetylated (33). Consistent with this observation the nonprotective 3E5 IgG3 bound to the modified GXM roughly 10 times more strongly than the other 3E5 Abs, and their relative strength of binding was IgG3 ≫ IgG1, IgG2a > IgG2b (Fig. 1*C*). All of the 4H3 switch variants bound to de-*O*-acetylated GXM, with the relative affinities being IgG3 > IgG1 > IgG2b (Fig. 1*D*), consistent with the finding that 4H3 mAbs do not prolong the life of infected animals (15). The ability of 3E5 IgG3 to bind the de-*O*-acetylated GXM with a higher affinity was surprising given that de-*O*-acetylation of GXM usually results in loss of binding of mAbs with identical V regions (13). This finding suggests that the C region may not only affect the relative fine specificities of V-region-identical isotype switch families, but may also allow IgG Abs of different subclasses but encoded by the same V region, to recognize different epitopes. Although it seemed less likely, an explanation for these results based on differences in avidity cannot be formally excluded.

Reactivity of mAbs 3E5 and 4H3 switch variants for KLH

To investigate whether the phenomenon of isotype-related changes in apparent specificity extended to non-GXM epitopes we assayed the binding of our 3E5 and 4H3 isotype switch families on additional unrelated Ags. The KLH is a highly glycosylated protein that is widely used as an immunologic carrier (34–37). Haptenic macromolecules have been conjugated to KLH using gluteraldehyde (25). KLH-g contains an epitope that has antigenic mimicry to a number of carbohydrate Ags including GXM (27). As observed with GXM, V region-identical Abs with different IgG C regions demonstrated differences in the relative strength of binding to KLH-g, which followed the order IgG1 ≫ IgG2b, IgG3 > IgG2a for the 3E5 set (Fig. 2*A*) and IgG1 > IgG2b, IgG3 for the 4H3 set (Fig. 2*B*). KLH-p destroys terminal glycol residues. Because Abs to polysaccharides often bind terminal sugar structures (38), periodate can selectively destroy the antigenicity of the surface carbohydrate molecules without affecting protein determinants or some of the internal carbohydrates (39). Although the mAbs in the 3E5 and 4H3 sets had lower affinity for KLH-p relative to KLH, significant variability between the different isotypes of each set was still apparent, with the relative strength in binding being IgG1 > IgG3 > IgG2a > IgG2b for 3E5 (Fig. 2*C*) and IgG1 > IgG2b > IgG3 for 4H3 (Fig. 2*D*). When comparing the

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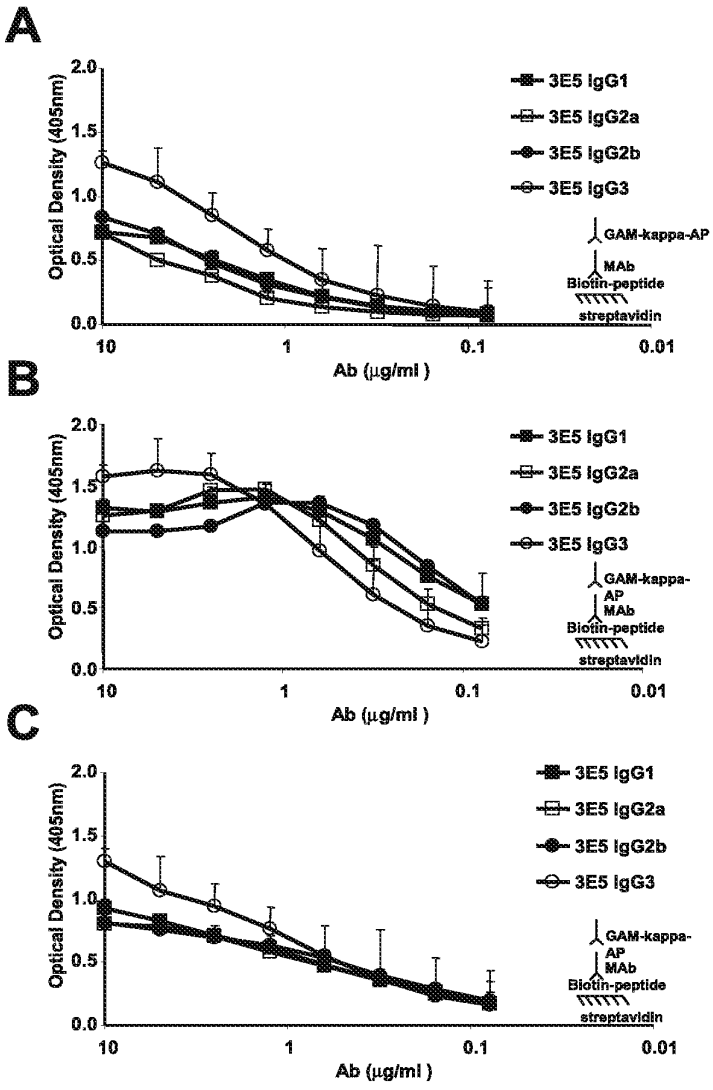


FIGURE 4. Reactivity of 3E5 mAbs with peptide mimetics by ELISA. Biotinylated peptides P601E (*A*), P206.1 (*B*), and PA1 (*C*) were immobilized onto streptavidin-coated plates. Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

binding of these Abs to KLH-g, KLH-p, and GXM, a clear shift in the highest binding isotype is apparent. Hence, for any given family of switch variants, the relative affinities of the isotype switch variants can shift, depending on the Ag.

Competition experiments

Ab competition experiments were done to further explore potential specificity differences between the various 3E5 and 4H3 isotypes. Abs that compete for binding may either recognize the same epitope, or bind to closely spaced epitopes and compete as a result of steric interference. However, when there is no competition between Abs one can conclude that they bind to different epitopes. We analyzed the ability of the 3E5 isotypes to compete with additional mAbs to GXM. mAb 12A1 (IgM) is also a member of the group II GXM-specific Abs, and is encoded by the same V region genes as the 3E5 switch variants, unlike 4H3. Although neither 4H3 IgG1 nor 4H3 IgG3 could compete with 3E5 for binding, as one would expect from Abs encoded by different V regions (data not shown), each of the 3E5 isotype switch variants competed with mAb12A1 (Fig. 3*A*), but demonstrated slight variations in their relative ability to inhibit the binding in the order IgG2b > IgG3 > IgG2a > IgG1.

Reactivity with anti-idiotypic mAbs

To examine whether the IgG subclass affects the 3E5 Id, we studied the reactivity of the 3E5 variants for an anti-idiotypic mAb known as 6A10, which was generated against the 2H1 mAb, a group II GXM-specific Ab, and interacts with both V_H and V_L of the Ag-combining site (21). mAb 6A10 binds the 3E5 switch variants with a relative efficacy of IgG3 ≫ IgG2b > IgG2a, IgG1 after the data obtained with mAb 6A10 was normalized for the amount of 3E5 mAb bound to the plate (Fig. 3*B*). These results point out that different C regions can alter Id reactivity of Abs with identical V regions.

Reactivity with peptide mimetics of GXM

Peptide mimetics of GXM bind to the Ag-combining site and can be used to probe for paratope changes that would be consistent with specificity changes. The peptides used were generated against mAb 2H1 (IgG1) and react with the binding site of mAb 3E5 (28)

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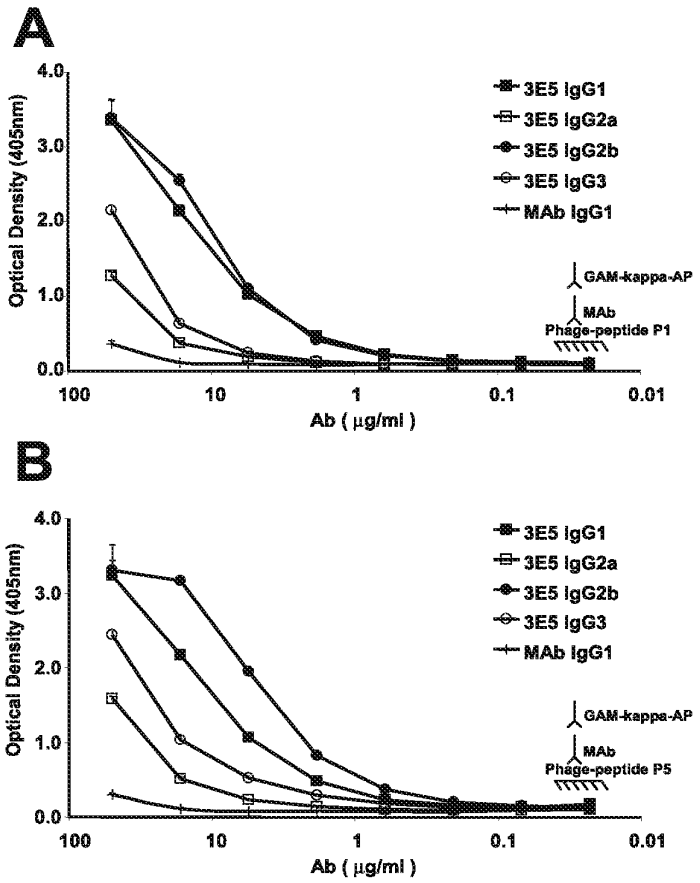


FIGURE 5. Reactivity of 3E5 mAb switch variants to phage-expressing peptides P1 (*A*) and P5 (*B*). Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

which uses the same family of V-region genes (17). Peptide mimetopes have proven useful in identifying small differences in fine specificity between mAbs with identical V regions (28). Each of the 3E5 isotypes showed reactivity with peptides P601E, P206.1, or PA1, but the binding curves were different consistent with differences in apparent affinity for the different peptides (Fig. 4, *A–C*). For peptide P601 (Fig. 4*A*), the 3E5 switch variants bound with a relative affinity of IgG3 > IgG2b, IgG1, IgG2a. For peptide P206.1 (Fig. 4*B*), the relative affinity was IgG2b, IgG1 > IgG2a > IgG3 for mAb concentrations <1 μg/ml, and prozone-like effects were apparent in the binding curve for IgG2b, IgG1, and IgG2a at mAb concentrations >1 μg/ml. For peptide PA1 (Fig. 4*C*), there was not much difference in binding for the various isotypes except that IgG3 produced higher signals at mAb concentrations >1 μg/ml.

Given that the peptides previously isolated with mAb 2H1 revealed differences in binding, we sought to isolate new peptides that would discriminate between the 3E5 isotypes by screening a peptide-expressing phage library with the 3E5 IgG3. Two phage clones, P1 and P5, were characterized by titering different numbers of transforming units of each phage clone against the 3E5 switch variants. The two phage clones expressed two new peptide mimetics known as P1 and P5 with sequences SPNQHTPPWMLK and IREPHVLCADR, respectively. The binding of phages expressing P1 and P5 to the 3E5 isotype variants was different for the 3E5 variants with IgG1 and IgG2b reacting more strongly than either IgG2a or IgG3 (Fig. 5, *A* and *B*). These synthetic biotin peptides behave differently with respect to their strength of binding than when the same peptide is expressed in the phage, and perhaps as a consequence, the difference among the different isotype switch variants is also not as evident as when the peptide was expressed in phage (Fig. 6, *A* and *B*). The 3E5 family of switch variants shows different binding affinity for bound GXM when competing with peptide P1, with a relative strength of binding of IgG1> IgG2a > IgG2b > IgG3 (Fig. 6*C*). In contrast, no significant differences were apparent with peptide P5 in competition experiments (data not shown). Because these peptides are monovalent and small, and contain only one epitope, the differences in apparent binding affinity for V-region-identical isotype variants in competition experiments cannot be ascribed to differences in avidity and must imply differences in paratope-epitope interaction

Immunofluorescence studies

Prior studies have shown that IF patterns are good indicators of Ab specificity (10, 31). Each of the 3E5 switch variants bound to *C. neoformans* cells in an annular binding pattern (Fig. 7). However, there were subtle differences in the localization of fluorescence in the capsule for each one of the Abs. For IgG1 (Fig. 7*A*) and IgG2a (Fig. 7*B*) we observed a brighter outer ring and fluorescence throughout the body of the cell. For IgG2b (Fig. 7*C*), we have an even thicker outer ring and fluorescence through out the body of the cell. For IgG3 we observe a thicker outer ring but no fluorescence on the body of the cell (Fig. 7*D*). The IF pattern observed for the 4H3 mAbs was punctate, and less differences were apparent by this technique between the different isotypes (Fig. 8).

Discussion

Resolving the question of whether the Ab C region contributes to specificity is of great importance for both fundamental and practical

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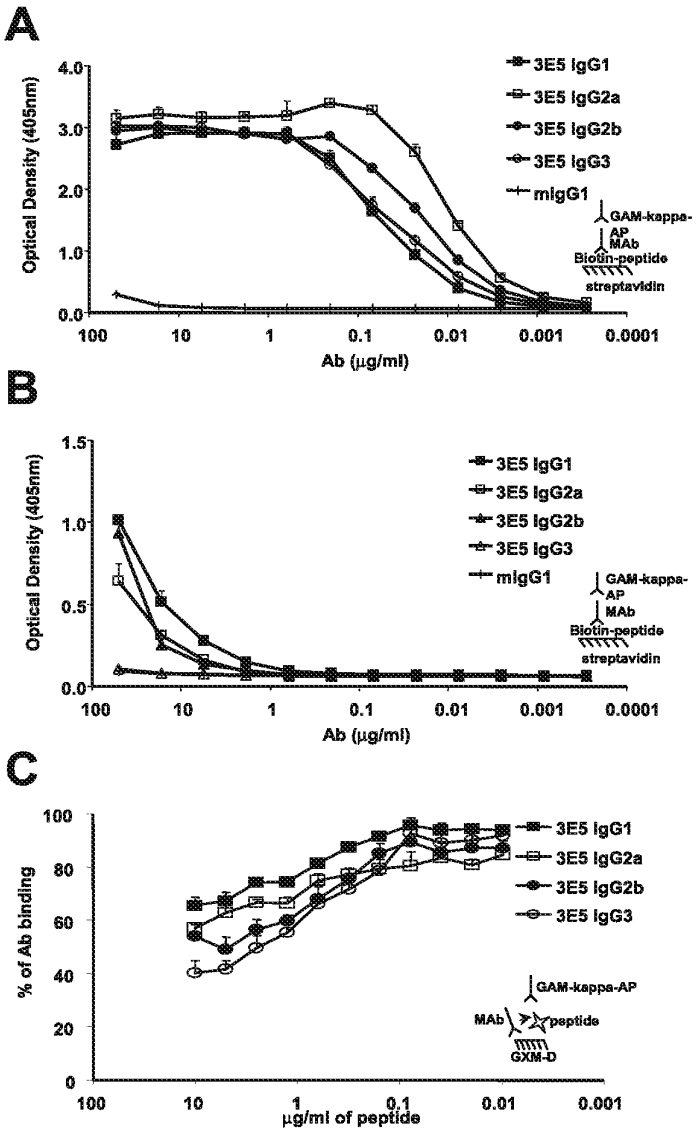


FIGURE 6. Reactivity of 3E5 mAbs to biotinylated peptides selected by the Ph.D. library P1 (*A*) and P5 (*B*). Competition binding assay between biotinylated peptide P1 (*C*) and the 3E5 family of switch variants to GXM. Points represent the average of three measurements, and brackets denote the SD. The details of each ELISA are diagrammed in the *inset* of each panel.

immunology. It has been long assumed that Ig C region domains do not play a significant role in the Ag-Ab interactions apart from their effects on avidity, which are mediated by Ab valency, and/or differences in hinge flexibility that affect the capacity of the Ig molecule to bind more than one epitope (40). The current view is that the primary and secondary Ab responses originate from the same B cell subset. These B cells are stimulated to undergo isotype-switching by T cell signals such that specificity is preserved as new isotypes are generated by the combination of V_H and C_H sequences (1). However, if the C region contributes in any way to specificity, one could imagine that isotype switching could result in Ig molecules with altered specificity despite identical V region sequences.

A variety of observations ranging from isotype-related Id restriction (41, 42) to differences in Ag binding (43–46) strongly suggest that the C region can influence V region molecular properties. Consistent with this view, is the suggestion for conformational signaling through the V_H/C_{H1} interface, which would impose structural and/or kinetic constrains in the Ab-Ag-binding site (47). Crystallographic studies indicate that the amino acids within the link between the variable domain and C_{H1} and C_L are flexible and allow spatial arrangements that facilitate the binding of Ag by Fab (48). Hence, changes in the C_H domain could affect the functional affinity and specificity of Ab through a complex mechanism involving Ab flexibility and cooperative interactions in the case of Abs bound to multivalent Ags (43). These observations, together the finding that IgA, IgG1, and IgM recombinant Abs are able to bind the same epitope but had significant binding differences (46), and our recent finding that V-region-identical chimeric Abs of different isotype had differences in fine specificity, make a circumstantial, yet compelling case for the notion that the C region can influence the interaction between the paratope and the epitope. In this study we explore this possibility with two sets of V-region-identical isotype variant mAbs that bind to distinct epitopes in GXM, use different V regions, and differ in protective efficacy (14, 17).

The Ag-binding properties of the 3E5 and 4H3 isotype variant sets were evaluated by ELISA using native and modified GXM, modified KLH, anti-Id mAbs, and peptide mimetics. In addition we conducted competition studies with other GXM-binding mAbs

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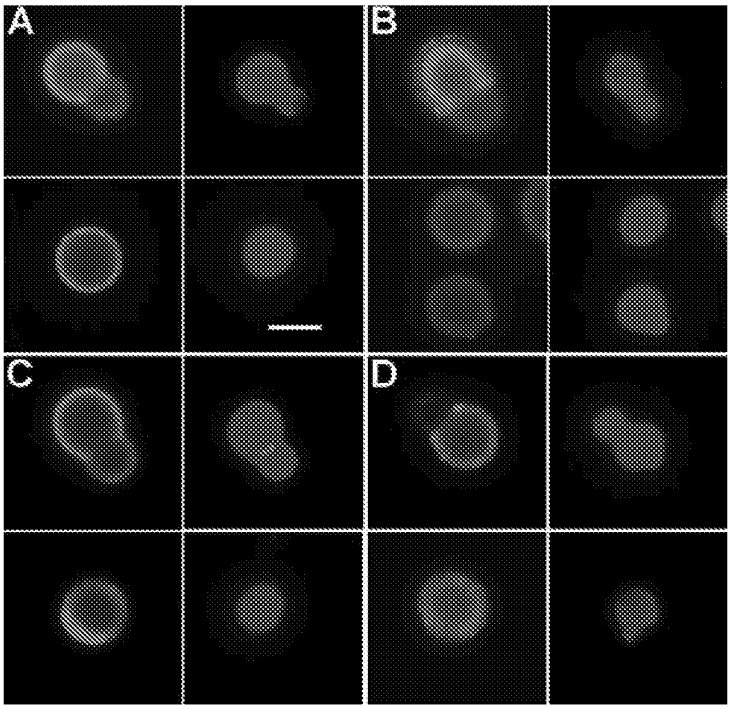


FIGURE 7. Indirect immunofluorescence of *C. neoformans* stained with 3E5 mAbs. 3E5 IgG1 (*A*), 3E5 IgG2a (*B*), 3E5 IgG2b (*C*), and 3E5 IgG3 (*D*). Green fluorescence represents Ab labeling against capsule, and blue fluorescence represents labeling to the cell wall of *C. neoformans* strain 24067. Cells shown are representative of the immunofluorescence patterns observed. Bar, 5 μm. Similar results were obtained by direct immunofluorescence using these mAbs labeled with Alexia Fluor 488 (data not shown).

and IF studies on *C. neoformans* cells. Each of these assays showed differences in binding for each of the two families of V-region-identical mAbs. The finding that the 3E5 family of switch variants manifest different efficacy in competition with other mAbs also suggests isotype-related differences in specificity. However, it is noteworthy that C region-associated differences in specificity were not sufficient to alter the binding of either the 3E5 or 4H3 switch variants sufficiently such that one would compete with one another. Although for each assay it is conceivable to imagine that some of the differences in relative binding of the two families of switch variants are due to avidity for the Ags used, this explanation is difficult to reconcile with the finding that the different isotypes did not show the same relative binding for the different Ags analyzed. In this regard, murine IgG3 was shown to have higher apparent affinity for polysaccharides than other IgG subclasses, presumably as a result of increased avidity resulting from constant-region mediated polymerization after Ag binding (14, 49).

If avidity were solely responsible for our observations, one might have expected IgG3 to consistently be the strongest binding isotype for those experiments that involved binding to polyvalent Ags, such as GXM and KLH. However, the data presented here shows that this was not the case. Differences in avidity cannot account for differences in binding to Ags with only one epitope such as peptides or to Ags that express either one epitope or are not polyvalent. For these Ags, the expectation was that the binding curves resulting from V-region-identical Abs would be identical, because only one Fab is expected to interact with the Ag. This is consistent with the findings by Pritsch and colleagues (46, 47) that monomeric IgA and IgA Fab display the same binding affinities. Furthermore, the MALDI-TOF mass spectrometry revealed no evidence for the formation of bimolecular or higher order complexes in the IgG isotype preparations, consequently we can exclude Ig aggregation as an explanation for our findings. In fact, the most straightforward interpretation of the data is that despite having the same primary structure, fine specificity of V-region-identical binding sites changes as they are expressed with different C regions.

Interestingly, the observation that the relative binding affinity between the isotypes changes depending on the Ag, suggests that the C region is not simply affecting the ability of the Ab to bind as a whole, but rather changing the fine specificity for particular Ags. A potential mechanism for this effect could involve isotype-related differences in the ability of V region-identical Abs to undergo adapted fit transitions upon interacting with different Ags. In this regard, a single Ab has been shown to bind different Ags as a function of V region structural transitions (50). Alternatively, the C region could confer a conformational change on the V region and its Ag-binding site. It is worth noting that the crystal structure of the 2H1 Ab with the PA1 peptide in its binding site was not different than without the peptide there, suggesting that this site is not easily changed at least by Ag binding (51). Furthermore, it is noteworthy that both isotype switching and somatic hypermutation share common molecular mechanisms, that can affect both apparent affinity and specificity, suggesting the possibility that these processes have synergistic effects in diversifying the Ab response.

We generated two new peptide mimetics of GXM by selecting a phage-display library with 3E5 isotypes. One peptide mimetic to which the mAbs 3E5 bound with a much stronger affinity had the motif WM that was identified in a prior study as common to many class II Ab-binding peptides (29). The other peptide had a sequence with no homology to previously identified GXM peptide mimetics. Peptide mimetics of class II mAbs bind in the Ab combining site and are useful in mapping the epitope specificities of closely related mAbs (29). The availability of a family of switch variants, with identical V region sequences, provides the opportunity to ascertain whether different isotypes have an effect in peptide and binding patterns specificities. Crystallographic studies of a class II mAb with a peptide mimetic in the binding grove revealed that the peptide interacts with only a few residues in the paratope (28, 51). Consequently, differences in binding of peptide mimetics for V-region-identical isotype switch variants must imply differences in the binding site structure resulting from the association of the V region with different C regions.

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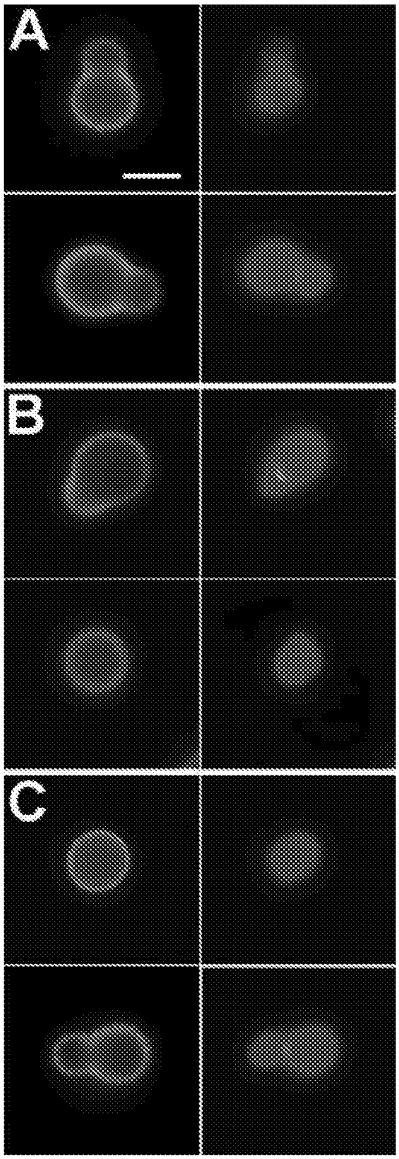


FIGURE 8. Indirect immunofluorescence of *C. neoformans* stained with 4H3 mAbs 4H3 IgGI (*A*), 4H3 IgG2b (*B*), and 3E5 IgG3 (*C*). Green fluorescence represents Ab labeling against capsule and blue fluorescence represents labeling to the cell wall of *C. neoformans* strain 24067. Cells shown are representative of the immunofluorescence patterns observed. Bar, 5 μm.

Anti-idiotypic Abs provide another means to study the influence of H chain C region on V region structure. The anti-idiotypic mAb 6A10 bound to the 3E5 mAbs with different reactivity, implying differences in the secondary structure of the V region protein chains. This observation is consistent with a variety of studies of Ag-stimulated B cells, which show that V regions that have similar, if not identical, Ag-binding specificity, do not necessarily share idiotypic determinants when associated with different H chains (9, 12). The finding that there is altered Id reactivity among the 3E5 mAb switch variants is consistent with, and supports, an explanation for the isotype-related differences in fine specificity involving V region structural differences that depended on the associated C region.

Prior studies using the 3E5 and 4H3 isotype variant sets have shown large isotype-related differences in the protective efficacy against *C. neoformans* in murine models of cryptoccocal infection (12, 15). The interpretation of those results assumed that V-region-identical Abs differing in isotype implied identical specificity and concluded that isotype was a critical parameter of Ab-mediated protection against *C. neoformans* through Fc-related properties, which included interaction with different FcR. The finding that 3E5 IgG3 also bound in an annular pattern supported this idea. Clearly, the results showing isotype-related differences in specificity indicate that this conclusion cannot be simply made by comparing the relative protective efficacy of isotype variants in one host, because changes in fine specificity may affect Ab efficacy. However, the observation that 3E5 IgG1 is protective in normal mice and not protective in CD4-deficient mice, combined with the finding that 3E5 IgG3 is not protective in normal mice but protective in CD8-deficient mice (12, 16) imply dependence of protective efficacy on isotype independent of any effects the C region may have on V region structure. It is noteworthy that when the 3E5 and 4H3 families were initially characterized (11, 19) we did not detect a significant difference in binding to GXM but those experiments used much higher Ab concentrations where binding differences are not apparent.

In summary, we observed changes in reactivity to GXM, peptide mimetopes, and anti-Id MAbs when the IgG3 3E5 mAb is switched to IgG1, IgG2a, and IgG2b. For each one of these Abs, the changes in fine specificity could be different; it may reflect changes or altered Fab structure, or differences in Ig surface properties (48, 52). It has been suggested that the C_{H1} domain of the H chain could influence the structure of the Ab-binding site to form a more stable Ab-Ag complex (47). The current study supports the idea that the H chain C domains influence the V region differences in Ab function and specificity (10, 43, 47, 53), and reveals a complex relationship between epitope specificity and isotype. The ability of C region type to influence V region structure has major implications for such central immunological concepts as the generation of diversity and the idiotypic regulation of immune responses. If isotype switching alters specificity and Id, then idiotypic network regulation would vary depending on the isotype expressed, a finding for which there is already experimental evidence in the literature (5, 7–10, 40, 54). The observation that isotype can alter fine specificity suggests a congruence in function between Ig class switching and somatic mutation, two mechanisms that rely on common molecular machinery. However, it is noteworthy that the changes in specificity associated with class switching are relatively minor and could represent a mechanism for broadening the reach of specificity of an immune response, without incurring the price of Ig loss associated with somatic mutation. Furthermore, it is conceivable that subtle alterations in specificity accompanying isotype switching serve a protective function for the host by reducing the likelihood of prozone-like effects (55) as increasing amounts of Ig are produced during an immune response. From a practical perspective, the findings that isotype can influence the fine specificity should be an important consideration in the development of genetically engineered Abs for therapeutic use because it is conceivable that humanization techniques or the synthesis of mouse-human chimeric Abs can alter the fine specificity of the original Ab.

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Guidance for Industry

Q1A(R2) Stability Testing of New Drug Substances and Products

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

November 2003
ICH

Revision 2

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Revision 2

Contains Nonbinding Recommendations

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Guidance for Industry[1]

Q1A(R2) Stability Testing of New Drug Substances and Products

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION (1) [2]

This guidance is the second revision of *Q1A Stability Testing of New Drug Substances and Products,* which was first published in September 1994 and revised in August 2001. The purpose of this revision is to harmonize the intermediate storage condition for zones I and II with the long-term condition for zones III and IV recommended in the ICH guidance *Q1F Stability Data Package for Registration Applications in Climatic Zones III and IV*. The changes made in this second revision are listed in the attachment to this guidance.

A. Objectives of the Guidance (1.1)

This guidance is intended to define what stability data package for a new drug substance or drug product is sufficient for a registration application within the three regions of the European Union (EU), Japan, and the United States. It does not seek to address the testing for registration in or export to other areas of the world. The guidance exemplifies the core stability data package for new drug substances and products, but leaves sufficient flexibility to encompass the variety of different practical situations that may be encountered due to specific scientific considerations and characteristics of the materials being evaluated. Alternative approaches can be used when there are scientifically justifiable reasons.

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[1] This guidance was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document was endorsed by the ICH Steering Committee at *Step 4* of the ICH process, February 2003. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan, and the United States.

[2] Arabic numbers reflect the organizational breakdown in the document endorsed by the ICH Steering Committee at Step 4 of the ICH process.

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B. Scope of the Guidance (1.2)

The guidance addresses the information to be submitted in registration applications for new molecular entities and associated drug products. This guidance does not currently seek to cover the information to be submitted for abbreviated or abridged applications, variations, or clinical trial applications.

Specific details of the sampling and testing for particular dosage forms in their proposed container closures are not covered in this guidance.

Further guidance on new dosage forms and on biotechnological/biological products can be found in ICH guidances *Q1C Stability Testing for New Dosage Forms* and *Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products*, respectively.

C. General Principles (1.3)

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions.

The choice of test conditions defined in this guidance is based on an analysis of the effects of climatic conditions in the three regions of the EU, Japan, and the United States. The mean kinetic temperature in any part of the world can be derived from climatic data, and the world can be divided into four climatic zones, I-IV. This guidance addresses climatic zones I and II. The principle has been established that stability information generated in any one of the three regions of the EU, Japan, and the United States would be mutually acceptable to the other two regions, provided the information is consistent with this guidance and the labeling is in accord with national/regional requirements.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. GUIDANCE (2)

A. Drug Substance (2.1)

1. General (2.1.1)

Information on the stability of the drug substance is an integral part of the systematic approach to stability evaluation.

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2. Stress Testing (2.1.2)

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance. The testing should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C) above that for accelerated testing), humidity (e.g., 75 percent relative humidity or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. The standard conditions for photostability testing are described in ICH *Q1B Photostability Testing of New Drug Substances and Products*.

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures. However, such examination may not be necessary for certain degradation products if it has been demonstrated that they are not formed under accelerated or long-term storage conditions.

Results from these studies will form an integral part of the information provided to regulatory authorities.

3. Selection of Batches (2.1.3)

Data from formal stability studies should be provided on at least three primary batches of the drug substance. The batches should be manufactured to a minimum of pilot scale by the same synthetic route as production batches and using a method of manufacture and procedure that simulates the final process to be used for production batches. The overall quality of the batches of drug substance placed on formal stability studies should be representative of the quality of the material to be made on a production scale.

Other supporting data can be provided.

4. Container Closure System (2.1.4)

The stability studies should be conducted on the drug substance packaged in a container closure system that is the same as or simulates the packaging proposed for storage and distribution.

5. Specification (2.1.5)

Specification, which is a list of tests, references to analytical procedures, and proposed acceptance criteria, is addressed in ICH *Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* and *Q6B*

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Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Biotechnological/Biological Products. In addition, specification for degradation products in a drug substance is discussed in ICH *Q3A Impurities in New Drug Substances*.

Stability studies should include testing of those attributes of the drug substance that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes. Validated stability-indicating analytical procedures should be applied. Whether and to what extent replication should be performed should depend on the results from validation studies.

6. Testing Frequency (2.1.6)

For long-term studies, frequency of testing should be sufficient to establish the stability profile of the drug substance. For drug substances with a proposed retest period of at least 12 months, the frequency of testing at the long-term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed retest period.

At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0, 3, and 6 months), from a 6-month study is recommended. Where an expectation (based on development experience) exists that the results from accelerated studies are likely to approach significant change criteria, increased testing should be conducted either by adding samples at the final time point or including a fourth time point in the study design.

When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time points, including the initial and final time points (e.g., 0, 6, 9, 12 months), from a 12-month study is recommended.

7. Storage Conditions (2.1.7)

In general, a drug substance should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its sensitivity to moisture. The storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment, and subsequent use.

The long-term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed retest period. Additional data accumulated during the assessment period of the registration application should be submitted to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the intermediate storage condition can be used to evaluate the effect of short-term excursions outside the label storage conditions (such as might occur during shipping).

Long-term, accelerated, and, where appropriate, intermediate storage conditions for drug substances are detailed in the sections below. The general case should apply if the drug substance

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is not specifically covered by a subsequent section. Alternative storage conditions can be used if justified.

a. General case (2.1.7.1)

Study	Storage condition	Minimum time period covered by data at submission
Long-term*	25°C ± 2°C/60% RH ± 5% RH or 30°C ± 2°C/65% RH ± 5% RH	12 months
Intermediate**	30°C ± 2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/75% RH ± 5% RH	6 months

* It is up to the applicant to decide whether long-term stability sturdies are performed at 25°C ± 2°C/60% RH ± 5% RH or 30°C ± 2°C/65% RH ± 5% RH.
** If 30°C ± 2°C/65% RH ± 5% RH is the long-term condition, there is no intermediate condition.

If long-term studies are conducted at 25°C ± 2°C/60% RH ± 5% RH and *significant change* occurs at any time during 6 months' testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. Testing at the intermediate storage condition should include all tests, unless otherwise justified. The initial application should include a minimum of 6 months' data from a 12-month study at the intermediate storage condition.

Significant change for a drug substance is defined as failure to meet its specification.

b. Drug substances intended for storage in a refrigerator (2.1.7.2)

Study	Storage condition	Minimum time period covered by data at submission
Long-term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C/60% RH ± 5% RH	6 months

Data from refrigerated storage should be assessed according to the evaluation section of this guidance, except where explicitly noted below.

If significant change occurs between 3 and 6 months' testing at the accelerated storage condition, the proposed retest period should be based on the real time data available at the long-term storage condition.

If significant change occurs within the first 3 months' testing at the accelerated storage condition, a discussion should be provided to address the effect of short-term excursions outside the label storage condition (e.g., during shipping or handling). This discussion can be supported, if appropriate, by further testing on a single batch of the drug substance for a period shorter than

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3 months but with more frequent testing than usual. It is considered unnecessary to continue to test a drug substance through 6 months when a significant change has occurred within the first 3 months.

c. Drug substances intended for storage in a freezer (2.1.7.3)

Study	Storage condition	Minimum time period covered by data at submission
Long-term	-20°C ± 5°C	12 months

For drug substances intended for storage in a freezer, the retest period should be based on the real time data obtained at the long-term storage condition. In the absence of an accelerated storage condition for drug substances intended to be stored in a freezer, testing on a single batch at an elevated temperature (e.g., 5°C ± 3°C or 25°C ± 2°C) for an appropriate time period should be conducted to address the effect of short-term excursions outside the proposed label storage condition (e.g., during shipping or handling).

d. Drug substances intended for storage below -20°C (2.1.7.4)

Drug substances intended for storage below -20°C should be treated on a case-by-case basis.

8. Stability Commitment (2.1.8)

When available long-term stability data on primary batches do not cover the proposed retest period granted at the time of approval, a commitment should be made to continue the stability studies postapproval to firmly establish the retest period.

Where the submission includes long-term stability data on three production batches covering the proposed retest period, a postapproval commitment is considered unnecessary. Otherwise, one of the following commitments should be made:

- If the submission includes data from stability studies on at least three production batches, a commitment should be made to continue these studies through the proposed retest period.
- If the submission includes data from stability studies on fewer than three production batches, a commitment should be made to continue these studies through the proposed retest period and to place additional production batches, to a total of at least three, on long-term stability studies through the proposed retest period.
- If the submission does not include stability data on production batches, a commitment should be made to place the first three production batches on long-term stability studies through the proposed retest period.

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The stability protocol used for long-term studies for the stability commitment should be the same as that for the primary batches, unless otherwise scientifically justified.

9. Evaluation (2.1.9)

The purpose of the stability study is to establish, based on testing a minimum of three batches of the drug substance and evaluating the stability information (including, as appropriate, results of the physical, chemical, biological, and microbiological tests), a retest period applicable to all future batches of the drug substance manufactured under similar circumstances. The degree of variability of individual batches affects the confidence that a future production batch will remain within specification throughout the assigned retest period.

The data may show so little degradation and so little variability that it is apparent from looking at the data that the requested retest period will be granted. Under these circumstances, it is normally unnecessary to go through the formal statistical analysis; providing a justification for the omission should be sufficient.

An approach for analyzing the data on a quantitative attribute that is expected to change with time is to determine the time at which the 95 percent, one-sided confidence limit for the mean curve intersects the acceptance criterion. If analysis shows that the batch-to-batch variability is small, it is advantageous to combine the data into one overall estimate. This can be done by first applying appropriate statistical tests (e.g., p values for level of significance of rejection of more than 0.25) to the slopes of the regression lines and zero time intercepts for the individual batches. If it is inappropriate to combine data from several batches, the overall retest period should be based on the minimum time a batch can be expected to remain within acceptance criteria.

The nature of any degradation relationship will determine whether the data should be transformed for linear regression analysis. Usually the relationship can be represented by a linear, quadratic, or cubic function on an arithmetic or logarithmic scale. Statistical methods should be employed to test the goodness of fit of the data on all batches and combined batches (where appropriate) to the assumed degradation line or curve.

Limited extrapolation of the real time data from the long-term storage condition beyond the observed range to extend the retest period can be undertaken at approval time if justified. This justification should be based, for example, on what is known about the mechanism of degradation, the results of testing under accelerated conditions, the goodness of fit of any mathematical model, batch size, and/or existence of supporting stability data. However, this extrapolation assumes that the same degradation relationship will continue to apply beyond the observed data.

Any evaluation should cover not only the assay, but also the levels of degradation products and other appropriate attributes.

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10. Statements/Labeling (2.1.10)

A storage statement should be established for the labeling in accordance with relevant national/regional requirements. The statement should be based on the stability evaluation of the drug substance. Where applicable, specific instructions should be provided, particularly for drug substances that cannot tolerate freezing. Terms such as *ambient conditions* or *room temperature* should be avoided.

A retest period should be derived from the stability information, and a retest date should be displayed on the container label if appropriate.

B. Drug Product (2.2)

1. General (2.2.1)

The design of the formal stability studies for the drug product should be based on knowledge of the behavior and properties of the drug substance, results from stability studies on the drug substance, and experience gained from clinical formulation studies. The likely changes on storage and the rationale for the selection of attributes to be tested in the formal stability studies should be stated.

2. Photostability Testing (2.2.2)

Photostability testing should be conducted on at least one primary batch of the drug product if appropriate. The standard conditions for photostability testing are described in ICH Q1B.

3. Selection of Batches (2.2.3)

Data from stability studies should be provided on at least three primary batches of the drug product. The primary batches should be of the same formulation and packaged in the same container closure system as proposed for marketing. The manufacturing process used for primary batches should simulate that to be applied to production batches and should provide product of the same quality and meeting the same specification as that intended for marketing. Two of the three batches should be at least pilot scale batches, and the third one can be smaller if justified. Where possible, batches of the drug product should be manufactured by using different batches of the drug substance.

Stability studies should be performed on each individual strength and container size of the drug product unless bracketing or matrixing is applied.

Other supporting data can be provided.

4. Container Closure System (2.2.4)

Stability testing should be conducted on the dosage form packaged in the container closure system proposed for marketing (including, as appropriate, any secondary packaging and

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container label). Any available studies carried out on the drug product outside its immediate container or in other packaging materials can form a useful part of the stress testing of the dosage form or can be considered as supporting information, respectively.

5. Specification (2.2.5)

Specification, which is a list of tests, references to analytical procedures, and proposed acceptance criteria, including the concept of different acceptance criteria for release and shelf life specifications, is addressed in ICH Q6A and Q6B. In addition, specification for degradation products in a drug product is addressed in ICH *Q3B Impurities in New Drug Products*.

Stability studies should include testing of those attributes of the drug product that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes, preservative content (e.g., antioxidant, antimicrobial preservative), and functionality tests (e.g., for a dose delivery system). Analytical procedures should be fully validated and stability indicating. Whether and to what extent replication should be performed will depend on the results of validation studies.

Shelf life acceptance criteria should be derived from consideration of all available stability information. It may be appropriate to have justifiable differences between the shelf life and release acceptance criteria based on the stability evaluation and the changes observed on storage. Any differences between the release and shelf life acceptance criteria for antimicrobial preservative content should be supported by a validated correlation of chemical content and preservative effectiveness demonstrated during drug development on the product in its final formulation (except for preservative concentration) intended for marketing. A single primary stability batch of the drug product should be tested for antimicrobial preservative effectiveness (in addition to preservative content) at the proposed shelf life for verification purposes, regardless of whether there is a difference between the release and shelf life acceptance criteria for preservative content.

6. Testing Frequency (2.2.6)

For long-term studies, frequency of testing should be sufficient to establish the stability profile of the drug product. For products with a proposed shelf life of at least 12 months, the frequency of testing at the long-term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed shelf life.

At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0, 3, and 6 months), from a 6-month study is recommended. Where an expectation (based on development experience) exists that results from accelerated testing are likely to approach significant change criteria, increased testing should be conducted either by adding samples at the final time point or by including a fourth time point in the study design.

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When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time points, including the initial and final time points (e.g., 0, 6, 9, 12 months), from a 12-month study is recommended.

Reduced designs (i.e., matrixing or bracketing), where the testing frequency is reduced or certain factor combinations are not tested at all, can be applied if justified.

7. Storage Conditions (2.2.7)

In general, a drug product should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its sensitivity to moisture or potential for solvent loss. The storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment, and subsequent use.

Stability testing of the drug product after constitution or dilution, if applicable, should be conducted to provide information for the labeling on the preparation, storage condition, and in-use period of the constituted or diluted product. This testing should be performed on the constituted or diluted product through the proposed in-use period on primary batches as part of the formal stability studies at initial and final time points, and if full shelf life, long-term data will not be available before submission, at 12 months or the last time point for which data will be available. In general, this testing need not be repeated on commitment batches.

The long-term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed shelf life. Additional data accumulated during the assessment period of the registration application should be submitted to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the intermediate storage condition can be used to evaluate the effect of short-term excursions outside the label storage conditions (such as might occur during shipping).

Long-term, accelerated, and, where appropriate, intermediate storage conditions for drug products are detailed in the sections below. The general case should apply if the drug product is not specifically covered by a subsequent section. Alternative storage conditions can be used if justified.

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a. General case (2.2.7.1)

Study	Storage condition	Minimum time period covered by data at submission
Long-term*	25°C ± 2°C/60% RH ± 5% RH or 30°C ± 2°C/65% RH ± 5% RH	12 months
Intermediate**	30°C ± 2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/75% RH ± 5% RH	6 months

* It is up to the applicant to decide whether long-term stability sturdies are performed at 25°C ± 2°C/60% RH ± 5% RH or 30°C ± 2°C/65% RH ± 5% RH.
** If 30°C ± 2°C/65% RH ± 5% RH is the long-term condition, there is no intermediate condition.

If long-term studies are condcuted at 25°C ± 2°C/60% RH ± 5% RH and *significant change* occurs at any time during 6 months' testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. The initial application should include a minimum of 6 months' data from a 12-month study at the intermediate storage condition.

In general, *significant change* for a drug product is defined as one or more of the following (as appropriate for the dosage form):

- A 5 percent change in assay from its initial value, or failure to meet the acceptance criteria for potency when using biological or immunological procedures
- Any degradation product' s exceeding its acceptance criterion
- Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation). However, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions.
- Failure to meet the acceptance criterion for pH
- Failure to meet the acceptance criteria for dissolution for 12 dosage units

b. Drug products packaged in impermeable containers (2.2.7.2)

Sensitivity to moisture or potential for solvent loss is not a concern for drug products packaged in impermeable containers that provide a permanent barrier to passage of moisture or solvent. Thus, stability studies for products stored in impermeable containers can be conducted under any controlled or ambient humidity condition.

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c. Drug products packaged in semipermeable containers (2.2.7.3)

Aqueous-based products packaged in semipermeable containers should be evaluated for potential water loss in addition to physical, chemical, biological, and microbiological stability. This evaluation can be carried out under conditions of low relative humidity, as discussed below. Ultimately, it should be demonstrated that aqueous-based drug products stored in semipermeable containers can withstand low relative humidity environments. Other comparable approaches can be developed and reported for nonaqueous, solvent-based products.

Study	Storage condition	Minimum time period covered by data at submission
Long-term *	25°C ± 2°C/40% RH ± 5% RH or 30°C ± 2°C/35% RH ± 5% RH	12 months
Intermediate**	30°C ± 2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/not more than (NMT) 25% RH	6 months

* It is up to the applicant to decide whether long-term stability sturdies are performed at 25°C ± 2°C/40% RH ± 5% RH or 30°C ± 2°C/35% RH ± 5% RH.
** If 30°C ± 2°C/35% RH ± 5% RH is the long-term condition, there is no intermediate condition.

When long-term studies are conducted at 25°C ± 2°C/40% RH ± 5% RH and significant change other than water loss occurs during the 6 months' testing at the accelerated storage condition, additional testing at the intermediate storage condition should be performed, as described under the general case, to evaluate the temperature effect at 30°C. A significant change in water loss alone at the accelerated storage condition does not necessitate testing at the intermediate storage condition. However, data should be provided to demonstrate that the drug product will not have significant water loss throughout the proposed shelf life if stored at 25°C and the reference relative humidity of 40 percent RH.

A 5 percent loss in water from its initial value is considered a significant change for a product packaged in a semipermeable container after an equivalent of 3 months' storage at 40°C/NMT 25 percent RH. However, for small containers (1 mL or less) or unit-dose products, a water loss of 5 percent or more after an equivalent of 3 months' storage at 40°C/NMT 25 percent RH may be appropriate if justified.

An alternative approach to studying at the reference relative humidity as recommended in the table above (for either long-term or accelerated testing) is performing the stability studies under higher relative humidity and deriving the water loss at the reference relative humidity through calculation. This can be achieved by experimentally determining the permeation coefficient for the container closure system or, as shown in the example below, using the calculated ratio of water loss rates between the two humidity conditions at the same temperature. The permeation

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coefficient for a container closure system can be experimentally determined by using the worst case scenario (e.g., the most diluted of a series of concentrations) for the proposed drug product.

Example of an approach for determining water loss:

For a product in a given container closure system, container size, and fill, an appropriate approach for deriving the water loss rate at the reference relative humidity is to multiply the water loss rate measured at an alternative relative humidity at the same temperature by a water loss rate ratio shown in the table below. A linear water loss rate at the alternative relative humidity over the storage period should be demonstrated.

For example, at a given temperature (e.g., 40°C), the calculated water loss rate during storage at NMT 25 percent RH is the water loss rate measured at 75 percent RH multiplied by 3.0, the corresponding water loss rate ratio.

Alternative relative humidity	**Reference relative humidity**	**Ratio of water loss rates at a given temperature**
60% RH	25% RH	1.9
60% RH	40% RH	1.5
65% RH	35% RH	1.9
75% RH	25% RH	3.0

Valid water loss rate ratios at relative humidity conditions other than those shown in the table above can also be used.

d. Drug products intended for storage in a refrigerator (2.2.7.4)

Study	**Storage condition**	**Minimum time period covered by data at submission**
Long-term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C/60% RH ± 5% RH	6 months

If the drug product is packaged in a semipermeable container, appropriate information should be provided to assess the extent of water loss.

Data from refrigerated storage should be assessed according to the evaluation section of this guidance, except where explicitly noted below.

If significant change occurs between 3 and 6 months' testing at the accelerated storage condition, the proposed shelf life should be based on the real time data available from the long-term storage condition.

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If significant change occurs within the first 3 months' testing at the accelerated storage condition, a discussion should be provided to address the effect of short-term excursions outside the label storage condition (e.g., during shipment and handling). This discussion can be supported, if appropriate, by further testing on a single batch of the drug product for a period shorter than 3 months but with more frequent testing than usual. It is considered unnecessary to continue to test a product through 6 months when a significant change has occurred within the first 3 months.

e. Drug products intended for storage in a freezer (2.2.7.5)

Study	Storage condition	Minimum time period covered by data at submission
Long-term	-20°C ± 5°C	12 months

For drug products intended for storage in a freezer, the shelf life should be based on the real time data obtained at the long-term storage condition. In the absence of an accelerated storage condition for drug products intended to be stored in a freezer, testing on a single batch at an elevated temperature (e.g., 5°C ± 3°C or 25°C ± 2°C) for an appropriate time period should be conducted to address the effect of short-term excursions outside the proposed label storage condition.

f. Drug products intended for storage below -20°C (2.2.7.6)

Drug products intended for storage below -20°C should be treated on a case-by-case basis.

8. Stability Commitment (2.2.8)

When available long-term stability data on primary batches do not cover the proposed shelf life granted at the time of approval, a commitment should be made to continue the stability studies postapproval to firmly establish the shelf life.

Where the submission includes long-term stability data from three production batches covering the proposed shelf life, a postapproval commitment is considered unnecessary. Otherwise, one of the following commitments should be made:

- If the submission includes data from stability studies on at least three production batches, a commitment should be made to continue the long-term studies through the proposed shelf life and the accelerated studies for 6 months.

- If the submission includes data from stability studies on fewer than three production batches, a commitment should be made to continue the long-term studies through the proposed shelf life and the accelerated studies for 6 months, and to place additional production batches, to a total of at least three, on long-term stability studies through the proposed shelf life and on accelerated studies for 6 months.

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- If the submission does not include stability data on production batches, a commitment should be made to place the first three production batches on long-term stability studies through the proposed shelf life and on accelerated studies for 6 months.

The stability protocol used for studies on commitment batches should be the same as that for the primary batches, unless otherwise scientifically justified.

Where intermediate testing is called for by a significant change at the accelerated storage condition for the primary batches, testing on the commitment batches can be conducted at either the intermediate or the accelerated storage condition. However, if significant change occurs at the accelerated storage condition on the commitment batches, testing at the intermediate storage condition should also be conducted.

9. Evaluation (2.2.9)

A systematic approach should be adopted in the presentation and evaluation of the stability information, which should include, as appropriate, results from the physical, chemical, biological, and microbiological tests, including particular attributes of the dosage form (e.g., dissolution rate for solid oral dosage forms).

The purpose of the stability study is to establish, based on testing a minimum of three batches of the drug product, a shelf life and label storage instructions applicable to all future batches of the drug product manufactured and packaged under similar circumstances. The degree of variability of individual batches affects the confidence that a future production batch will remain within specification throughout its shelf life.

Where the data show so little degradation and so little variability that it is apparent from looking at the data that the requested shelf life will be granted, it is normally unnecessary to go through the formal statistical analysis; providing a justification for the omission should be sufficient.

An approach for analyzing data of a quantitative attribute that is expected to change with time is to determine the time at which the 95 percent one-sided confidence limit for the mean curve intersects the acceptance criterion. If analysis shows that the batch-to-batch variability is small, it is advantageous to combine the data into one overall estimate. This can be done by first applying appropriate statistical tests (e.g., p values for level of significance of rejection of more than 0.25) to the slopes of the regression lines and zero time intercepts for the individual batches. If it is inappropriate to combine data from several batches, the overall shelf life should be based on the minimum time a batch can be expected to remain within acceptance criteria.

The nature of the degradation relationship will determine whether the data should be transformed for linear regression analysis. Usually the relationship can be represented by a linear, quadratic, or cubic function on an arithmetic or logarithmic scale. Statistical methods should be employed to test the goodness of fit on all batches and combined batches (where appropriate) to the assumed degradation line or curve.

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Limited extrapolation of the real time data from the long-term storage condition beyond the observed range to extend the shelf life can be undertaken at approval time if justified. This justification should be based, for example, on what is known about the mechanisms of degradation, the results of testing under accelerated conditions, the goodness of fit of any mathematical model, batch size, and/or existence of supporting stability data. However, this extrapolation assumes that the same degradation relationship will continue to apply beyond the observed data.

Any evaluation should consider not only the assay but also the degradation products and other appropriate attributes. Where appropriate, attention should be paid to reviewing the adequacy of the mass balance and different stability and degradation performance.

10. Statements/Labeling (2.2.10)

A storage statement should be established for the labeling in accordance with relevant national/regional requirements. The statement should be based on the stability evaluation of the drug product. Where applicable, specific instruction should be provided, particularly for drug products that cannot tolerate freezing. Terms such as *ambient conditions* or *room temperature* should be avoided.

There should be a direct link between the label storage statement and the demonstrated stability of the drug product. An expiration date should be displayed on the container label.

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GLOSSARY (3)

The following definitions are provided to facilitate interpretation of the guidance.

Accelerated testing: Studies designed to increase the rate of chemical degradation or physical change of a drug substance or drug product by using exaggerated storage conditions as part of the formal stability studies. Data from these studies, in addition to long-term stability studies, can be used to assess longer term chemical effects at nonaccelerated conditions and to evaluate the effect of short-term excursions outside the label storage conditions such as might occur during shipping. Results from accelerated testing studies are not always predictive of physical changes.

Bracketing: The design of a stability schedule such that only samples on the extremes of certain design factors (e.g., strength, package size) are tested at all time points as in a full design. The design assumes that the stability of any intermediate levels is represented by the stability of the extremes tested. Where a range of strengths is to be tested, bracketing is applicable if the strengths are identical or very closely related in composition (e.g., for a tablet range made with different compression weights of a similar basic granulation, or a capsule range made by filling different plug fill weights of the same basic composition into different size capsule shells). Bracketing can be applied to different container sizes or different fills in the same container closure system.

Climatic zones: The four zones in the world that are distinguished by their characteristic, prevalent annual climatic conditions. This is based on the concept described by W. Grimm (*Drugs Made in Germany*, 28:196-202, 1985 and 29:39-47, 1986).

Commitment batches: Production batches of a drug substance or drug product for which the stability studies are initiated or completed postapproval through a commitment made in the registration application.

Container closure system: The sum of packaging components that together contain and protect the dosage form. This includes primary packaging components and secondary packaging components if the latter are intended to provide additional protection to the drug product. A packaging system is equivalent to a container closure system.

Dosage form: A pharmaceutical product type (e.g., tablet, capsule, solution, cream) that contains a drug substance generally, but not necessarily, in association with excipients.

Drug product: The dosage form in the final immediate packaging intended for marketing.

Drug substance: The unformulated drug substance that may subsequently be formulated with excipients to produce the dosage form.

Excipient: Anything other than the drug substance in the dosage form.

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Expiration date: The date placed on the container label of a drug product designating the time prior to which a batch of the product is expected to remain within the approved shelf life specification, if stored under defined conditions, and after which it must not be used.

Formal stability studies: Long-term and accelerated (and intermediate) studies undertaken on primary and/or commitment batches according to a prescribed stability protocol to establish or confirm the retest period of a drug substance or the shelf life of a drug product.

Impermeable containers: Containers that provide a permanent barrier to the passage of gases or solvents (e.g., sealed aluminum tubes for semi-solids, sealed glass ampoules for solutions).

Intermediate testing: Studies conducted at 30°C/65% RH and designed to moderately increase the rate of chemical degradation or physical changes for a drug substance or drug product intended to be stored long-term at 25°C.

Long-term testing: Stability studies under the recommended storage condition for the retest period or shelf life proposed (or approved) for labeling.

Mass balance: The process of adding together the assay value and levels of degradation products to see how closely these add up to 100 percent of the initial value, with due consideration of the margin of analytical error.

Matrixing: The design of a stability schedule such that a selected subset of the total number of possible samples for all factor combinations is tested at a specified time point. At a subsequent time point, another subset of samples for all factor combinations is tested. The design assumes that the stability of each subset of samples tested represents the stability of all samples at a given time point. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same container closure system, and, possibly in some cases, different container closure systems.

Mean kinetic temperature: A single derived temperature that, if maintained over a defined period of time, affords the same thermal challenge to a drug substance or drug product as would be experienced over a range of both higher and lower temperatures for an equivalent defined period. The mean kinetic temperature is higher than the arithmetic mean temperature and takes into account the Arrhenius equation.

When establishing the mean kinetic temperature for a defined period, the formula of J. D. Haynes *(J. Pharm. Sci.,* 60:927-929, 1971) can be used.

New molecular entity: An active pharmaceutical substance not previously contained in any drug product registered with the national or regional authority concerned. A new salt, ester, or noncovalent bond derivative of an approved drug substance is considered a new molecular entity for the purpose of stability testing under this guidance.

Pilot scale batch: A batch of a drug substance or drug product manufactured by a procedure fully representative of and simulating that to be applied to a full production scale batch. For

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solid oral dosage forms, a pilot scale is generally, at a minimum, one-tenth that of a full production scale or 100,000 tablets or capsules, whichever is larger.

Primary batch: A batch of a drug substance or drug product used in a formal stability study, from which stability data are submitted in a registration application for the purpose of establishing a retest period or shelf life, respectively. A primary batch of a drug substance should be at least a pilot scale batch. For a drug product, two of the three batches should be at least pilot scale batch, and the third batch can be smaller if it is representative with regard to the critical manufacturing steps. However, a primary batch may be a production batch.

Production batch: A batch of a drug substance or drug product manufactured at production scale by using production equipment in a production facility as specified in the application.

Retest date: The date after which samples of the drug substance should be examined to ensure that the material is still in compliance with the specification and thus suitable for use in the manufacture of a given drug product.

Retest period: The period of time during which the drug substance is expected to remain within its specification and, therefore, can be used in the manufacture of a given drug product, provided that the drug substance has been stored under the defined conditions. After this period, a batch of drug substance destined for use in the manufacture of a drug product should be retested for compliance with the specification and then used immediately. A batch of drug substance can be retested multiple times and a different portion of the batch used after each retest, as long as it continues to comply with the specification. For most biotechnological/biological substances known to be labile, it is more appropriate to establish a shelf life than a retest period. The same may be true for certain antibiotics.

Semipermeable containers: Containers that allow the passage of solvent, usually water, while preventing solute loss. The mechanism for solvent transport occurs by absorption into one container surface, diffusion through the bulk of the container material, and desorption from the other surface. Transport is driven by a partial pressure gradient. Examples of semipermeable containers include plastic bags and semirigid, low-density polyethylene (LDPE) pouches for large volume parenterals (LVPs), and LDPE ampoules, bottles, and vials.

Shelf life (also referred to as expiration dating period): The time period during which a drug product is expected to remain within the approved shelf life specification, provided that it is stored under the conditions defined on the container label.

Specification: See ICH Q6A and Q6B.

Specification, Release: The combination of physical, chemical, biological, and microbiological tests and acceptance criteria that determine the suitability of a drug product at the time of its release.

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Specification, Shelf life: The combination of physical, chemical, biological, and microbiological tests and acceptance criteria that determine the suitability of a drug substance throughout its retest period, or that a drug product should meet throughout its shelf life.

Storage condition tolerances: The acceptable variations in temperature and relative humidity of storage facilities for formal stability studies. The equipment should be capable of controlling the storage condition within the ranges defined in this guidance. The actual temperature and humidity (when controlled) should be monitored during stability storage. Short-term spikes due to opening of doors of the storage facility are accepted as unavoidable. The effect of excursions due to equipment failure should be addressed and reported if judged to affect stability results. Excursions that exceed the defined tolerances for more than 24 hours should be described in the study report and their effect assessed.

Stress testing (drug substance): Studies undertaken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing.

Stress testing (drug product): Studies undertaken to assess the effect of severe conditions on the drug product. Such studies include photostability testing (see ICH Q1B) and specific testing of certain products (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

Supporting data: Data, other than those from formal stability studies, that support the analytical procedures, the proposed retest period or shelf life, and the label storage statements. Such data include (1) stability data on early synthetic route batches of drug substance, small-scale batches of materials, investigational formulations not proposed for marketing, related formulations, and product presented in containers and closures other than those proposed for marketing; (2) information regarding test results on containers; and (3) other scientific rationales.

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REFERENCES (4)[3]

ICH Q1B Photostability Testing of New Drug Substances and Products

ICH Q1C Stability Testing for New Dosage Forms

ICH Q3A Impurities in New Drug Substances

ICH Q3B Impurities in New Drug Products

ICH Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products

ICH Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances

ICH Q6B Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Biotechnological/Biological Products

[3] We update guidances periodically. To make sure you have the most recent version of a guidance, check the CDER guidance page at http://www.fda.gov/cder/guidance/index.htm

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ATTACHMENT
List of Revision 2 Changes

The revisions to this *Q1A* guidance result from adoption of the ICH guidance *Q1F Stability Data Package for Registration Applications in Climatic Zones III and IV*. The following changes were made.

1. The intermediate storage condition has been changed from 30°C ± 2°C/60% RH ± 5% RH to 30°C ± 2°C/65% RH ± 5% RH in the following sections:

   - II.A.7.a (2.1.7.1) Drug Substance - Storage Conditions - General case
   - II.B.7.a (2.2.7.1) Drug Product - Storage Conditions - General case
   - II.B.7.c (2.2.7.3) Drug products packaged in semipermeable containers
   - Glossary (3) *Intermediate testing*

2. 30°C ± 2°C/65% RH ± 5% RH has been added as a suitable alternative long-term storage condition to 25°C ± 2°C/60% RH ± 5% in the following sections:

   - II.A.7.a (2.1.7.1) Drug Substance - Storage Conditions - General case
   - II.B.7.a (2.2.7.1) Drug Product - Storage Conditions - General case

3. 30°C ± 2°C/35% RH ± 5% RH has been added as a suitable alternative long-term storage condition to 25°C ± 2°C/40% RH ± 5% and the corresponding example for the ratio of water-loss rates has been included in the following section:

   - II.B.7.c (2.2.7.3) Drug products packaged in semipermeable containers

Midstream switch of the intermediate storage condition from 30°C ± 2°C/60% RH ± 5% RH to 30°C ± 2°C/65% RH ± 5% RH can be appropriate provided that the respective storage conditions and the date of the switch are clearly documented and stated in the registration application.

It is recommended that registration applications contain data from complete studies at the intermediate storage condition 30°C ± 2°C/65% RH ± 5% RH, if applicable, by three years after the date of publication of this revised guideline in the respective ICH tripartite region.

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Review

Instability, stabilization, and formulation of liquid protein pharmaceuticals

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Abstract

One of the most challenging tasks in the development of protein pharmaceuticals is to deal with physical and chemical instabilities of proteins. Protein instability is one of the major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs. Protein

Abbreviations: ADA, adenosine deaminase; ADH, alcohol dehydrogenase; AcP, acylphosphatase; BDNF, brain-derived neurotrophic factor; BGG, bovine γ-globulin; BSA, bovine serum albumin; BSF, bovine serum fetuin; CD, circular dichroism spectroscopy; CE, capillary electrophoresis; CMC-Na, carboxymethyl cellulose sodium; rhCNTF, recombinant human ciliary neurotrophic factor; rConIFN, recombinant consensus α-interferon; DGK, diacylglycerol kinase; DMSO, dimethylsulfoxide; rhDNase, recombinant human deoxyribonuclease; DSC, differential scanning calorimetry; hEGF, human epidermal growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; rFVIII, recombinant factor VIII; pdFIX, plasma-derived factor IX; rFIX, recombinant factor IX; rFXIII, recombinant factor XIII; FTIR, Fourier transform infrared spectroscopy; GA, glucoamylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte colony-stimulating factor; GDH, glutamate dehydrogenase; met-hGH, methionyl human growth hormone; pGH, porcine growth hormone; GRF, growth hormone releasing factor; GdnHCl, guanidine hydrochloride; HP-β-CD, hydroxypropyl-β-cyclodextrin; IFN-β, interferon-β; IFN-γ, interferon-γ; hIGF-I, recombinant human insulin-like growth factor I; IL-1β, interleukin-1β; IL-1R, interleulin-1 receptor; IL-2, interleukin-2; rhIL-1ra, recombinant human interleukin-1 receptor antagonist; IR, infrared spectroscopy; rhKGF, recombinant human keratinocyte growth factor; LDH, lactate dehydrogenase; *phm*-MDH, pig heart mitochondrial malate dehydrogenase; LMW-UK, low molecular weight urokinase; rhM-CSF, recombinant human macrophage colony-stimulating factor; rhMGDF, recombinant human megakaryocyte growth and development factor; MS, mass spectroscopy; rhNGF, recombinant human nerve growth factor; NMR, nuclear magnetic resonance spectroscopy; tPA, tissue plasminogen activator; PAGE, polyacrylamide gel electrophoresis; hPAH, human phenylalanine hydroxylase; PE40, 40 kD segment of *Pseudomonas* exotoxin (PE); PEG, polyethylene glycol; *ml*-PEPC, maize leaf phosphoenolpyruvate carboxylase; PFK, phosphofructokinase; huPrP(90-231), recombinant protein corresponding to the human prion protein domain (residues 90-231); PVA, polyvinylalcohol; PVP, polyvinylpyrrolidone; rhPTH, recombinant human parathyroid hormone; RP-HPLC, reversed phase HPLC; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; SEC-HPLC, size exclusion HPLC; TGF, transforming growth factor; TMAO, trimethylamine *N*-oxide; TP40, Cys-replaced (with Ala in PE40) mutant of TGF-α-PE40; rhTPO, recombinant human thrombopoietin; YEI, yeast external invertase.

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pharmaceuticals usually have to be stored under cold conditions or freeze-dried to achieve an acceptable shelf life. To understand and maximize the stability of protein pharmaceuticals or any other usable proteins such as catalytic enzymes, many studies have been conducted, especially in the past two decades. These studies have covered many areas such as protein folding and unfolding/denaturation, mechanisms of chemical and physical instabilities of proteins, and various means of stabilizing proteins in aqueous or solid state and under various processing conditions such as freeze-thawing and drying. This article reviews these investigations and achievements in recent years and discusses the basic behavior of proteins, their instabilities, and stabilization in aqueous state in relation to the development of liquid protein pharmaceuticals. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Denaturation; Unfolding; Melting; Aggregation; Degradation; Preferential interaction

1. Introduction

The advent of recombinant DNA technology has led to a worldwide zeal to develop protein pharmaceuticals in the past two decades. These protein pharmaceuticals or pharmaceutical candidates include functional regulators and supplements, enzyme activators and inhibitors, poly- and monoclonal antibodies, and various vaccines. In comparison with small chemical drugs, protein pharmaceuticals have high specificity and activity at relatively low concentrations. These features have made protein pharmaceuticals indispensable in combating human diseases.

Due to advances in analytical separation technology, recombinant proteins can now be purified to an unprecedented level (Bond et al., 1998). Highly purified protein pharmaceuticals significantly reduce the known and unknown potential side or even toxic effects. However, one of the most challenging tasks remains in the development of protein pharmaceuticals: dealing with physical and chemical instabilities of proteins. Protein instability is one of the two major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs (Wang, 1996). Protein pharmaceuticals usually have to be stored under cold conditions or even freeze-dried to a solid form to achieve an acceptable shelf life.

In search for ways of stabilizing proteins, scientists turned their attention to nature for an answer. It is well known that certain natural organisms can grow well at extreme temperatures. Hyperthermophilic organisms (hyperthermophiles) such as anaerobic, methanogenic, or sulfate-reducing archaebacteria grow at temperatures near or above 100°C (Huber et al., 1989; Adams, 1993, 1994). Proteins in these organisms function normally at high temperatures. For example, enolase and α-glucosidase in hyperthermophilic *Pyrococcus furiousus* have optimum activity, at >90 and >105°C, respectively (Costantino et al., 1990; Peak et al., 1994). The most thermostable proteins found so far have half-lives in excess of 10 min at 130°C (Daniel et al., 1996). The mechanisms responsible for the high molecular stability of thermophilic proteins include increased hydrophobic interactions, greater molecular packing, more H-bonds, more salt-bridging, loss of surface loops, more helix-forming amino acids, restricted N-terminus mobility, etc. (Vieille and Zeikus, 1996; Cowan, 1997; Vogt and Argos, 1997). Extrinsic factors (not primary structure-related) have also contributed to protein stabilization. One of these is the high cellular content of sugars, salts, or other organic solutes/osmolytes, such as α-glutamate, di-myo-inositol-phosphate and its isomer, β-mannosylglycerate, and di-glycerol-phosphate (Huber et al., 1989; Rupley and Careri, 1991; Martins and Santos, 1995; Martins et al., 1997; Ramakrishnan et al., 1997).

The identification of intrinsic and extrinsic factors that contribute to the stabilization of thermophilic proteins has provided valuable information for stabilizing protein pharmaceuticals and for designing more stable mutant proteins. Yet the structural differences among different proteins are so significant that generalization of universal stabilization strategies has not been successful. Very often, proteins have to be evaluated individually and stabilized on a trial-and-error basis.

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To understand and maximize the stability of protein pharmaceuticals or any other usable proteins such as various catalytic enzymes, many studies have been conducted in the past few decades. These studies have been reviewed with emphasis on general protein stability (Jaenicke, 1991; Kristjánsson and Kinsella, 1991), mechanisms of chemical and physical instabilities of proteins (Manning et al., 1989), mechanisms and prevention of major protein degradation pathways (Cleland et al., 1993), and various means of stabilizing proteins in aqueous or solid state and under various processing conditions such as freeze-thawing or drying (Gianfreda and Scarfi, 1991; Arakawa et al., 1993; Timasheff, 1993; Manning et al., 1995; Wong and Parascrampuria, 1997). This article reviews these investigations and achievements in recent years and discusses the basic behavior of proteins, their instabilities, and stabilization in aqueous state in relation to the development of liquid protein pharmaceuticals.

2. Basic protein behavior and properties

Protein pharmaceuticals, unlike small drug molecules, have high molecular weight (> 5 kD). Their large size, compositional variety, and amphipathic characteristics constitute specific behavior such as folding, conformational stability, and unfolding/denaturation. Understanding proteins' basic behavior may help toward their stabilization.

2.1. Protein folding and its related forces and stability

Biologically active proteins are properly folded. The number of possible conformations of a folded polypeptide chain with an average domain size is about 10^{80} (Jaenicke, 1991). The three-dimensional folded state of a protein is a fluctuating state of a limited number of preferred conformations (Tang and Dill, 1998). The most stable (least energy) conformation of a protein is usually the native state (Darnell et al., 1986). Under native conditions the vast majority of protein molecules exist in their unique native state (N). A tiny fraction must also occupy all possible higher energy states as dictated by the Boltzmann relationship (Bai and Englander, 1996).

2.1.1. Protein Folding Process

Folding of newly-synthesized polypeptides in cells requires the assistance of so-called molecular chaperone proteins (Hendrick and Hartl, 1995). These proteins bind unfolded or partially folded polypeptides in their central cavity and promote folding by ATP-dependent cycles of release and rebinding. The molecular chaperone GroEL facilitates protein folding by preventing protein aggregation and correcting protein misfolding (Golbik et al., 1998). Protein folding is generally a highly cooperative process, in which only the native and unfolded states are stable (Goto and Fink, 1989).

When a protein folds, about 80% of nonpolar side chains (Ala, Val, Ile, Leu, Met, Phe, Trp, Cys) are buried in the interior of protein molecules out of contact with water. For example, folding of RNase T1, a compact globular protein (104 aa), removes about 85% of nonpolar residues from contact with water (Thomson et al., 1989). More than 80% of amino acids in globular proteins exist in α-helix/β-sheet or in the turns connecting them (Pace et al., 1996).

The rate of protein folding is usually high. Many small proteins can fold in milliseconds or less (Dobson and Hore, 1998). Some may fold at a slower rate. For example, the folding of a bacteria protein MerP (72 aa) by diluting the protein solution containing 3 M guanidine hydrochloride (GdnHCl) shows two exponential phases. The initial phase is fast with a rate constant of 1.2/s, but the second phase is slow with a rate constant of 0.053/s, accounting for about 20% of the folding signal (Aronsson et al., 1997). Folding of proteins involving Pro isomerization is also relatively slow.

2.1.2. Major forces involved in protein folding

Many forces are involved in protein folding. These include hydrophobic interactions, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding, intrinsic propensities, and van der Waals forces. Hydrophobic interactions are repulsive interactions between water and

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non-polar residues in proteins, leading to minimal hydration of the hydrophobic core. These interactions are strongly disfavored and associated with a large increase in heat capacity (Dill, 1990). A hydrogen bond is the strong dipole–dipole attraction between covalently-bonded hydrogen atoms and other strongly electronegative atoms such as oxygen and nitrogen. It is primarily a linear arrangement of donor, hydrogen, and acceptor. Hydrogen bonds between amide hydrogen and carbonyl oxygen make up 68% of the total hydrogen bonds in globular proteins (Pace et al., 1996).

Among all the forces involved in protein folding, the apparent dominant force is hydrophobic interaction (Jaenicke, 1990; Kristjánsson and Kinsella, 1991). The dominant opposing force is the loss of non-local conformational entropy (Dill et al., 1989; Dill, 1990). The difference in heat capacity of a protein in the native folded and denatured states (ΔC_p) is a measure of hydrophobic stabilization of the protein, and the large ΔC_p on thermal denaturation of proteins supports the major role of hydrophobic interactions in protein stabilization (Wang et al., 1996a). Recently, it was found that the contribution of electrostatic interactions in proteins at neutral conditions to the free energy difference between the folded and unfolded states is close to 0, indicating that the main driving forces for protein folding under these conditions are hydrophobic and hydrogen-bonding interactions (Dimitrov and Crichton, 1997).

Hydrophobic interaction, albeit dominant, needs to be balanced to maintain protein activity. In studying the stability of mutant Rop proteins, Munson et al. (1996) demonstrated that underpacking the hydrophobic core with small amino acids like Ala not only loses protein activity but also decreases protein stability. On the other hand, overpacking the hydrophobic core with only large amino acids like Leu stabilizes proteins but protein activity is lost. This indicates that both favorable steric interaction and burial of sufficient hydrophobic volume and surface area are important to stabilize a protein.

Protein molecular packing may be the most applicable factor that leads to the unique structures of most globular proteins (Richards, 1997). The packing in native proteins is so well arranged that all solvent molecules are essentially excluded and the protein interior is more like a crystalline solid than a non-polar liquid (Jaenicke, 1991). Glu, Lys, and Arg, with three or four rotatable bonds, are almost invariably located on the protein surface and use their flexibility to help ensure exposure of their charged groups to the solvent. However, buried polar residues do not necessarily destabilize a protein if they can form stable hydrogen bonds (Pace et al., 1996) and/or form stable intramolecular salt bridges, enhancing the structural rigidity (Vieille and Zeikus, 1996).

Interaction forces in proteins can also be divided into two types: local (short-ranged or secondary) and non-local (long-ranged or tertiary) (Chan and Dill, 1991). Long-range interactions happen between two residues that are separated by at least ten residues (Dosztányi et al., 1997). Long-range interactions such as parallel and antiparallel sheets are mainly responsible for polymer collapse to compact states whereas local forces are mainly responsible for helix formation such as hydrogen bonding. It seems that long-range interactions (non-local forces) control overall protein stability. The dominant role of long-range interactions in protein stability is supported by recent identification of so-called elements of stabilization center (SC) in proteins. By long-range interactions, these SCs, mainly consisting of hydrophobic residues that are less flexible, tend to connect more sheets to each other, and are primarily responsible for stabilization of protein structures (Dosztányi et al., 1997). If different proteins have similar structural folds, their conformational stability may still be very different due to the difference in their primary sequences (Chiti et al., 1998).

2.1.3. Protein folding and free energy change

The folded state of proteins has conformational stability, which is defined as the free energy change, $\Delta G_{f\rightarrow u}$ (or simply ΔG), for the unfolding/denaturation reaction under physiological conditions (Pace, 1990). The larger the $\Delta G_{f\rightarrow u}$, the more stable the protein. The folded state of proteins is only marginally more stable than the unfolded state since its $\Delta G_{f\rightarrow u}$ is small. The $\Delta G_{f\rightarrow u}$

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for proteins has been reported in the following ranges: 21–63 kJ/mol (Kristjánsson and Kinsella, 1991); 5–20 kcal/mol (Volkin and Klibanov, 1989); or 5–10 kcal/mol (Jaenicke, 1990; Pace et al., 1996).

The low $\Delta G_{f\rightarrow u}$ values indicate that conformational stability of a protein in aqueous solution is equivalent to a few H-bonds or ion pairs. A single hydrogen bond can lower the protein's free energy by 0.5–2 kcal/mol and an ion pair by 0.4–1.0 kcal/mol (Vogt and Argos, 1997). After analyzing more than a dozen mutant proteins, Pace et al. (1996) found that average free energy gain is 1.1–1.6 kcal/mol per hydrogen bond and 1.18 kcal/mol per $-CH_2-$ for hydrophobic effect. These values suggest significant contribution of hydrogen bonding to $\Delta G_{f\rightarrow u}$ relative to that from hydrophobic effect, however, if the polar groups must be buried to form intramolecular hydrogen bonds, the net gain is only about 0.6 kcal/mol per hydrogen bond. In comparison, the strength of a hydrogen bond in water is about 5 kcal/mol, and the energy of van der Waals interaction amounts to about 1 kcal/mol at 25°C (Darnell et al., 1986).

2.2. Protein unfolding/denaturation

In solution, the folded state of any protein is not infinitely stable (Shortle, 1996). It may unfold/denature into an inactive form—a process of protein conformational changes. These conformational changes are due to predominant intermolecular protein–solvent interactions over intramolecular interactions, which keeps the folded state (Jaenicke, 1990). On the other hand, solvent-induced inactivation of proteins may occur without disruption of protein conformation (tertiary structure) (Cowan, 1997).

2.2.1. Protein unfolding process

Protein unfolding can be described generally by a single transition step between the completely folded and unfolded states since any intermediate state is highly unstable and only exists in negligible amounts (Chan and Dill, 1991; Jaenicke, 1991). This is true at least for small globular proteins (Kristjánsson and Kinsella, 1991). Others may have more than one unfolding process such as human placental alkaline phosphatase (Hung and Chang, 1998).

The following equation describes the two-state model:

$$N\ (\text{native}) \rightleftharpoons U\ (\text{unfolded/denatured}) \Rightarrow A\ (\text{aggregated})$$

or

$$P_{\text{folded}} \rightleftharpoons P_{\text{unfolded}} \Rightarrow A.$$

For most proteins, the unfolded state (U) is insoluble and favors aggregation. Under certain conditions, a particular U state exists, which is highly compact and has significant amounts of residual secondary structures such as thermally-unfolded RNase T1 (Pancoska et al., 1996) and acid-unfolded apomyoglobin (Staniforth et al., 1998). This particular U state is termed molten globule (Goto and Fink, 1989; Shortle, 1996).

The N state may unfold reversibly or, depending on the condition, irreversibly to the U state. For example, unfolding of wild-type barnase and some of its mutants are reversible based on the repeatability of DSC endotherms or CD transitions on rescanning (Johnson et al., 1997). Thermal treatment of IFN-β-1a (Runkel et al., 1998) and β-galactosidase (Yoshioka et al., 1994a) causes irreversible denaturation of both proteins and eventually leads to formation of aggregates (A).

Proteins unfold locally and globally. Local and global unfolding occur concurrently and independently. Increasing denaturant concentration or temperature can selectively promote global unfolding because global unfolding exposes more surface and gives rise to higher chain entropy and enthalpy (at high temperatures) than local unfolding (Bai et al., 1994).

An important thermodynamic parameter in two-state transition is the change in heat capacity, ΔC_p. The large and positive ΔC_p observed in protein denaturation is due primarily to exposure of nonpolar groups (Pace et al., 1996; Johnson et al., 1997). The molar enthalpy of protein denaturation, ΔH, at low temperatures may be either positive or negative but increases markedly with temperature. Since the driving force for protein unfolding is the increased conformational entropy

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in aqueous solution, $\Delta S_{f\rightarrow u}$ is expected to be positive.

2.2.2. Protein unfolding/melting temperature

Proteins unfold above certain temperatures. During a thermal unfolding process, the temperature at which 50% of protein molecules are unfolded ($\Delta G = 0$ at this time) is defined as the unfolding (or melting/denaturation/transition) temperature (T_m). Thermal unfolding of a protein is usually endothermic. The T_m of many proteins have been determined (Table 1) and appear mostly in the range of 40–80°C. When the level of protein hydration decreases, T_m may increase sharply due to destabilization of the unfolded state (Rupley and Careri, 1991).

The higher the T_m, the greater the thermal resistance of a protein. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from hyperthermophilic bacterium *Thermotoga maritima* has a melting temperature of 109°C (Jaenicke, 1996). However, there is no particular relationship between T_m and protein stability as measured by $\Delta G_{f\rightarrow u}$ (Dill et al., 1989). For example, the T_m of serum albumin from different sources has the following order: human (59.7°C) > dog (59.5°C) > rabbit (57.8°C) > rat (57.6°C) > bovine (56.8°C), yet their temperatures of maximum stability (ΔG) are all similar at about 20°C (Kosa et al., 1998). The ΔGs of three SH3 domains of the Tec family of tyrosine kinases are below 12–16 kJ/mol, but their melting temperatures are relatively high between 69 and 80°C (Knapp et al., 1998).

A protein may have two or more melting temperatures, depending on the experimental conditions and analytical techniques used (Table 1). For example, Kosa et al. (1998) demonstrated that dog and rabbit albumin have one transition, but human, bovine, and rat albumin have two transitions, suggesting the presence of a stable intermediate. The apical domain (residues 191–376) of GroEL protein shows two reversible melting temperatures at 35 and 67°C by far UV CD, which are attributed to unfolding of the C-terminal helices and the domain core, respectively. Protein huPrP(90–231) has one melting temperature in the presence of GdnHCl at pH 5.0 and 7.2, but shows two transition temperatures at pH 3.6 and 4.0 with a stable unfolding intermediate (Swietnicki et al., 1997). By DSC, interleukin-1 receptor (IL-1R, 50 kD) shows two unfolding transitions near 48°C and 65°C, but after deconvolution, three melting transition peaks are identified, representing three different domains in the protein (Remmele et al., 1998).

Multimeric, chimeric, or modular proteins often have more than one melting temperature. For example, native recombinant human placental factor XIII (rFXIII) is a non-covalent dimer and each subunit consists of 3 thermolabile domains (56 kD, N-terminal) and two thermostable domains (24 kD, C-terminal). The intact protein melts in two distinct temperature regions by DSC: 69°C at pH 8.6, representing three thermolabile domains; and 90°C in 2 M GdnHCl, representing two thermostable domains (Kurochkin et al. 1995). The chimeric protein toxin sCD4(178)-PE40 (sCD4-PE40), consisting of HIV binding domains of the T-cell membrane protein (CD4) and the cytotoxic domains of *Pseudomonas* exotoxin A (PE-40), has two transition temperatures originating from both components. In addition, unfolding of the less stable PE-40 induces unfolding of the more stable CD4 component because the free form of CD4 denatures at a higher temperature (56°C) than that in the protein complex (46°C) at pH 6.5 (Davio et al., 1995).

2.3. Mesophilic versus thermophilic proteins

In describing basic properties of proteins, it is necessary to mention thermophilic proteins, a different class of proteins having much higher thermostability than that of mesophilic counterparts like those in humans. Mesophilic proteins usually retain their native structures over a narrow range of temperatures from about 5 to 50°C. For thermophilic proteins, the upper limit of thermostability is usually 20 to 30°C higher, corresponding to an increase in protein stability by 5–7 kcal/mol. A ΔG of this order may be achieved by 1 or 2 additional salt bridges inside the protein globule, several additional hydrogen bonds, or 7–10 additional CH_3- groups in the hydrophobic nucleus of the protein (Mozhaev and Martinek, 1984).

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Table 1
Melting temperatures (T_m) of proteins

Proteins	Protein solution compositions	Methods	T_m (°C)	References
AcP (98 aa)	0.4 mg/ml in 50 mM acetate, pH 5.5	CD (222 nm)	57	Chiti et al., 1998
HSA (66 kD)	20 μM in 100 mM phosphate, pH 7.4	Fluorescence (λ_{em} = 345 nm)	62	Farruggia et al., 1997
	88 μM in 100 mM phosphate, pH 7.4	DSC	63	Picó, 1995
	0.1 mM in 67 mM phosphate, pH 7.4	DSC	60	Kosa et al., 1998
BSF (48 kD)	2.5 mg/ml in 50 mM phosphate, pH 6	DSC	60	Wang et al., 1996a
	2.5 mg/ml in 50 mM phosphate, pH 7	DSC	54	Wang et al., 1996a
	2.5 mg/ml in 50 mM phosphate, pH 8	DSC	48	Wang et al., 1996a
α-Chymotrypsin	0.3 mg/ml in 10 mM phosphate, pH 6.0	UV (281 nm)	47	Lozano et al., 1997
	0.3 mg/ml in 10 mM phosphate, pH 7.0	UV (281 nm)	44	Lozano et al., 1997
	0.3 mg/ml in 10 mM phosphate, pH 8.0	UV (281 nm)	42	Lozano et al., 1997
Cytochrome c	2 mg/ml in 600 mM NaCl	DSC	77	Lo and Rahman, 1998
rhDNase	10 mg/ml in water, pH 6.8	DSC	67	Chan et al., 1996
Elastase	20 mg/ml in 10 mM acetate, pH 5.0	DSC	66	Chang et al., 1993
aFGF	100 μg/ml in PBS, pH 7.2	CD (228 nm)	45	Volkin et al., 1993
bFGF	1 mg/ml in phosphate-citrate-borate, pH 4	DSC	50	Wang et al., 1996b
	1 mg/ml in phosphate-citrate-borate, pH 9	DSC	64	Wang et al., 1996b
GA (82 kD)	1.8 mg/ml in 50 mM phosphate, pH 6	DSC	64	Wang et al., 1996a
	1.8 mg/ml in 50 mM phosphate, pH 7	DSC	62	Wang et al., 1996a
	1.8 mg/ml in 50 mM phosphate, pH 8	DSC	58	Wang et al., 1996a
rhGH (22 kD)	0.18 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	89	Bam et al., 1998
	1 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	81	Bam et al., 1998
	3 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	79	Bam et al., 1998
IgG (mouse)	3.3 mg/ml in 5 mM phosphate, pH 6.0	DSC	74	Vermeer et al., 1998
Recombinant IFN-β-1a	100 μg/ml in 100 mM Na_2HPO_4, 200 mM NaCl, pH 7.2	UV (280 nm)	67	Runkel et al. 1998
Recombinant IFN-β-1a, deglycosylated	100 μg/ml in 100 mM Na_2HPO_4, 200 mM NaCl, pH 7.2	UV (280 nm)	63	Runkel et al. 1998
Lysozyme	In 0.1 M NaCl, 0.1 M acetate, pH 5.4	CD	66	Shoichet et al., 1995
	In 50 mM citrate, pH 4.0	DSC	74	Liu and Sturtevant, 1996
	In 50 mM citrate and 1 M sucrose, pH 4.0	DSC	80	Liu and Sturtevant, 1996

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Table 1 (continued)

Proteins	Protein solution compositions	Methods	T_m (°C)	References
M-CSF	1.2 mg/ml in 20 mM PolyB buffer	DSC	87	Schrier et al., 1993
wt-hPAH (452 aa)	14-18 mg/ml in 20 mM HEPES buffer, pH 7.4	IR, peak ratio of 1619 to 1650 cm^{-1}	57	Chehin et al. 1998
RNase A	In 0.04 M glycine, pH 2.8	UV (287 nm)	41	Lin and Timasheff, 1996
	In 0.03 M MES, pH 5.8	UV (287 nm)	60	Lin and Timasheff, 1996
	In 0.03 potassium phosphate, pH 6.7	UV (287 nm)	64	Lin and Timasheff, 1996
	2 mg/ml in 600 mM NaCl	DSC	62	Lo and Rahman, 1998
	0.1 mg/ml in water	CD (222 nm)	65	Tsai et al., 1998a
	0.2 mg/ml in 20 mM citric acid, pH 2.3	CE	36	McIntosh et al., 1998
	0.2 mg/ml in 20 mM citric acid, pH 3.1	CE	49	McIntosh et al., 1998
	0.2 mg/ml in 20 mM citric acid, pH 3.1	CD (222 nm)	50	McIntosh et al., 1998
	About 2 mg/ml in 50 mM glycine, pH 2.8	DSC	48	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate, pH 6.0	DSC	65	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate and 1 M sucrose, pH 6.0	DSC	70	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate and 1 M glycine, pH 6.0	DSC	68	Liu and Sturtevant, 1996
RNsae H	25 μg/ml in 5 mM HEPES and 0.8 M GdnHCl, pH 8	CD (222 nm)	30	Goedken and Marqusee, 1998
	25 μg/ml in 5 mM HEPES, 0.8 M GdnHCl, and 1 mM $MnCl_2$, pH 8	CD (222 nm)	41	Goedken and Marqusee, 1998
wt-RNase T1	About 1 mg/ml in 30 mM MOPS, pH 7	Optical rotation (295 nm)	48	Shirley et al., 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	UV (286 nm)	48	Thomson et al. 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	CD (238 nm)	47	Thomson et al., 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	CD (284 nm)	48	Thomson et al., 1989
Thrombin	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 6.5	DSC	57	Boctor and Mehta, 1992
	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 7.9	DSC	54	Boctor and Mehta, 1992
	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 9.9	DSC	48	Boctor and Mehta, 1992
TP40 (40kD)	0.1mg/ml, pH 7.2	Fluorescence	42	Sanyal et al., 1996
	0.1mg/ml, pH 7.2	CD	55	Sanyal et al., 1996
	1 mg/ml, pH 7.2	DSC	48	Sanyal et al., 1996
Trypsin	1.5 mg/ml in HCl solution, pH 2.5	DSC	52	Boctor and Mehta, 1992
YEI (240 kD)	2 mg/ml in 50 mM phosphate, pH 6.0	DSC	63	Wang et al., 1996a
	2 mg/ml in 50 mM phosphate, pH 7.0	DSC	52	Wang et al., 1996a
	2 mg/ml in 50 mM phosphate, pH 8.0	DSC	48	Wang et al., 1996a

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There are seemingly two basic mechanisms responsible for enhanced thermal stability of thermophilic proteins. First, thermophilic proteins may possess structural characteristics different from those of mesophilic proteins. These structural characteristics include (1) increased hydrophobic interactions; (2) formation of extra hydrogen bonds and/or salt bridges; (3) more compact protein structures (may have crystal-like density); and (4) presence of few Cys, high content of Arg, and low content of Lys (Mozhaev and Martinek, 1984; Vieille and Zeikus, 1996; Vogt and Argos, 1997). The second mechanism of stabilization is favorable interactions of proteins with other cellular components or accumulated low-molecular-weight compounds/osmolytes as thermoprotectants. The interaction reduces the contact area of nonpolar fragments in proteins with water, leading to a decrease in free energy of the system and stabilization of proteins. These osmolytes include potassium salt, sugars, α-glutamate, cyclic 2, 3-diphosphoglycerate (DPG), 2-*O*-β-mannosylglycerate, di-glycerol-phosphate, and di-myo-inositol-1,1′(3,3′)-phosphate (Mozhaev and Martinek, 1984; Huber et al., 1989; Rupley and Careri, 1991; Martins and Santos, 1995; Martins et al., 1997).

Is there a major force that contributes the most to stability of thermophilic proteins? Many thermophilic proteins show good correlation between stability and high hydrophobicity as measured by the hydrophobic index, a ratio of the volumes of polar to nonpolar amino acids (Mozhaev and Martinek, 1984). Detailed analysis indicates that protein stability depends not on total content of hydrophobic amino acid residues but on those inside the protein. Recent findings suggest that hydrogen bonding may play a major role in stabilizing thermophilic proteins. By comparing a group of thermostable proteins, Vogt and Argos (1997) recently concluded that increasing hydrogen bonding density at the protein surface is a major factor for increased thermal stability. After analyzing 16 protein families containing a total of 56 proteins from thermophilic, mesophilic, and thermophobic sources, Vogt et al. (1997) found that hydrogen bonding can provide the most general explanation for thermal stability in proteins because over 80% of the protein families show correlation between thermostability and an increase in the number of hydrogen bonds.

Secondary structure also contributes to enhanced stability of thermophilic proteins (Vogt and Argos, 1997). The α-helix and loops/turns are stabilized in thermophilic proteins. Ala residues (with high helix propensity) are prevalent in helical structures, and loops are often shorter in thermozymes (Vieille and Zeikus, 1996). Two facets of intrahelical interactions—the intrinsic helical propensities of amino acids and side chain interactions, are found to be the main contributors to protein thermostability, and an enhanced total stability of α-helices is a general feature of many thermophilic proteins (Petukhov et al., 1997).

3. Protein instability and its influencing factors and analytical monitoring

One of the most troubling and challenging tasks in the development of liquid protein pharmaceuticals is to deal with their physical and chemical instabilities. The most common physical instability is protein aggregation, which can be induced and/or affected by a variety of factors and chemical transformations. Careful examination of these stability-influencing factors may help to prevent or mitigate certain stability problems. In addition, selection of proper and adequate analytical methods for efficient and accurate monitoring of protein instability may ensure successful development of quality protein products.

3.1. Protein aggregation—a major event of physical instability

Under certain conditions (or simply with time), the secondary, tertiary, and quaternary structure of a protein may change and lead to protein unfolding and/or aggregation, a major event of physical instability. Protein aggregates may have no or reduced activity, reduced solubility, and altered immunogenicity. Presence of any insoluble aggregates in a protein pharmaceutical is generally not acceptable for product release.

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3.1.1. Mechanisms of protein aggregation

Protein aggregation in many cases results from intermolecular association of partially denatured protein chains (Fields et al., 1992). Recent evidence suggests that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific coaggregation (Speed et al., 1996). The aggregation process can be roughly divided into three steps: initiation, propagation, and termination (Roefs and De Kruif, 1994).

Proteins aggregate to minimize thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues of proteins. Hydrophobic interaction, the reluctance of nonpolar groups to be exposed to water, is considered to be the major driving force for both protein folding and aggregation. Both protein aggregation and folding represent a balance of exposed and buried hydrophobic surface areas (Patro and Przybycien, 1996). The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior (Fields et al., 1992).

Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, vortexing, surface/interface adsorption, etc. These factors can increase the hydrophobic surface area of proteins, causing aggregation. For example, recombinant human keratinocyte growth factor (rhKGF) undergoes slow unfolding at elevated temperatures, leading to immediate aggregation and precipitation (Chen et al., 1994a). Recombinant porcine growth hormone (pGH) at 0.5 mg/ml precipitates with time at 63°C (Charman et al., 1993). Vortexing hGH (22 kD) solutions (0.5 mg/ml at pH 7.4) for 1 min denature 67% as insoluble aggregates (Katakam et al., 1995). These influencing factors will be discussed in detail in Section 3.3.

Protein aggregation may result from chemical degradations or modifications and subsequent exposure of the hydrophobic surface(s). Proteins can directly form covalent aggregates such as insulin (Strickley and Anderson, 1997) or aggregate indirectly such as human relaxin after oxidation of His and Met residues (Li et al., 1995a). Both physical and chemical aggregations may occur simultaneously. For example, bFGF in citrate buffer (pH 5) forms intact and truncated dimers and trimers after storage at 25°C for 7 weeks (Shahrokh et al., 1994a). Freeze-dried β-galactosidase aggregates and forms insoluble precipitates via covalent disulfide bonding during storage, while non-covalent interaction causes aggregation but forms soluble precipitates in solution (Yoshioka et al., 1993). Protein aggregates, like proteins, may have isomeric forms such as IL-1ra dimers (Chang et al., 1996a).

Protein aggregation may start from a single protein molecule (unimolecular/intramolecular process) or more than one protein molecule (multimolecular/intermolecular process). Unimolecular processes include β-elimination and intrachain disulfide scrambling or formations such as IL-1ra in aqueous solution (Chang et al., 1996a). Examples of multimolecular aggregation include thiol-disulfide interchange for bovine serum albumin, thiol-catalyzed disulfide exchange for insulin, other covalent aggregation such as ribonuclease A, and non-covalent aggregation such as tetanus toxoid (Costantino et al., 1994a). If the percentage of precipitates/aggregates increases with increasing protein concentrations, multimolecular aggregation/precipitation processes may be involved such as aggregation of hIGF-I (Charman et al., 1993; Fransson et al., 1996).

Protein aggregation may or may not follow first-order kinetics (Pikal et al., 1991). The formation of IL-1ra dimers in aqueous solution during storage follows first-order kinetics (Chang et al., 1996a). Both heat and denaturant-induced aggregations of recombinant human interferon-γ (IFN-γ) apparently follow first-order kinetics (Kendrick et al., 1998a,b). At 60°C, the aggregation/precipitation of IL-1β follows apparent first-order behavior to 30% drug remaining, but at or below 55°C, it deviates and becomes biphasic (slow and fast) (Gu et al., 1991). Biphasic aggregation has also been observed for aFGF in phosphate buffer (pH 7.4) at 50°C.

Many agents can be used to probe possible mechanisms of protein aggregation. Denaturants such as sodium dodecyl sulfate (SDS), GdnHCl, and urea can be used to determine whether protein aggregation is covalent in nature. If

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protein aggregates can be dissolved in these agents, formation of these aggregates is probably non-covalent. Otherwise, covalent/chemical aggregation is suggested. Examples of non-covalent aggregation evidenced by this method include chymotrypsinogen (Allison et al., 1996), insulin (Sluzky et al., 1991), and IL-1β (Gu et al., 1991). Similarly, reducing agents such as DTT or β-mercaptoethanol can be used to determine any involvement of disulfide bonds in protein aggregation. Since these reducing agents may have limited dissolution capability, denaturants are often included for complete dissolution of protein aggregates.

Nevertheless, there are cases in which experimental results may lead to inaccurate or even incorrect interpretation. For example, the insoluble aggregates of hGH can be completely dissolved in either 1 or 2% SDS. SEC-HPLC analysis of the solubilized aggregates indicates presence of both protein monomers and dimers in 1% SDS, suggesting possible covalent aggregation, but only monomers are found in 2% SDS, suggesting non-covalent aggregation (Katakam et al., 1995). This example illustrates that denaturants may not be able to dissolve non-covalent protein aggregates depending on their concentrations. As a matter of fact, non-covalent insulin fibrils cannot be dissolved in 7 M urea from pH 2 to 8, 5 M GdnHCl, 50% acetonitrile, or detergents (Brange et al., 1997). The failure of these agents to dissolve insulin fibrils suggests that these substances do not have access to the interfaces of insulin molecules due to strong hydrophobic interactions in fibrils.

Protein aggregation may be probed by computer simulation. Agitation or hydrophobic surface-induced aggregation of insulin has been modeled by computer simulation and the model predicts rather reliably the effect of protein concentration, agitation rate, and hydrophobic surface area on insulin aggregation (Sluzky et al., 1991, 1992). Recently, the colloid aggregation process has been simulated by a cluster–cluster aggregation (CCA) process. The heat-induced aggregation of BSA at pH 7.0 seems to fit the reaction-limited CCA model; at pH 5.1, it fits diffusion-limited CCA model (Hagiwara et al., 1996).

3.1.2. Reversibility of Protein Aggregation

Protein aggregation can be reversible or irreversible. Generally speaking, aggregation is irreversible if the aggregates cannot be resolubilized in denaturing and reducing agents. The aggregation and precipitation of apomyoglobin formed in 0.5–4 M urea is reversible, as the precipitates can be dissolved in 8 M urea and further dilution leads to protein refolding (De Young et al., 1993). Thermally-induced protein aggregation is often irreversible. Thrombin in solution aggregates and loses its clotting activity irreversibly when heated to 85°C (Boctor and Mehta, 1992). Thermally-induced precipitation of recombinant pGH is irreversible (Charman et al., 1993).

What determines reversibility of protein aggregation? Patro and Przybycien (1996) simulated the structure of reversible protein aggregates as a function of protein surface characteristics and protein–protein interactions. They demonstrated that the structure and morphology of protein aggregates are profoundly affected by the surface characteristics of protein monomers. Both the extent and distribution of hydrophilic and hydrophobic sites on protein surface affect aggregate properties kinetically and thermodynamically. Although physical properties of reversible and irreversible aggregates are similar, reversible aggregates are more stable energetically, more ordered (or crystal-like), and less dense than irreversible counterparts. An increase in the extent of monomer hydrophobic surface area can result in decreased solvent-exposed hydrophobic surface area and lower free energy, forming preferably reversible aggregates.

3.1.3. Activity of protein aggregates

Protein aggregates usually have no or reduced activity. Bovine pancreas ribonuclease A (RNase A, 13.7 kD) at 1.5 mg/ml forms soluble aggregates in 0.1 M phosphate buffer (pH 10) during storage at 45°C, and the amount of aggregates corresponds qualitatively to loss of enzyme activity (Townsend and DeLuca, 1990). The specific antiviral activity of interferon-β-1b (IFN-β-1b) aggregates is three times lower than that of the intact protein (Runkel et al., 1998).

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Table 2
Favored pH conditions for some degradation reactions in proteins/peptides

Degradations	Proteins/peptides	Reaction sites	Favored pH conditions	References
Cleavage	bFGF	Asp-X, esp.X = Pro	Very acidic	Shahrokh, et al., 1994a
Deamidation	hEGF	Asn^{1}	Neatral to alkaline	Son and Kwon, 1995
Deamidation	bFGF	Asn-X	Neutral to alkaline	Shahrokh, et al., 1994a
Deamidation	Insulin	Asn^{A21}	pH < 5	Strickley and Anderson, 1997
Deamidation	RNase A Lysozyme	Asn-X, Gln-X	High pHs	Kristjánsson and Kinsella, 1991
Oxidation (by H_2O_2)	rhPTH	Met^{8} and Met^{18}	10	Nabuchi et al., 1995
Oxidation (by ascorbic acid/$CuCl_2$ /O_2)	Relaxin	His^{A12}, Met^{B4}, Met^{B25}, etc.	5 > 6 > 7 > 8	Li et al., 1995a
Oxidation (by ascorbic acid/$FeCl_3$ /O_2)	His-Met	Met	7–8	Li et al., 1993
Succinimidation	bFGF	Asx-Gly	4–5	Shahrokh et al., 1994a

Protein aggregation may or may not accompany major conformational changes. Interleukin-1 receptor antagonist (IL-1ra) in solution can form non-covalent but irreversible dimers during storage, and the activity of the non-covalent dimer is about one-third of the native monomer, even though the dimer has almost the same structure as the native form by CD and IR spectroscopy (Chang et al., 1996a).

3.2. Chemical instabilities of proteins

Many chemical reactions are responsible for inactivation of protein drugs. In many cases, a couple of reactions can happen simultaneously in proteins, making separation and identification of protein degradation products very difficult. For example, bFGF undergoes multiple degradations in solution, including multimerization, succinimidation, hydrolysis, and aggregation (Wang et al., 1996b). Lyophilized insulin, after forming a cyclic anhydride intermediate at low pH, may react with water to form desamido insulin or react with other amino acids to form two different dimers (Asp^{A21}-Phe^{B1} and Asp^{A21}-Gly^{A1}) (Strickley and Anderson, 1997). The eventual degradation product distribution (between desamido insulin or covalent dimers) may depend on water content of the formulation, pH of the reconstituted solution, and nature of the excipients in the formulation. Many proteins show changes in degradation pathways at different pHs and temperatures. The degradation products (aggregates and C-terminal fragments) of recombinant human macrophage colony-stimulating factor (M-CSF) change significantly at different pHs (Schrier et al., 1993). Recombinant human thrombopoietin (rhTPO) shows different shapes of k_{obs}-pH profiles and different patterns of bands on SDS-PAGE at 5, 30, and 37°C (Senderoff et al., 1996). Therefore, to prevent proteins from chemical inactivation, the dominant reaction should first be identified and inhibited. This can be achieved to a certain degree by adjusting the formulation pH away from favorable ranges (Table 2).

The location of labile amino acids in a protein is critical in determining their chemical reactivity. Since a very limited number of water molecules exist inside a protein, presence of chemically labile amino acids may not be a problem if they are localized inside and have limited mobility (Mozhaev and Martinek, 1984). Chemical reactions of many amino acids in proteins require certain local molecular flexibility; and thus the rate of reaction may be higher in denatured proteins or small peptides with high flexibility

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than in native proteins. For example, the reactivity (disulfide exchange) of the sulphydryl group in β-lactoglobulin-A is very slow in its native state but increases significantly when the protein gradually unfolds with increasing urea concentration from 4 to 8 M (Apenten, 1998). Native protein conformation, therefore, needs to be protected to prevent or inhibit potential chemical degradations.

Powell (1996) tried to correlate the reactivity of protein deamidation, hydrolysis, and oxidation in aqueous solutions with protein sequence structure by analyzing the hydropathy/flexibility (hydroflex) plots for 73 proteins. The analysis shows that the primary reaction in proteins at pH 4.5–7.5 occurs largely at Asn and Asp within these motifs: -Asn-Gly-, -Asn-Ser-, -Asp-Gly-, and, to a lesser extent, -Gln-Gly-, -Asp-Pro-, and -Met- (the hot-spot sites). Reactions at non-hot-spot sites can also occur if the site conformation is favorable (hydrophilicity and flexibility) or if the reaction is catalyzed by contaminating proteases. Hydropathy appears to be a better predictor of protein degradation than does calculated flexibility.

Chemical reactions may not always affect protein conformation or activity depending on the location of transformed residues. Due to the terminal location of Met^{B4} and Met^{B25} in recombinant human relaxin, oxidation at these two residues does not change the protein bioactivity (Nguyen et al., 1993). The Met^{1} mono-oxidized recombinant human leptin (16 kD) does not show any detectable changes in tertiary structure and retains its full potency as compared to native form (Liu et al., 1998). Similarly, when 63% of human epidermal growth factor (hEGF, 6 kD) is deamidated, its mitogenic activity is completely unchanged, indicating that the deamidation site is not critical in determining the mitogenic activity. Other deamidated products having essentially the same biological activity as the intact proteins include insulin (Brange et al., 1992b) and rIL-2 (Sasaoki et al., 1992). In some cases, degraded proteins may have even higher activities such as rhTPO during storage at 5, 30, and 37°C (Senderoff et al., 1996).

3.2.1. Deamidation

Deamidation appears to be the most common degradation in protein pharmaceuticals. In many cases, it is a major degradation pathway in proteins, such as recombinant human deoxyribonuclease (rhDNase) (Shire, 1996; Chen et al., 1998) and rhVEGF at pH 5–6 (Goolcharran et al., 1998). The deamidated rhDNase has only about 40–50% of the original activity.

Several deamidation mechanisms have been reported and discussed (Cleland et al., 1993; Xie and Schowen, 1999). Asn and Gln are the two amino acids susceptible to deamidation in proteins and Asn is much more labile (Powell, 1994; Li et al., 1995b; Daniel et al., 1996). Deamidation of Asn in proteins and peptides in an aqueous solution can proceed at a much higher rate than hydrolysis of a peptide bond (Daniel et al., 1996).

The rate, mechanism, and location of deamidation in peptides or proteins are pH-dependent. Deamidation of Asn-X appears favored mostly at neutral or alkaline conditions (Son and Kwon, 1995; Daniel et al., 1996). Maximum stability of Asn residues within peptides is found between pH 2-5. Between pH 5 and 12, the reaction proceeds rapidly and entirely through a cyclic imide (succinimide) intermediate, while slow deamidation at pH 1–2 seems to bypass the succinimide intermediate (Daniel et al., 1996). The rate of deamidation at Asn^{74} in bovine pancreatic DNase increases with increasing pH from 5.0 to 7.7 (Shire, 1996). In insulin, however, deamidation originates from a rate-limiting intramolecular nucleophilic attack at the C-terminal Asn^{A-21} with a cyclic anhydride intermediate to form desamido insulin (Brange et al., 1992b; Darrington and Anderson, 1995; Strickley and Anderson, 1997). Since the unionized carboxyl group at the C terminal is the catalyzing group, increasing solution pH inhibits deamidation of Asn^{A-21}. Therefore, the reaction is favored at low pH (< 5). In neutral solutions, deamidation in insulin takes place predominantly at Asn^{B-3} residue (Brange et al., 1992b; Darrington and Anderson, 1995).

The relative position of Asn and/or Gln in proteins may determine their relative rate of deamidation. hEGF has three deamidation

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residues (Asn^1, Asn^{32}, and Gln^{43}) and Asn^1 is the most labile one in neutral and alkaline pH due to its relatively high mobility in the protein (Son and Kwon, 1995). In tPA, only three Asn-X sequences (Asn^{58}-Gly^{59}, Asn^{77}-Ser^{178}, and Asn^{37} -Ser^{38}) are deamidated; and all three sites are located on the protein surface and have more-than-average local sequence mobility (Paranandi et al., 1994).

Neighboring amino acids at deamidation sites in peptides or proteins may affect the rate of deamidation. Cross and Schirch (1991) have studied the rate of non-enzymatic deamidation of a series of pentapeptides. They have found that in neutral and alkaline solutions, amino acid residues on the amino side of Asn have little or no effect on the rate of deamidation regardless of its charge or size, but residues on the carboxyl side of Asn affect the rate significantly. Increasing the size and branching in the side chain of the residue decreases the rate of deamidation as much as 70-fold relative to Gly. Therefore, the most labile sequence seems to be Asn-Gly (Powell, 1994, 1996). However, under acidic conditions, the rate of deamidation of Asn residue is not affected by neighboring amino acids in these pentapeptides (Cross and Schirch, 1991). In tPA, 7 Asn-X sequences are identified to have more-than-average local sequence mobility, but only three of them show detectable deamidation, suggesting possible influence of neighboring amino acids on the rate of deamidation in addition to local flexibility (Paranandi et al., 1994). Local flexibility/mobility of proteins can be plotted for comparison based on hydrophobicity–volume product for consecutive quintuplets of amino acid residues (Ragone et al., 1989).

The rate of deamidation in proteins is also influenced by secondary structure of proteins. Xie and Schowen (1999) recently reviewed secondary structure and protein deamidation and indicated that both α-helical and β-turn secondary structures tend to stabilize Asn residues against deamidation. A good example is the deamidation of Asn^8 in several growth hormone releasing factor (GRF) analogues, which decreases with increasing helical content in aqueous methanol solutions (Stevenson et al., 1993).

3.2.2. Oxidation

The side chains of His, Met, Cys, Trp, and Tyr residues are potential sites of oxidation (Manning et al., 1989; Li et al., 1995b,c; Daniel et al., 1996). Oxidation at these sites can be catalyzed by trace amount of transition metal ions (site-specific process) or enhanced by oxidants or upon exposure to light (non-site-specific process). The site specificity is due to generation of and oxidation by reactive oxygen species at specific metal-binding sites (Li et al., 1995a; Zhao et al., 1997). The complicated oxidation mechanisms and related major oxidation products have been reviewed elsewhere (Cleland et al., 1993; Stadtman, 1993; Li et al., 1995c).

The most easily oxidizable sites are thio groups on Met and Cys. Met residues in proteins can be easily oxidized by atmospheric oxygen such as hGH in a vial containing only 0.4% oxygen. The easy oxidation of Met residue occurs probably because Met (also Gln) is one of the most flexible of nonpolar or mildly polar residues in proteins (Richards, 1997). Nevertheless, it was concluded that oxidation at Met residues in most proteins is not as significant as deamidation and hydrolysis (Powell, 1996).

The thioether group of Met can be oxidized into two possible forms: sulfoxide and sulfone. Met is first oxidized reversibly to sulfoxide, which can be further oxidized irreversibly to sulfone under harsh conditions. Like deamidation, the rate of oxidation depends on the position of oxidizable groups in a protein. For example, Met sulfoxides can form at both Met^{18} and Met^8 in recombinant human parathyroid hormone (rhPTH) in the presence of H_2O_2, but Met^{18} is more susceptible to oxidation (Nabuchi et al., 1995). There are four oxidizable Met residues in recombinant human leptin, and their relative reactivity is $Met^1 > Met^{69} > > Met^{55} \cong Met^{137}$ (Liu et al., 1998).

The formulation pH may affect the rate of oxidation by changing the oxidation potential of oxidants, the affinity of binding between catalytic metal ions and the ionizable amino acids, and the stability of oxidation intermediates. In a few cases, the rate of oxidation is enhanced at alkaline pHs. rhPTH is oxidized most at pH 10 by H_2O_2

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(Nabuchi et al., 1995). The relative rate of oxidation of human relaxin in ascorbate/Cu (II) solution is pH $8 > 7 > 6 > 5$ (Li et al., 1995a). The oxidation rate of human insulin-like growth factor I (hIGF-I, 7.65 kD) in solution increases as the solution pH is increased from 6.0 to 7.5. The pH dependence of hIGF-I oxidation was attributed to formation of a phosphorylated sulfonium ion between Met and phosphate as an oxidation intermediate (Fransson and Hagman, 1996). In a different case, changing the solution pH in the range of 3–8 fails to change significantly the rate of oxidation of recombinant human relaxin by H_2O_2 (Nguyen et al., 1993).

Oxidation could dominate other degradation pathways in a protein. There are several potential degradation reactions in hIGF-I, including oxidation of Met^{59}, deamidation of Asn^{26} and Gln^{15}, and reduction of three disulfide bridges (Cys^{6}-Cys^{48}, Cys^{47}-Cys^{52}, and Cys^{18}-Cys^{61}) and oxidation of Met is found to be the major degradation pathway (Fransson et al., 1996). Similarly, oxidation is the major degradation pathway for KGF-2 (Kaushal et al., 1998).

3.2.3. Disulfide bond breakage and formation

Disulfide bonds are often critical in controlling both protein activity and stability. α-Interferon has two disulfide crosslinks between 1 and 98 and between 29 and 138. Selective breaking of the 1–98 crosslink results in no loss of activity, while breaking of the other results in almost complete loss of activity (Evans and Grassam, 1986). Ribonuclease T1 has two disulfide bonds, which stabilize the protein. When the two disulfide bonds are broken, its T_m is reduced by about 40°C and its conformational stability is decreased by over 9 kcal/mol (Thomson et al., 1989).

Free Cys residues in proteins can be oxidized easily to form disulfide bond linkages or cause thio-disulfide exchanges, causing protein aggregation or polymerization, such as basic fibroblast growth factor (bFGF) (Shahrokh, et al. 1994a; Wang et al., 1996b). However, if these cysteinyl residues are buried within the tertiary structure of proteins, they are much less reactive without much concern. Thio-disulfide exchange in a protein is a reaction between an ionized thiol group (thiolate anion) and a disulfide bond. The rate of thiol-disulfide exchange depends on the extent of ionization of the nucleophilic thiol, and therefore, generally increases as the reaction pH increases until pK of the nucleophilic thiol group is exceeded (Darby and Creighton, 1997).

Even though a protein does not have free Cys residues, disulfide bond scrambling may still occur, causing protein aggregation. This is the case for lyophilized insulin, which has only three disulfide bonds but still forms disulfide-scrambled aggregates via β-elimination of an intact disulfide during storage (Costantino et al., 1994b). β-Elimination was also observed for IL-1ra in aqueous solution (Chang et al., 1996a).

3.2.4. Hydrolysis

Amino acids, the components of proteins, are subject to acid and base hydrolysis. Most peptide bonds are stable except those in -X-Asp-Y- sequence (Manning et al. 1989; Li et al., 1995b; Vieille and Zeikus, 1996). The Asp-Y bond may be at least 100 times more labile than other peptide bonds in dilute acid (Li et al., 1995b). Cleavage is particularly rapid at Asp-Gly and Asp-Pro (Powell, 1994, 1996). A major degradation pathway of rhM-CSF in an acidic solution is peptide cleavage at two sites: Asp^{169}-Pro^{170} and Asp^{213}-Pro^{214} (Schrier et al., 1993). During hydrolysis, Asp forms succinimide intermediate, which is similar to that for Asn (Daniel et al., 1996). In addition, formation of cyclic anhydride intermediate is also possible, especially when the C-flanking residue of Asp is Pro such as bFGF (Shahrokh et al., 1994a).

In many cases, hydrolysis is a continuation after deamidation of Asn residues. Insulin first forms *iso*-Asp and Asp derivatives at Asn^{A-21} and/or Asn^{B-3} depending on the solution pH during storage. These derivatives are further hydrolyzed and the rate of hydrolysis at B3 position is independent of the insulin strength between 40 and 400 IU/ml (Brange et al., 1992b).

3.2.5. Isomerization

All amino acids have the potential of racemization except Gly. Natural or deamidation-derived

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Asp-X peptide bonds can easily undergo a reversible isomerization between Asp and *iso*-Asp via a cyclic imide (succinimide) intermediate. The succinimide intermediate is usually not stable, and significant hydrolysis may occur in hours such as tissue plasminogen activator (tPA) (Paranandi et al., 1994).

Many cases of protein isomerization have been reported, such as bFGF (Shahrokh et al., 1994a), insulin (Brange et al., 1992b), and tPA (Paranandi et al., 1994). Incubation of tPA at pH 7.3 and 37°C for 14 days can result in formation of 0.77 mol of *iso*-aspartate derivative per mol of tPA due to multiple deamidations at Asn^{58}-Gly^{59}, Asn^{177}-Ser^{178}, and Asn^{37} -Ser^{38} (Paranandi et al., 1994).

Like deamidation, the rate of Asp isomerization is strongly influenced by its location and mobility in a protein. The *iso*-aspartate formation is most likely to occur in relatively unstructured domains of intact proteins or in domains susceptible to transient unfolding (Johnson et al., 1989). Methionyl recombinant human growth hormone (met-hGH) at pH 7.4 forms isoaspartate derivatives only at Asp^{130} (Asp-Gly) and Asn^{149} (Asn-Ser) during storage at 37°C, even though a couple of Asn and Asp residues exist in the protein. The selective *iso*-aspartate formation at Asp^{130} occurs because Asp^{130} resides in a domain, which has conformational flexibility similar to that of a short synthetic peptide (Johnson et al., 1989).

3.2.6. Succinimidation

Formation of succinimide intermediates may precede deamidation of Asn and isomerization of Asp in proteins and peptides. In fact, formation of succinimide is the cause of *iso*-aspartate derivative formation in proteins. Asn deamidates via succinimide formation at neutral and alkaline conditions, but formation of succinimide at Asp-Gly linkages in proteins may occur at an optimum pH of 4–5, such as bFGF (Shahrokh et al., 1994a).

Like other chemical degradations, the rate of succinimide formation is strongly influenced by neighboring groups of the labile residue(s) and protein conformation. Stephenson and Clarke (1989) studied the rate of succinimide formation in a series of synthetic peptides (Val-Tyr-Pro-X-Y-Ala) at pH 7.4. They demonstrated that the rate of succinimide formation of the asparaginyl (X) peptides is 13.1–35.6 times faster than those of the aspartyl peptides when Y is glycyl, seryl, or alanyl residue. The relative rate of succinimide formation is glycyl > seryl > alanyl peptides. Recently, Gietz et al. (1998) identified two degradation products in hirudin, which are succinimides at Asp^{53} and Asp^{33}. Both of these two sites are located in two flexible segments of the molecule.

3.2.7. Non-disulfide crosslinking

Proteins may form covalent dimers or polymers by non-disulfide pathways. Insulin has been shown to form such dimers and polymers during storage (Brange et al., 1992a). The rate-limiting generation of a cyclic anhydride intermediate is involved in formation of both dimeric and deamidated insulin (Darrington and Anderson, 1995). The covalent insulin dimer appears to form mainly through transamidation reactions involving Asn^{A-21} and Phe^{B-1} residues. The relative amounts of the two insulin reaction products (deamidated and dimeric insulin) changes with pH between 2.0 and 5.5. Increasing the pH decreases the formation of [desamido $^{A-21}$] insulin with a concurrent increase in desamido^{A-21}-Phe^{B-1} dimer. The formation of insulin dimer is negligible below pH 4 at insulin concentration of about 10 μM, increases sharply with increasing pH, and becomes dominant near pH 5. Recently, it has been found that the C-terminal Asn in insulin, after forming a cyclic anhydride intermediate, may form two different dimers with either Phe (Asp^{A21}-Phe^{B1}) or Gly (Asp^{A21}-Gly^{A1}) in both aqueous and lyophilized formulations (Strickley and Anderson, 1997).

Another non-disulfide pathway is the formaldehyde-mediated crosslinking proposed by Schwendeman et al. (1995). This crosslinking pathway apparently causes significant aggregation of lyophilized tetanus and diphtheria toxoids during storage and thus raises concerns for storing formalin-treated vaccines in both liquid and solid forms.

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3.2.8. Deglycosylation

One of the functions of carbohydrate moieties in proteins is to protect proteins from thermal and hydrolytic inactivation (Lis and Sharon, 1993). The effect of glycosylation on stability of proteins is highly protein-dependent. Wang et al. (1996a) studied the effect of deglycosylation on the stability of five glycoproteins, including yeast external invertase, bovine serum fetuin, glucoamylase from *Aspergillus niger*, chicken egg white ovotransferrin, and avidin. The amounts of saccharides in these proteins vary from 2.2 to 50%. Deglycosylation of the five model glycoproteins decreases the protein stability as evidenced by decreased T_m and denaturation enthalpy (ΔH), even though protein conformations are not significantly affected.

Natural IFN-β is a 166 amino acid glycoprotein and has multiple functions. Runkel et al. (1998) demonstrated that deglycosylation of recombinant IFN-β-1a with PNGase F decreases the protein activity, and the loss of activity is primarily due to formation of insoluble aggregates upon carbohydrate removal. Deglycosylation also makes the protein more sensitive to thermal denaturation. At a protein concentration of 100 μg/ml, deglycosylated IFN-β-1a denatures at 63°C while glycosylated IFN-β-1a denatures at 67°C. Similarly, RNase A at 85 μM in 50 mM Tris–HCl buffer (pH 8.0) unfolds at 60.4°C while RNase B, a carbohydrated derivative at Asn^{34}, unfolds at 61.9°C under the same condition (Arnold and Ulbrich-Hofmann, 1997).

3.2.9. Maillard reaction

Sugars are often used as protein stabilizers in both liquid and solid formulations (see Section 4.2). Unfortunately, reducing sugars may react with amino groups in proteins forming carbohydrate adduct, especially at high temperatures. This extremely complex browning pathway is known as the Maillard reaction (Paulsen and Pflughaupt, 1980).

Maillard reactions are widely present in the food industry (Chuyen, 1998). Thermal treatment of milk easily leads to formation of β-lactoglobulin-lactose adduct via Lys residues (Fogliano et al., 1998). Although there have not been many reports on Maillard reactions in the development of liquid protein pharmaceuticals, this reaction may present potential formulation issues. Tarelli et al. (1994) demonstrated that lysine vasopressin undergoes rapid glycation in the presence of reducing sugars in both aqueous and solid formulations and that the N-terminal adduct can form rapidly even at −20°C.

3.3. Factors affecting protein stability

Protein stability is a result of balancing between destabilizing and stabilizing forces. The destabilizing forces are mainly due to the large increase in entropy of unfolding, and the stabilizing forces are provided by a few non-covalent interactions as discussed in Section 2.1. Disruption of any of these interactions will shift the balance and destabilize a protein. Many factors can disrupt this delicate balance.

3.3.1. Temperature

The most important factor affecting protein stability is temperature. Unfortunately, there is no general mechanism to describe the effect of temperature on the structure and function of proteins due to their structural complications. In general, the higher the temperature, the lower the protein stability.

Proteins are usually stable in a certain temperature range. Several globular proteins, including basic pancreatic trypsin inhibitor, α-chymotrypsin, cytochrome c, lysozyme, metmyoglobin, RNase A, and trypsin, have maximum free energy change $\Delta G_{f\rightarrow u}$ (8–17 kcal/mol) in a temperature range between 10 and 40°C, indicating that both high and low temperatures outside this range may destabilize or denature the proteins (Jaenicke, 1991). Under 3000 bar (to prevent freezing), RNase undergoes both cold (−22°C) and heat (40°C) denaturation (Zhang et al., 1995). Similarly, two extreme denaturation temperatures, lower than 0°C and higher than 50°C, exist for serum albumin in solution (Kosa et al., 1998).

Thermodynamically, protein stability comes from two large but opposing forces: enthalpic and entropic forces. Both are temperature-dependent. The enthalpic forces (the intramolecular interac-

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tions) are stabilizing, and the entropic forces (the dissipative forces, loss of conformational entropy of native state) are destabilizing (Kristjánsson and Kinsella, 1991). High temperature-induced loss of protein stability ($\Delta\Delta G < 0$) seems to result from a decrease in free energy of the denatured state instead of an increase in free energy of the native state (Shortle, 1996). The free energy of the denatured state decreases rapidly with increasing temperature mainly due to increased entropy ($-T\Delta S$). When temperature increases, electrostatic interactions are not affected much, hydrogen bonding is weakened, and hydrophobic interactions are strengthened (to a certain point) due to its entropic origin (Jaenicke, 1990). Hydrophobic interaction increases until the temperature reaches about 110°C, when water does not solvate non-polar groups in proteins (Jaenicke, 1990; Kristjánsson and Kinsella, 1991). The ΔH of protein unfolding also increases with increasing temperature and changes from negative to positive (Jaenicke, 1990). Therefore, low temperature denaturation is mainly enthalpy-driven (Jaenicke, 1991; Shortle, 1996).

High temperatures cause denaturation of many protein pharmaceuticals. Examples include the rapid formation of bovine insulin fibrils (Brange et al., 1997), aggregation/precipitation of rhKGF (Chen et al., 1994a), and precipitation of pGH (Charman et al., 1993). High temperature is also responsible for almost complete aggregation of recombinant consensus α-interferon (rConIFN) at 0.5 mg/ml after ultrasonic nebulization in a Microstat device for 10 min (Ip et al., 1995).

Protein denaturation at high temperatures can be reversible depending on experimental conditions. Both thermal and chemical (urea) denaturation of muscle acylphosphatase (AcP, 98 aa) are reversible (Chiti et al., 1998). Patatin, the major potato tuber protein (40 kD), unfolds at a high temperature (91°C) but refolds partially upon cooling (20°C). Heating native serum albumin to 85°C causes reversible denaturation (approximately 80–90% recovery), but heating for a longer period (10 h) at 60°C (pasteurization condition) causes irreversible denaturation (Kosa et al., 1998).

High temperatures also accelerate chemical degradations, such as increased hydrolysis of Asp residues, deamidation of Asn or Gln residues in RNase A and lysozyme at high temperatures (Kristjánsson and Kinsella, 1991), and formation of covalent insulin oligo- and polymers at temperatures ≥ 25°C (Brange et al., 1992a). Degradation mechanisms in proteins easily change with temperature. At 60°C, the aggregation of interleukin 1β (IL-1β) in aqueous solution follows apparent first-order behavior to 30% drug remaining, but at or below 55°C, the aggregation deviates from apparent first-order and becomes biphasic (slow and fast) (Gu et al., 1991).

3.3.2. Formulation pH

At extreme pHs, far away from p*I* of proteins, electrostatic repulsions between like charges in proteins increase, resulting in a tendency to unfold (Goto and Fink, 1989; Volkin and Klibanov, 1989; Dill, 1990). The process of unfolding leads to a reduction of charge density, thus lowering electrostatic free energy (Chan and Dill, 1991). A minor factor contributing to the unfolding tendency is the decreased capability of salt bridge formation at extreme pHs. In addition, the pKa values of charged groups in the folded state are different from those in the unfolded state. Changes in pH lead to titration of groups only in the unfolded form, which causes destabilization of overall native protein structure (Chiti et al., 1998). pH-dependent unfolding has been found to be due only to a small number of groups with anomalous pKa's (Dimitrov and Crichton, 1997). These ionizable groups with anomalous pKa's may stabilize or destabilize a protein in different pH ranges, as those in hen egg-white lysozyme (Yang and Honig, 1993).

Proteins are often stable in a narrow pH range such as pH 6.5–7.0 for recombinant factor VIII SQ (FVIII SQ) (Fatouros et al. 1997a); 6–7 for low molecular weight urokinase (LMW-UK, 33 kD) (Vrkljan et al., 1994); and 4.5–5 for relaxin (Nguyen and Shire, 1996). Protein refolding to a wrong conformation is often seen at a pH close to its p*I* (Kristjánsson and Kinsella, 1991). pH-induced denaturation of proteins can be reversible, such as porcine pancreatic elastase, which dena-

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tures on lowering the pH to less than 5.0 but renatures completely by immediately adjusting the pH back to 5.0 (Chang et al., 1993).

Certain proteins may have stable folded structures at extreme pHs. Apomyoglobin has five conformational states: native (N), acidic (A), acid-unfolded (U_A), basic (B), and base-unfolded (U_B) (Goto and Fink, 1989). Apomyoglobin starts to unfold at pH 5 with loss of helical structure and exposure of Trp residues as monitored by CD and fluorescence. At pH 2, the protein is maximally unfolded (U_A) to an extent similar to that unfolded in 6 M GdnHCl. Further increase in HCl concentration results in refolding to A state. The conformation of U_A state varies from fully unfolded to a partially folded structure (Goto and Fink, 1994). The acidic transition under low salt conditions is reversible ($N \leftrightharpoons U \leftrightharpoons N$), while reversibility of the alkaline transition depends on the length of time under high-pH conditions (Goto and Fink, 1989).

The rate of protein aggregation can be strongly affected by pH, such as the aggregation of bFGF (Wang et al., 1996b), deoxy hemoglobin (Kerwin et al., 1998), IL-1β (Gu et al., 1991), human relaxin (Li et al., 1995a), and bovine pancreas RNase A (Townsend and DeLuca, 1990; Tsai et al., 1998a). At pH 2.5, bovine insulin forms a significant amount of fibrils within a few hours at 37°C but is very stable without agitation for many months at pH 7. As pH increases from < 2 to 7, monomeric insulins gradually form dimeric, tetrameric, and eventually hexameric insulins. The monomeric form has the highest tendency to form fibrils due to its large exposed hydrophobic area as compared with other forms (Brange et al., 1997).

Formulation pH, like temperature, may affect both physical and chemical stability of proteins. Different chemical degradations may be facilitated at different pHs (Table 2). This explains why degradation products are different at different pHs for the same protein, such as hirudin (Gietz et al., 1998). Hydrolysis can easily occur at Asp residues under mild acidic condition (Volkin and Klibanov, 1989). Deamidation of Asn and Gln residues readily takes place under strongly acidic, neutral, and basic conditions. Under basic conditions, many reactions can occur, such as peptide bond hydrolysis, deamidation, hydrolysis of Arg to ornithine, β-elimination and racemization, and double bond formation. The pH effect on chemical stability may depend on the presence of other excipients. For example, a V-shaped chemical (oxidation and deamidation) degradation-pH profile has been demonstrated for hGH with minimum degradation at a pH of about 7.5, but the minimum is shifted to a pH of about 7 when formulated with dextran 40 (Pikal et al., 1991).

The destabilizing effect of pH on proteins can be estimated by determining of a variety of parameters. These parameters include (1) $\Delta G_{f \to u}$ of proteins such as RNase T1 (Thomson et al., 1989), (2) hydrodynamic radius of proteins such as β-lactamase (28.5 kD) (Goto and Fink, 1989), and (3) T_m of proteins such as yeast external invertase (YEI), bovine serum fetuin, glucoamylase (Wang et al., 1996a), myoglobin (Chan and Dill, 1991), rhDNase (Chan et al., 1996), bFGF (Wang et al., 1996b), RNase A (Liu and Sturtevant, 1996; McIntosh et al., 1998), and thrombin (Boctor and Mehta, 1992). The pH effect on protein function and stability can also be probed by computerized molecular dynamics simulation (Baptista et al., 1997).

3.3.3. *Adsorption*

Proteins can be adsorbed to many surfaces and interfaces, such as container surfaces, and air/water interfaces. Protein adsorption in air/water interfaces starts with creation of an area for anchoring the protein molecule, followed by subsequent reorientation and rearrangement of the adsorbed molecules at the interface (Damodaran and Song, 1988). The severity of adsorption is protein-dependent and does not seem to depend on the size and p*I* of proteins (Burke et al., 1992). Evidence suggests that secondary structure of a protein may change significantly at the adsorption surface such as IgG (Vermeer et al., 1998). Therefore, surface adsorption may result in loss and/or destabilization of proteins. At 1 μg/ml, the adsorptive loss of aFGF to surfaces of several containers (untreated glass, siliconized glass, sulfur-treated glass, Purcoat glass, polyester,

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polypropylene, and nylon) are in the range of 20–40% or 0.2–0.8 μg protein/cm^2 (Volkin and Middaugh, 1996). Interleukin-2 (IL-2), at 0.1 mg/ml in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, has been shown to lose about 90% of its activity after being pumped through silicone rubber catheter tubing (60 × 0.1 cm) in 24 h (Tzannis et al., 1996). The majority (> 80%) of the activity loss is due to adsorption-induced protein denaturation (Tzannis et al., 1997).

Protein surface adsorption is usually concentration-dependent and may reach a maximum (at least for certain proteins) above certain protein concentrations. The adsorption of bovine serum albumin (BSA) is linearly proportional to its concentration (up to 0.5%) in membrane filters made of pleated nylon, polyvinylidene difluoride, or cellulose acetate. Burke et al. (1992) examined surface adsorption of six proteins, including alcohol dehydrogenase (ADH), β-amylase, lactate dehydrogenase (LDH), α-chymotrypsinogen A, thyroglobulin, and IgG. Except LDH, adsorption of all other proteins to surfaces of containers (untreated glass, nylon, polyester + 0.3%, and polypropylene) appears saturated at approximately 5 μg/ml at a surface/solution volume ratio of 2.4 cm^2/ml. At this concentration, the amount of protein adsorbed is less than 0.5–1 μg/ml (about 10–15% of the total protein).

The type of container or membrane significantly influences protein adsorption to its surface. The adsorption of 14 model proteins has been examined in a variety of containers made of untreated glass, siliconized glass, sulfur-treated glass, Purcoat-treated glass, nylon, polypropylene, polyester 5X0, and polyester + 0.3%. Among these different containers, the untreated glass, silicone-treated glass, and polypropylene seem to have the least protein binding to their surfaces, and sulfur-treated glass and polyester containers seem to have higher adsorption for certain proteins (Brose and Waibel, 1996). Storing 1 ml of transforming growth factor-β_1 (TGF-β_1) at 1.0 μg/ml in 30 mM citrate buffer (pH 2.5) at 4°C for 11 days drops the protein concentration to 0.39, 0.37, and < 0.10 μg/ml, respectively, in polypropylene, siliconized glass, and untreated glass containers due to surface adsorption (Gombotz et al., 1996). BSA can be adsorbed to pleated nylon filter at 220 μg/cm^2/wt%, but at 120 μg/cm^2/wt% to filters made of polyvinylidene difluoride (PVDF) and cellulose acetate (CA).

The rate of protein adsorption can be very rapid. At 22 and 45°C, the percentages of recombinant human granulocyte colony-stimulating factor (rhG-CSF) adsorbed to PVC surface are, respectively, 93 and 97% of maximum after 10 min of equilibration (Johnston, 1996).

3.3.4. Salts

The effect of salts on protein stability is complex, partly because of the complex ionic interactions on fully exposed surfaces, and in fully or partially buried interior of proteins. Salts may stabilize, destabilize, or have no effect on protein stability depending on the type and concentration of salt, nature of ionic interactions, and charged residues in proteins (Kohn et al., 1997). For example, the T_m (67.4°C) of recombinant human deoxyribonuclease (rhDNase) decreases to 65.3°C in the presence of 75 mg/ml of NaCl but increases to 70.1°C at 200 mg/ml (Chan et al., 1996). Increasing $(NH)_2SO_4$ concentration from 50 to 200 mM increases aggregation of KGF during storage at 45°C, but NaCl has the opposite effect within the same concentration range (Zhang et al., 1995).

Salts can affect electrostatics in a protein in two ways: by non-specific (Debye-Hückel) electrostatic shielding, and by specific ion binding to the protein. At low concentrations, salts affect electrostatic shielding and weaken ionic repulsion/attractions as counterions. Therefore, this shielding effect may be either stabilizing when there are major repulsive interactions leading to protein unfolding, or destabilizing when there are major stabilizing salt bridges or ion pairs in the protein. At high concentrations, electrostatic shielding is saturated; the dominant effect of salt, like other additives, is on solvent properties of the solution. The net effect on stability of a protein would be determined by the relative effect on free energy of unfolded and native states (Goto and Fink, 1989). The stabilizing salts seem to increase surface tension at water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing pref-

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erential hydration of proteins (Kristjánsson and Kinsella, 1991; Kohn et al., 1997).

The salt effect strongly depends on the solution pH, which dictates charged state of ionizable groups. Recently, in a study on stability of de novo designed two-stranded α-helical coiled coils, it has been found that stability of a designed protein (QQx), which has no intra or interhelical ionic interaction but contains 5 Lys residues for maintaining solubility, increases gradually with increasing KCl concentration (up to 1 M) against urea-induced denaturation (Kohn et al., 1997). The effect is the same at both pH 3 and 7, since there is no ionized state in this pH range. However, stability of another designed protein (KEx), which contains Glu and Lys at positions that form interhelical ionic interactions, increases drastically with increasing KCl concentration at pH 3. This is because at this pH, Lys is the only charged residue at interaction positions, and KCl can electrostatically shield the destabilizing repulsion by interacting with charged Lys. Addition of 50 mM $LaCl_3$ increases slightly the stability of QQx at both pH 3 and 7 but decreases the stability of KEx due to La^{3+} binding to glutamate residues and disrupting interhelical ion pairs at pH 7. However, at pH 3, $LaCl_3$ stabilizes KEx since Glu residues are largely protonated at this pH and can not bind to La^{3+}. The destabilizing effect of salts on proteins has been reported in many cases. Increasing ionic strength of insulin solution from 0.04 to 0.11 by addition of NaCl increases insulin fibrillation, possibly by shielding repulsive forces between similarly charged groups (Brange et al., 1997). Probably by similar shielding effect, NaCl causes thermal aggregation of RNase at pH 3 (Tsai et al., 1998b). Increasing NaCl concentration from 120 to 360 mM also increases the mean particle (aggregate) size of hepatitis A virus (HAV) in solution from about 30 nm to greater than 120 nm in 24 h at room temperature (Volkin et al., 1996).

In many cases, salts do not play a major role in altering chemical instability of proteins or peptides based on a limited number of studies. It has been found that the rate of deamidation of Asn residues in a series of pentapeptides is not affected by ionic strength (Cross and Schirch, 1991). The rate of oxidation of Met in hIGF-I at 0.13 mM does not change whether or not sodium chloride (145 mM) is included at pH 6.0 (Fransson and Hagman, 1996). Similarly, the rate of oxidation of recombinant human relaxin by hydrogen peroxide does not change significantly at NaCl concentrations ranging from 0.007 to 0.21 M in 0.01 M acetate buffer at pH 5.0 (Nguyen et al., 1993). However, addition of 0.1 M KCl inhibits intracellular SH and S-S exchange in bovine mercaptalbumin at pH 8.6 (Kuwata et al., 1994).

3.3.5. Metal ions

Depending on the type and concentration, metal ions may destabilize or stabilize a protein. Since the negative counter ions may also significantly affect protein stability either positively or negatively, contribution of metal ions to protein stability should be carefully interpreted.

Some proteins are stable only in the presence of certain metal ions. The number of stabilizing metal ions required in each protein molecule is protein-dependent, and the metal ions may or may not be mutually replaceable for protein stability. It has been found that maximum activity of murine adenosine deaminase (ADA) is at 1:1 molar ratio of zinc (Zn^{2+}) or cobalt (Co^{2+}) to ADA. Both higher and lower ratios can inhibit the protein activity, and no measurable activity of ADA is left in the presence of other metals ions (at 1:1, 10:1 or 100:1 ratio), including Ca^{2+}, Cd^{2+}, Cu^{2+}, Cu^{+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, or Mg^{2+} (Cooper et al., 1997). While Ca^{2+} (up to 100 mM) stabilizes rhDNase, other metal ions such as Mg^{2+}, Mn^{2+} and Zn^{2+} destabilize it (Chan et al., 1996). The stabilizing effect of Ca^{2+} on rhDNase is presumably due to direct binding of Ca^{2+} to the protein and preventing breaking of a disulfide bridge (Chen et al., 1998). A number of metal ions have been tested for stabilization of FVIII SQ, including Ca^{2+}, Sr^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Mn^{2+}. While Ca^{2+} or Sr^{2+} increases the stability of the protein, Fe^{+2} at 1 or 10 mM and Cu^{+2} or Zn^{+2} at 10 mM sharply destabilize FVIII SQ (Fatouros et al., 1997a).

Metal ions may significantly affect protein stability without affecting much of its secondary structure. Although ADA (apoenzyme) is not ac-

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tive (less than 0.1% of the holoenzyme activity) in the absence of Zn^{2+} or Co^{2+}, CD analysis of the protein indicates that secondary structures are the same with or without these metal ions (Cooper et al., 1997). Similarly, removal of exogenous Ca^{2+} by EGTA-treatment has little effect on the secondary structure of rhDNase based on IR determination in the amide III region in either aqueous or lyophilized state (Chen et al., 1998).

Trace amount of metal ions in protein formulation may catalyze oxidation in proteins mainly via the Fenton pathway (Stadtman, 1993). These labile residues include Met, Cys, His, Trp, Tyr, Pro, Arg, Lys, or Thr (Stadtman, 1993; Li et al., 1995b,c). The catalysis depends on concentration of the metal ions. For example, addition of 0.15 ppm (w/w) chloride salts of Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, or Zn^{2+} does not affect the oxidation rate of Met in hIGF-I at 0.13 mM in 50 mM sodium phosphate (pH 6.0) at room temperature, but when the salt concentration increases to 1 ppm, Fe^{3+} causes a significant increase in oxidation (Fransson and Hagman, 1996). The metal-catalyzed oxidation can be facilitated in the presence of a reducing agent such as ascorbate or RSH (Stadtman, 1993; Li et al., 1995c). Metal ions, oxygen, and reducing agents can generate reactive oxygen species (ROS) to oxidize proteins. The ascorbate/Cu(II)/O_2 system easily oxidizes papain (Kanazawa et al., 1994), brain-derived neurotrophic factor (BDNF) (Jensen et al., 1998), His in hGH (Zhao et al., 1997), His and Met in human relaxin (Li et al., 1995a), and the His-containing fragment of relaxin (Khossravi and Borchardt, 1998). The dual effect of ascorbic acid might explain why it slightly inhibits oxidation of recombinant human nerve growth factor (rhNGF) but destabilizes FVIII SQ (Österberg and Fatouros, 1994) and increases oxidation of recombinant human ciliary neutotrophic factor (rhCNTF) in the presence of peroxides (Knepp et al., 1996).

3.3.6. Chelating agents

In close relation to metal ions, chelating agents such as EDTA and citric acid may destabilize a protein by binding to the protein and/or its critical metal ions or stabilize the protein by binding to any harmful metal ions.

Since transition metal ions can catalyze protein oxidation, metal ion-chelating agents should be able to protect a protein from metal-catalyzed oxidation. EDTA at 0.1 mM can completely inhibit oxidation of relaxin induced by the ascorbic acid/$CuCl_2$/O_2 system (Li et al., 1995a). At 0.15 mM and 1 mM, it also effectively inhibits oxidation of Cys in aFGF (Wang et al., 1996b) and bFGF (Volkin and Middaugh, 1996).

In many cases, however, the effect of chelating agents is more complex. The net effect depends on the metal ions, oxidation mechanism, and type and concentration of the chelating agent (Stadtman, 1993). It has been found that EDTA at 0.02 mM suppresses formation of Met sulfoxide in His-Met catalysed by the ascorbic acid/$FeCl_3$/O_2 system at pH 7.4 but increases the overall degradation (Li et al., 1993). In a recent study, some polyaminocarboxylate metal chelators including EDTA failed to inhibit oxidation of Met- or His-containing heptapeptides induced by the dithiothreitol/Fe_3Cl/O_2 system but significantly changed the distribution of oxidation products (Zhao et al., 1996).

3.3.7. Shaking and shearing

Proteins can be denatured by shaking or shearing. Shaking creates hydrophobic air/water interface, which results in alignment of protein molecules at the interface, unfolding to maximize exposure of hydrophobic residues to air and to initiate aggregation (Volkin and Klibanov, 1989). The hydrophobic surfaces causing protein aggregation during shaking can be either gaseous or solid (Sluzky et al., 1991, 1992). Similarly, shearing also exposes hydrophobic areas of proteins, initiating aggregation.

Examples of shaking-induced protein instability include aggregation of rFXIII (Kreilgaard et al., 1998), hGH (Katakam et al., 1995; Katakam and Banga, 1997; Bam et al., 1998), hemoglobin (Kerwin et al., 1998), and insulin (Thurow and Geisen, 1984; Sluzky et al., 1991; Brange et al., 1997). More than 50% of urease activity is lost at 0.33 mg/ml in phosphate buffer (pH 7.0) after shaking for 24 h, and 77% of rIL-2 activity is lost at 0.2 μg/ml in PBS (pH 7.0) after shaking for 8 h at 4°C (Wang and Johnston, 1993a). Air-jet nebu-

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lization of rConIFN at pH 6.3 for 25 min at 40 psig causes 70% aggregation (Ip et al., 1995).

Proteins may tolerate shearing inactivation to a different degree. Catalase loses about 50% of its activity after exposure to a shear of 7×10^6, but plasma fibrinogen loses the same amount of its clotting activity after exposure to a shear of 5×10^8 (Charm and Wong, 1970a,b). Similarly, about 30% of rhGH forms aggregates at a shear of 10^5, while rhDNase is stable at a shear of 2×10^7 when these proteins are homogenized (Maa and Hsu, 1997). This is because rhGH is more surface active and may easily accumulate at the homogenization-induced air/water interface, accelerating aggregation. Other factors such as the rigidity of protein structure and the number of hydrophobic residues exposed on the protein surface might have contributed to the different level of shear tolerance.

The effect of shear can be evaluated by pumping a protein solution through a capillary tube. The amount of shear experienced by the protein can be expressed as the product of shear rate (S) and residence time (t): $S*t = (8/3)(L/r)$, where L is the length and r is the radius of the tube (Charm and Wong, 1970a).

3.3.8. Protein denaturants

GdnHCl, urea, NaSCN (thiocyanate), and SDS are often-used denaturants. These denaturants bind preferentially to proteins, disrupting both hydrophobic interaction and hydrogen bonds, and reducing protein chemical potential and free energy of denaturation (Darnell et al., 1986; Volkin and Klibanov, 1989; Timasheff, 1993; Allison et al., 1996). While GdnHCl and urea may interact weakly with the hydrophobic surface of a protein, SDS probably interacts strongly with it, causing protein denaturation, since a denatured state exposes more hydrophobic surface for binding (Shortle, 1996). The denaturant concentration for complete protein denaturation is usually 6 M for GdnHCl and 8 M for urea, although lower concentrations can be as effective depending on the protein.

GdnHCl is a more potent denaturant than urea. RNase H can be completely denatured in 2 M GdnHCl, but 4 M urea is needed for complete denaturation (Goedken and Marqusee, 1998). For rhDNase, 0.5 M GdnHCl lowers its T_m from 67.4 to 61°C, but 2 M urea is needed to achieve the same effect (Chan et al., 1996). The denatured proteins by GdnHCl or urea may be reversible, such as diacylglycerol kinase (DGK) (Clarke and Waltho, 1997).

Denatured proteins are usually soluble in a denaturant solution because of increased solvation of nonpolar amino acids (Chan and Dill, 1991). Exceptions do exist. For example, the rate and extent of horse apomyoglobin aggregation increase with increasing urea concentrations (up to 2.4 M) (De Young et al., 1993). This may be due to the possible effect of protein-dependent preferential exclusion of urea at low concentrations (Timasheff, 1998). In fact, protein activation or stabilization by low concentrations of a denaturant has been reported. GdnHCl at concentrations up to 0.6 M stimulates activity of human placental alkaline phosphatase (Hung and Chang, 1998) and at concentrations up to 0.3 M stabilizes RNase T1 against thermal and urea-induced unfolding (Mayr and Schmid, 1993). The stabilizing effect of GdnHCl is apparently due to the contribution of stabilizing effect of Cl^- as observed for apomyoglobin (Staniforth et al., 1998).

It should be noted that cyanate may spontaneously form from urea, and an 8 M urea solution contains approximately 0.02 M cyanate, which can react with both amino and sulphydryl groups in proteins, causing irreversible inactivation (Volkin and Klibanov, 1989). Therefore, urea should be prepared fresh or cyanate ions should be removed when used in probing protein stability. In IR studies NaSCN is a preferable denaturant since it does not absorb in the amide I region (Allison et al., 1996).

3.3.9. Non-aqueous solvents

A folded protein in an aqueous solution has hydrophobic regions sequestered from and hydrophilic areas in contact with the aqueous environment. When the polarity of an aqueous solvent decreases by adding a non-aqueous solvent, protein hydrophobic cores tend to dissipate in contact with the solvent, and the protein hydration shell may be disrupted, leading to destabiliza-

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tion and unfolding of the protein. For example, addition of 60% (v/v) glycerol in 10 mM Tris–HCl buffer (pH 7.4) decreases $\Delta G_{f\rightarrow u}$ of ferric horse heart myoglobin by about 10 kcal/mol (Barteri et al., 1996). Therefore, non-aqueous solvents reduce free energy of the unfolded state by solvating the exposed nonpolar residues (Dill, 1990). In two-phase organic/aqueous systems, proteins unfold/denature predominantly at the interface, which depends on both the interfacial tension and interfacial surface area (Cowan, 1997).

Non-aqueous solvent-induced inactivation of proteins by disruption of water residues at the active site may occur without disruption of the tertiary structure. Therefore, protein inactivation by non-aqueous solvents may be reversible. For instance, inactivation of certain proteins by dimethylsulfoxide (DMSO) is fully reversible upon immediate dilution with aqueous solution (Chang et al., 1991).

While non-aqueous solvents generally destabilize proteins, some at low concentrations may have the opposite (stabilizing) effect, such as polyhydric alcohol and some polar, aprotic solvents like DMSO and dimethylformamide (DMF) (Volkin and Klibanov, 1989). Four organic solvent additives, polyethylene glycol (PEG), 2-methyl-2,4-pentanediol (MPD), DMSO, and trimethylamine N-oxide (TMAO), have been found to induce preferential hydration of lysozyme and BSA (Kita et al., 1994). The T_m of IL-1R is increased from 48.1 to 48.6°C in the presence of 5.1 mg/ml ethanol but decreased to 43.8°C at 50 mg/ml (Remmele et al., 1998). The stabilization effect by low concentrations of non-aqueous solvents is probably due to the mixed solvent system more resembling the protein's cellular environment (Butler, 1979a,b).

Proteins suspended in certain non-aqueous solvents may be as stable as in solid state. Recently, it has been found that plasma-derived factor IX (pdFIX) suspended in perfluorodecalin with 0.0003% moisture does not lose protein activity within 24 weeks as observed in a lyophilized formulation at 37°C, while that suspended in soybean oil, methoxyflurane, octanol, or PEG 400 rapidly loses activity to a different degree within the same period (Knepp et al., 1998). When pdFIX powder containing 10% moisture is suspended in perfluorodecalin, the resultant suspension is not as stable. This may be explained as the strong dependency of non-aqueous solvent-induced denaturation on availability of free water. In the absence of free water, protein conformation is highly rigid and compact, and resists unfolding processes (Cowan, 1997). Due to the inflexibility, certain proteins in non-aqueous solvents can resist thermal denaturation as well as chemical degradation (Tuena de Gómez-Puyou and Gómez-Puyou, 1998).

3.3.10. Protein concentration

Protein aggregation is generally concentration-dependent. The mean-field lattice model predicts that proteins will aggregate/precipitate at sufficiently high concentrations (Fields et al., 1992). It has been suggested that increasing protein concentration to higher than 0.02 mg/ml may facilitate potential protein aggregation (Ruddon and Bedows, 1997).

Accelerated aggregation of proteins at high concentrations has been reported in many cases. Examples include aFGF (Won et al., 1998), β-lactoglobulin (Roefs and De Kruif, 1994), IL-1β in the range of 0.1–0.5 mg/ml (Gu et al., 1991), LMW-UK in the range of 0.2–0.9 mg/ml (Vrkljan et al., 1994), and apomyoglobin in the range of 4–12 mg/ml in the presence of 2.4 M urea (De Young et al., 1993). The size of protein aggregates may also increase with increasing protein concentrations such as β-lactoglobulin (Roefs and De Kruif, 1994).

The effect of protein concentration on its aggregation may depend on the mechanism of aggregation and the experimental conditions. The formation of bovine insulin fibrils increases with increasing protein concentrations from 0.75 to 5% during storage at pH 2.5 and 21°C, and it takes less than 10 h to produce the same amount of fibrils at 5% as that in 15 days at 0.75% (Brange et al., 1997). In contrast, bovine insulin at 0.1 mg/ml at pH 7.4 aggregates more readily than at 0.6 mg/ml during shaking (Sluzky et al., 1991, 1992). This is attributable to the more favorable formation of insulin hexamers at 0.6 mg/ml, which are less susceptible to hydrophobic surface-

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induced adsorption/aggregation than insulin monomers.

In some cases, protein concentration also affects chemical degradations to a certain degree. Increasing insulin concentration increases formation of covalent insulin oligomers and polymers in neutral solution at 37 or 45°C (Brange et al., 1992a). However, the rate constant for oxidation of Met in hIGF-I (k_{ox}) decreases when the protein concentration is increased from 0.26 to 1.4 mM at either 30 or 50°C, presumably due to the limited amount of oxygen available for oxidation in the solution.

On the other hand, concentrated protein solutions can be more resistant against freezing-induced protein aggregation and loss of activity such as labile LDH (Carpenter et al., 1990, 1997). There are at least three explanations. First, the amount of protein accumulated at ice-water interface is finite and therefore, interface-induced protein denaturation is limited in concentrated protein solutions. Second, protein unfolding may be inhibited by steric repulsion of neighboring protein molecules (Allison et al., 1996). Last, protein–protein interactions may change monomers to active and more stable dimers or multimers (Mozhaev and Martinek, 1984).

3.3.11. Source and purity of proteins

Stability of proteins may be significantly different depending on the manufacturer and source of proteins. The rate of formation of high-molecular-weight products in human insulin preparations in neutral solutions can differ up to 100% among different manufacturers (Brange et al., 1992a). One of the major influencing factors is the purity of proteins. Any presence of trace amount of enzymes, metal ions, or other contaminants can potentially affect protein stability. Thus, protein purification is one of the most critical steps in controlling protein quality.

Very often, the extent and mechanism of protein degradation is different depending on the purification scheme such as rhTPO (Senderoff et al., 1996). Recombinant and plasma-derived proteins may require different purification schemes, which may influence protein stability. Recombinant factor IX (rFIX) is essentially free of contamination from non-factor IX products, but many high-purity pdFIX preparations exhibit significant amounts of non-factor IX contaminants, including other coagulation proteins, which may potentially affect the protein stability (Bond et al., 1998).

3.3.12. Protein morphism

Protein morphism is also a factor influencing protein stability. Crystalline drugs are generally more stable chemically than amorphous forms, especially for small molecules. The formation of high-molecular-weight insulin in a suspension of amorphous zinc insulin appears faster than that in crystalline zinc insulin at 37°C (Brange et al., 1992a). The total hydrolytic transformation at B3 position of the amorphous (or soluble) insulin is considerably higher than that of the crystalline form (Brange et al., 1992b). On the contrary, freeze-dried amorphous insulin is far more stable than crystalline insulin against deamidation and dimerization at all water content up to 15%. While the rate of degradation of crystalline insulin increases with increasing water content, the rate of amorphous form is essentially independent of water content change in the same range (Pikal and Rigsbee, 1997).

3.3.13. High pressure

High pressure can cause protein unfolding because the volume of protein–solvent systems is smaller in the unfolded state. In other words, unfolded proteins are more compressible than folded ones. For example, 40% of staphylococcal nuclease at <10 μM unfolds under 1000 bar in 10 mM Bis–Tris buffer (pH 4.5) containing 1.25% xylose (Frye and Royer, 1997). Bovine pancrease RNase A unfolds completely under 4000 bar (Zhang et al., 1995; Prehoda et al., 1998). Increasing the pressure from 0 to 2000 bar significantly inhibits activity of H^+-ATPase and increases its aggregation (Tsai et al., 1998c).

3.4. Analytical techniques in monitoring protein instability

To monitor protein instability, a protein activity assay is indispensable. Often, a bioassay is

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needed based on the protein's function, such as the clot lysis assay for tissue plasminogen activator (tPA) (Hsu et al. 1995). A good activity assay should be reproducible and sensitive enough to detect small changes in protein activity. In reality, a protein activity assay is often variable, unable to detect small activity changes, labor-intensive, and unable to reveal any intricate changes in the structure of proteins. Therefore, other analytical techniques are used, preferably in conjunction with an activity assay, to characterize a protein and monitor its instability. The available analytical methods for assessing protein formulations have been discussed elsewhere (Jones, 1994). Table 3 lists the major analytical techniques and their applications in monitoring protein instability.

3.4.1. Monitoring the protein unfolding process

Proteins can be unfolded chemically or thermally. The often-used chemicals for protein unfolding studies include urea and GdnHCl. The denaturant-induced unfolding process can be slow depending on the protein, and on the type and concentration of denaturant. It has been reported that at least 6 h are needed to unfold human placental alkaline phosphatase (Hung and Chang, 1998). Different denaturants may also unfold a protein differently. Apomyoglobin from *Aplysia limacina* shows two transitions on titration with GdnHCl but only one with urea as monitored by far-UV CD (Staniforth et al., 1998).

Many physical techniques can be used to monitor an unfolding event, including circular dichroism (CD), fluorescence, optical rotation, or UV (Thomson et al., 1989; Lau and Bowie, 1997; Swietnicki et al., 1997). The signal of hydrogen exchange (HX) in proteins, as monitored by nuclear magnetic resonance spectroscopy (NMR), can also be used in these studies (Bai et al., 1994; Zhang et al., 1995). These techniques are based on signal differences between the folded and unfolded states. Two useful parameters in chemical unfolding studies can be determined: C_{half}, the concentration of a denaturant causing unfolding of 50% protein molecules, and $\Delta G_{f\rightarrow u}$, the free energy change of unfolding. Both parameters can be used to examine the effect of formulation excipients on protein stability (Barteri et al., 1996; Foord and Leatherbarrow, 1998). To obtain $\Delta G_{f\rightarrow u}$, the fraction of unfolded protein, F_u, is first calculated using the following equation, assuming a two-state transition:

$$F_u = (y_f - y_{obs})/(y_f - y_u).$$

In this equation, y_{obs} is the observed variable signal in the presence of different denaturant concentrations, and y_f and y_u are, respectively, the signal of completely folded and unfolded conformations. ΔG at the equilibrium state can be calculated using the following equation (Thomson et al., 1989; Pace, 1990):

$$\Delta G = -RT \ln[(F_u)/(1 - F_u)]$$
$$= -RT \ln[(y_f - y_{obs})/(y_{obs} - y_u)],$$

where R is the gas constant and T is the absolute temperature. The $\Delta G_{f\rightarrow u}$ is obtained by extrapolating the unfolding curve (ΔG versus denaturant concentration) at 0 denaturant concentration.

What causes the signal difference between the folded and unfolded states of proteins? Protein unfolding exposes hydrophobic amino acid residues. Solvent exposure of these residues such as Tyr or Trp changes their environment and often results in a signal change. The change in UV or fluorescence signal can be negative or positive depending on protein sequence and solution properties. For example, unfolding of α-chymotrypsin at 0.3 mg/ml at pH 7.0 decreases its UV absorption by about 10% at 281 nm and fluorescence intensity by about 70% with a shift of fluorescence λ_{max} from 331 to 347 nm (Lozano et al., 1997). The signal decrease results from an increase in polarity of the environment of Trp in the protein upon unfolding as observed for apomyoglobin (Staniforth et al., 1998). The intrinsic fluorescence of human albumin decreases substantially upon unfolding due to loss of energy transfer (several Tyr residues transferring excitation energy to Trp residues in the native state) (Farruggia et al., 1997). However, exposure of buried Tyr to solvent upon unfolding of cutinase increases its UV absorbance significantly at 250 nm at pH 9.6 due to ionization of Tyr (Melo et al., 1997). Positive change in fluorescence signal has also been reported. The fluorescence intensity of soluble protein MerP (72 aa) increases about 10 times at

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Table 3
Analytical techniques in protein characterization and instability monitoring

Analytical techniques	Major applications	Protein examples	References
Analytical centrifugation	Protein aggregation	Rop proteins	Munson et al., 1996
CE	Protein degradation	Hirudin	Gietz et al., 1998
	Determination of T_m	RNase	McIntosh et al., 1998
CD	Estimation of secondary structures	α-Spectrin	Prieto et al., 1997
	Determination of T_m	AcP	Chiti et al. 1998
	Probing protein conformation	β-lactoglobulin	Matsuura and Manning, 1994
	Determination of multimers	Fragment of ribosomal protein L9	Kuhlman et al., 1997
DSC	Determination of T_g	hGH	Pikal et al., 1991
	Determination of T_m	aFGF	Tsai et al., 1993
	Protein unfolding	BSA	Giancola et al., 1997
Electron paramagnetic resonance (EPR)	Ligand-protein interactions	rhGH, rhIFN-γ	Bam et al., 1995
Fluorescence	Protein unfolding/interaction	MerP	Aronsson et al., 1997
	Determination of T_m	HSA	Farruggia et al., 1997
	Probing protein conformation	α-Antitrypsin	Kwon and Yu, 1997
HPLC-ion exchange	Protein degradation and aggregation	hGH	Zhao et al., 1997
HPLC-reversed phase	Protein degradation and aggregation	hGH	Pikal et al., 1991
	Estimation of contamination	rhPTH	Nabuchi et al., 1995
HPLC-size exclusion	Protein degradation and aggregation	hGH	Pikal et al., 1991
	Estimation of contamination	β-Galactosidase	Yoshioka et al., 1993
IR	Estimation of secondary structures	Chymotrysinogen	Allison et al., 1996
	Determination of T_m	hPAH	Chehin et al. 1998
	Probing protein confirmation	IL-1ra	Chang et al., 1996b
Karl Fischer	Water determination	Insulin formulation	Strickley and Anderson, 1996, 1997
Light scattering	Protein aggregation	Human relaxin	Li et al., 1995a
MS	Determination of molecular weight, degradation products and contaminants	bFGF	Shahrokh et al., 1994a
NMR	Determination of 3-D and secondary structures	IL-6	Xu et al., 1997
	Protein relaxation and softening	BSA, BGG	Yoshioka et al., 1997, 1998
	Protein unfolding	RNase	Zhang et al., 1995
Raman spectroscopy	Determination of secondary structures	Insulin	Yeo et al. 1994
Refractometry	Ligand–protein interactions	rhGH, rhIFN-γ	Bam et al., 1995
UV/visible spectroscopy	Determination of T_m	RNase	Thomson et al., 1989
	Protein aggregation	aFGF	Tsai et al., 1993
	Estimation of contamination	hGH	Perlman and Nguyen, 1992
	Probing protein conformation	IL-2, Insulin	Butler, 1979a,b; Brewster et al., 1991

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300 nm upon unfolding (Aronsson et al., 1997). Similar increase in fluorescence signal was also observed for Trp RNase A mutant at 350 nm (Sendak et al., 1996). Effective quenching of Tyr and Trp in the folded proteins causes the large signal increase upon unfolding. Sometime the signal change is due to a simple solute effect rather than conformational change. A 30% drop in fluorescence intensity of DGK at 330 nm is observed upon SDS denaturation, and the same extent of signal change is also seen for free Trp (Lau and Bowie, 1997). To maximize detection sensitivity, a signal ratio may be used, such as the ratio of fluorescence intensity at 350 nm to that at 330 nm in the study of rFXIII unfolding (Kurochkin et al., 1995).

Proteins may have more than one unfolding process if there is a stable protein intermediate, which may not be detectable depending on the techniques used. Two unfolding processes are observed for human, bovine, rat, dog, and rabbit albumin when they are denatured by GdnHCl and monitored by CD, but only human, bovine, or rat albumin shows two transitions when monitored by fluorescence (Kosa et al., 1998). Similarly, the UV absorption of DGK drops biphasically with increasing concentration of denaturant SDS, suggesting two unfolding transitions, but CD measurement at 222 nm only shows a single denaturation phase (Lau and Bowie, 1997). The failure of CD to detect an intermediate is probably due to a variety of factors affecting CD determination, such as protein concentration (causing improportional changes in secondary structure), pH, solvent property, and ionic strength (Farnsworth et al., 1997; Hu et al., 1997).

Fluorescence spectroscopy is often used to probe protein unfolding (Eftink, 1994). A red shift in fluorescence spectroscopy is commonly observed during protein unfolding because Trp or Tyr residues are in less hydrophobic environments (De Young et al., 1993; Volkin et al., 1996). An often-used agent in the investigation of protein unfolding is Bis-ANS, whose fluorescence is pH-independent and very weak in water but increases greatly upon binding to unfolding-exposed hydrophobic sites in proteins. Due to this property, Bis-ANS has been used recently to probe the mechanism of α_1-antitrypsin polymerization (James and Bottomley, 1998).

Both primary and second-derivative amide I IR spectra can be used to probe pH or temperature-induced protein unfolding or conformational changes in proteins such as hemoglobin (Dong and Caughey, 1994) and insulin (Pikal and Rigsbee, 1997). By monitoring changes in band intensity of native β-sheet structure at 1638 cm^{-1} and an aggregate band at 1619 cm^{-1}, human FXIII has been found to denature irreversibly as the temperature increases from 25 to 85°C (Dong et al., 1997). IR spectra of proteins in solution can be significantly different from those obtained in solid state. Insulin in solution shows a sharper and stronger IR helix band than solid insulin, and the spectrum of denatured insulin (spectrum at 85°C) resembles that of solid insulin (Pikal and Rigsbee, 1997).

3.4.2. Determination of protein melting/unfolding temperature (T_m)

Those physical methods used to determine $\Delta G_{f\rightarrow u}$ can be used similarly to determine protein melting/unfolding temperature (T_m), such as CD, fluorescence, optical rotation, or UV. T_m can be easily located at $\Delta G = 0$ on the ΔG versus T curve or directly determined from the signal–temperature curve (Farruggia et al., 1997).

Differential scanning calorimetry (DSC) is one of the frequently-used techniques to determine protein T_m. Proteins unfold with an endothermic peak, but aggregation is exothermic by DSC. If the two events happen approximately at the same time, a peak would be the sum of the two events. The first event may be overwhelmed by the second, such as the single exothermic peak observed for rhKGF unfolding and aggregation (Chen et al., 1994a). In this case, modulated DSC may be considered as a way to differentiate overlapping thermal events due to its higher resolution (Craig and Royall, 1998; Royall et al., 1998).

There are several other factors that can potentially affect determination of T_m. The first factor is the determination method to be used. Different methods can give significantly different T_m values

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for the same protein. Detailed examples are listed in Table 1. Temperature-ramping rate during determination of T_m may have a significant effect. For example, lowering the temperature-ramping rate reduces the T_m of rhKGF, suggesting a kinetically controlled denaturation process (Chen et al., 1994a). Vermeer et al. (1998) recently demonstrated that at 0.5°C/min, mouse IgG shows both a melting and an aggregation peak by DSC but at 0.1°C/min, only the melting peak can be observed. Protein concentration may or may not affect determination of T_m. While the initial unfolding temperature of rhKGF is independent of protein concentration (Chen et al., 1994a), that of IFN-β-1a decreases from about 77 to 68°C when the protein concentration is increased from 10 to 100 μg/ml (Runkel et al., 1998). The T_m independence on protein concentration may imply a rate-limiting protein unfolding.

Recently, infrared spectroscopy (IR) and capillary electrophoresis (CE) have also found their application in determination of T_m. By IR, the T_m of human phenylalanine hydroxylase (hPAH) has been estimated based on temperature-induced changes in the intensity ratio of the amide I band at 1619 and 1650 cm^{-1} (Chehin et al., 1998). Similarly, the T_m of RNase has been determined by CE based on changes in the protein's electrophoretic mobility at different temperatures (McIntosh et al., 1998).

3.4.3. Estimation of protein secondary structures

IR is one of oldest methods and remains the most extensively-used technique today for studying secondary structure of proteins (Susi and Byler, 1986; Dong et al., 1995b; Allison et al., 1996; D'Auria et al., 1997). The commonly-used band for determination of protein secondary structure is the amide I region (1620–1690 cm^{-1}), although the amide III band (1200–1330 cm^{-1}) has also been used in this regard. The amide I region covers different C=O stretching frequencies arising from different secondary structures (α-helix, β-sheet, turn, and unordered structures) and has no major interfering bands from other structures in proteins, whereas the amide III band has better resolution for secondary structures and no water interference (Cooper and Knutson, 1995). A good example of using the amide III band is the estimation of secondary structure of some dozen different proteins in both liquid and solid states by Griebenow and Klibanov (1995).

IR absorption bands are generally broad and consist of overlapping components representing different structures. Two methods have been used for resolution enhancement: Fourier self-deconvolution and second or fourth derivation (Goormaghtigh et al., 1994b). Both techniques work equally well in resolving overlapping components (Dong et al., 1995b). However, some believe that analysis of derivative spectra should be discouraged because derivation does not preserve the integrated areas of individual components (Surewicz et al., 1993). Nevertheless, the two resolution-enhancing methods can be conveniently used on modern instruments of Fourier transform infrared spectroscopy (FTIR), which also offer high sensitivity (high S/N ratio), greater accuracy, and higher speed (Susi and Byler, 1986; Surewicz et al. 1993). Secondary structure estimation after resolution enhancement seems rather accurate. Dong et al. (1990) determined the second-derivative IR spectra of 12 globular proteins in aqueous solutions, and the relative amounts of different secondary structures in all proteins were nearly identical (mostly $\pm 5\%$) to those obtained by crystallography.

Unfortunately, water molecules absorb strongly in the amide I region (about 1640 cm^{-1}), interfering with data interpretation. There are two ways to overcome this obstacle: careful subtraction of water absorption or use of D_2O as the solvent (Goormaghtigh et al., 1994a). To make reliable subtraction, higher protein concentrations (>10 mg/ml) are recommended to increase the protein signal and a CaF_2 (or BaF_2) cell with a path length of 10 μm or less should be used to control the total sample absorbance within 1 unit (Cooper and Knutson, 1995). Replacing water with D_2O as the solvent not only eliminates water interference in the amide I region but also stabilizes proteins in some cases (Bai et al., 1994). For example, human FXIII is apparently more stable in D_2O than in H_2O (Dong et al., 1997). The higher stability of proteins in D_2O is believed to be due to stronger hydrogen bonds formed by deuterium

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than those formed by hydrogen. This suggests that IR spectra of proteins in D_2O may not be the same as those in water. As a matter of fact, subtle differences in the secondary structure in bovine β-lactoglobulin variants are found in the two solvents (Dong et al., 1995a).

Another often-used method to determine the relative composition and changes of secondary structure in a protein is CD. A broad negative peak at about 218 nm is characteristic of a β-sheet structure, and two negative peaks at 208 and 222 nm are characteristic of a α-helix structure (Chen et al., 1994a; Farnsworth et al., 1997; Hu et al., 1997; Swietnicki et al., 1997). Although CD is generally less sensitive than IR, the two methods are highly complementary and, if possible, should be used together (Surewicz et al., 1993). Their relative contribution can be illustrated in the following examples. In the comparison of the secondary structures of two generic bovine β-lactoglobulin variants in D_2O solution, the CD spectra of the two proteins are nearly identical, but the IR spectra are significantly different (Dong et al., 1995a). In a different investigation, about 20–35% random or unordered elements in human FXIII in an aqueous solution are detected by CD but not observed by FTIR, although detailed analysis indicates that the secondary structures determined by the two methods are very similar (Dong et al., 1997).

Several factors can affect the appearance of the characteristic peaks in CD spectra, such as protein concentration, temperature, pH, and ionic strength. Increasing protein concentration may be associated with increased content of β-sheet (Matsuura and Manning, 1994). High ionic strength may reduce helicity of a protein (destabilization), if charge–charge interactions make a net favorable contribution to helix stability (Kuhlman et al., 1997). Although a double ellipticity minima at 208 and 222 nm is of a typical helical conformation, this may not be obvious if the helical content is very low and/or there are interfering aromatic residues such as Tyr (Prieto et al., 1997).

Lastly, NMR can also be used to determine secondary structures of proteins. It can provide accurate estimation of both the extent and location of the secondary structures (Reid et al., 1997). A particular use of NMR is the determination of secondary structural propensities of residues in unfolded or partially-folded proteins (Dyson and Wright, 1998).

3.4.4. Protein aggregation

In addition to polyacrylamide gel electrophoresis (PAGE) and analytical centrifugation, size exclusion HPLC (SEC-HPLC) is an often-used method in monitoring protein aggregation. Unfortunately, the size of a protein or its aggregates can be overestimated by SEC-HPLC if the protein is not spherical (such as highly-coiled proteins) and its Stokes radius is greater than that of a globular protein (Kuhlman et al., 1997). In addition, if a protein has carbohydrates or interacts with the column, the elution profile of the protein may change, leading to erroneous estimation of its molecular weight. To solve these issues, SEC-HPLC can be coupled with both light scattering and refractive index detectors to determine the size of a protein or its aggregates. The size calculated by the signal ratio of the two detectors is not affected by the above factors (Wen et al., 1996).

While SEC-HPLC can only determine the total amount of aggregates based on size, reversed phase HPLC (RP-HPLC) may detect both covalent and non-covalent aggregates such as hGH (Perlman and Nguyen, 1992) or different isoforms of protein aggregates such as bFGF dimers (Shahrokh et al. 1994b). Since a protein sample is usually filtered through a 0.2 μm filter to remove any particulates before analysis, the HPLC method is limited for determination of soluble aggregates. In addition, a protein may unfold or refold during HPLC analysis depending on the protein and the nature of the mobile phase, which may change the retention time of the protein. For example, denatured bFGF partially refolds into native protein during analysis, which changes the retention time and peak shape of the denatured protein (Shahrokh et al. 1994b). Therefore, prevention of unfolding/refolding by addition of certain modifiers in the mobile phase may be needed in these cases.

The turbidimetric method is often used to estimate the amount of protein aggregates by measuring optical density of samples based on light scattering in near UV or visible region, where

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proteins do not have any absorption (Eckhardt et al., 1994). A variety of wavelengths has been chosen and used, including 300 nm for urokinase (Vrkljan et al., 1994), 350 nm for aFGF (Tsai et al., 1993; Volkin et al., 1993), average optical density between 340 and 360 nm for met-hGH and tPA (Hsu et al. 1991, 1995), 450 nm for pGH (Charman et al. 1993), and 500 nm for IL-1ra (Chang et al., 1996b). This method has been proved to be more sensitive than HPLC in detecting early formation of aggregates (Wang et al., 1996b). In addition, the turbidity of a protein solution can be linear in a narrow concentration range for accurate quantitation, such as IL-1ra in the range of 0.1–0.3 mg/ml (Chang et al., 1996b). On the other hand, this method can be variable. For example, it has been found that the turbidimetric variation in determination of LMW-UK aggregation in phosphate buffer (pH 6.5) upon heat treatment is usually less than 10% but increases up to 30% when the LMW-UK solution contains other polymer additives (Vrkljan et al., 1994).

A simpler method for estimation of the extent of protein aggregation is to compare visually the level of sample cloudiness using seven categories of visual appearance: clear, slightly opalescent, opalescent, very opalescent, slightly cloudy, cloudy, and very cloudy (Eckhardt et al., 1994). The requirement of using this method is a uniform distribution of all the aggregates.

Recently, IR has been used extensively in protein denaturation/aggregation studies. A common feature of thermally-induced (or lyophilization-induced) protein aggregation is the formation of an intermolecular hydrogen-bonded antiparallel β-sheet structure, which is represented by a low-frequency band around 1620 cm^{-1} and an associated weaker high-frequency band around 1685 cm^{-1} (Dong et al., 1995b). For example, an aggregation band has been found at 1619 cm^{-1} for human FXIII (Dong et al., 1997) and hPAH (Chehin et al., 1998), and at 1620 cm^{-1} for recombinant human IFN-γ (Kendrick et al., 1998b). This structural transition occurs regardless of the initial composition of the secondary structure of native proteins. Therefore, these bands can be used to monitor and quantify aggregation in both aqueous and solid states. Similarly, reversibility of protein aggregation may be determined by monitoring an aggregate band. By this method, the temperature-induced aggregation of human FXIII in both D_2O and H_2O was found irreversible (Dong et al., 1997).

Another IR method for estimating protein aggregation is to compare overall spectral change of proteins, as the change may correlate with protein aggregation. The overall spectral change can be measured by fractional area overlapping of the second-derivative spectrum of a protein sample with that of a reference. Using this method, Allison et al. (1996) demonstrated that any decrease in fractional spectral area overlap of freeze-dried chymotrypsinogen directly correlates with the amount of aggregates formed after rehydration of the solid.

3.4.5. Determination of protein degradation products

A variety of analytical methods is available to determine protein degradation products. Both SEC-HPLC and PAGE can detect protein degradation products or certain fragments but these methods are usually inadequate in detecting subtle structural changes in proteins such as oxidation and deamidation. Generally, RP-HPLC is the preferred method in these cases. RP-HPLC has been widely used to separate and quantitate deamidated proteins such as GRF (Stevenson et al., 1993), insulin (Darrington and Anderson, 1995), and tPA (Paranandi et al., 1994), oxidized proteins such as hIGF-1 (Fransson et al., 1996), human leptin (Liu et al., 1998), and rhPTH (Nabuchi et al., 1995), hydrolyzed proteins such as bFGF (Shahrokh et al., 1994a), insulin (Brange et al. 1992b) and rhM-CSF (Schrier et al., 1993), isomerized and oxidized (Met^{125}) hGH (Johnson et al., 1989; Perlman and Nguyen, 1992), and succinimidated bFGF (Shahrokh et al., 1994a).

However, RP-HPLC itself is usually not capable of identifying protein degradation products. To achieve identification, RP-HPLC-separated degradation products have to be collected and further analyzed by other analytical methods such as mass spectroscopy (MS), peptide mapping and sequencing, and amino acid analysis (Johnson et

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al., 1989; Schrier et al., 1993; Shahrokh et al., 1994a; Nabuchi et al., 1995; Liu et al., 1998). A more efficient technique in use is the coupled RP-HPLC and MS (LC-MS). This technique has been used in the identification of hydrolyzed bFGF (Shahrokh et al., 1994a), and different mono-, di- and tri-oxidized human leptin (Liu et al., 1998). In addition to HPLC, capillary electrophoresis has also been coupled to MS (CE-MS) for highly sensitive analysis of proteins and peptides (Figeys and Aebersold, 1998). MS alone has been a very powerful tool in identification of protein degradation products as well as molecular weight determination (Nguyen et al., 1995). The use of tandem MS (MS–MS) may allow direct identification of partial to complete sequence for peptides up to 25 amino acids, sites of deamidation, and isomerization (Carr et al., 1991).

3.4.6. Probing protein tertiary/quaternary structure

The ultimate goal of structural studies on proteins is to gain insight into the protein's three-dimensional structure at a high-resolution level. Two delicate techniques can be used to probe protein structures: X-ray crystallography and NMR. Both methods have advantages and shortcomings. X-ray crystallography has more accuracy but needs highly-ordered crystals and a large amount of material in solid state. NMR can be applied for solutions containing an adequate amount of protein (typically millimolar) but is usually limited to small proteins (< 20 kD) (Middaugh, 1990). Recent advances in multidimensional NMR methodology can now permit structural determination of larger proteins up to 60 kD (Clore and Gronenborn, 1998). Other indirect techniques for probing structures of large proteins by NMR include determination of the rate of hydrogen exchange (Raschke and Marqusee, 1998) or structures of 'dissected' protein subunits or domains (Campbell and Downing, 1998).

Other methods of probing protein tertiary structure include CD in the near UV region, fluorescence spectroscopy, and second-derivative UV spectroscopy. However, these methods generally do not provide detailed information about structure. Nevertheless, CD has been used to demonstrate destabilization of methionyl pGH in the presence of either poloxamer 407 (Pluronic F127) or 188 (Pluronic F68) (Charman et al., 1995), and to monitor changes in the tertiary structure of β-lactoglobulin upon heating (Matsuura and Manning, 1994). Second-derivative UV spectroscopy in combination of SEC-HPLC has been used to monitor the relative solvent exposure of Tyr residues in IL-2 mutants (Ackland et al., 1991).

4. Stabilization and formulation of liquid protein pharmaceuticals

Proteins in extremophilic organisms can tolerate one or more of the following stresses: low or high temperatures, high hydrostatic pressure, high salinity, and extreme pHs, even though their building blocks are exclusively the canonical 20 natural amino acids. Therefore, significant room exists to stabilize any unstable protein pharmaceuticals. The central issue in protein stabilization is preservation of the functional state of proteins under various stressful conditions.

4.1. Means and mechanisms of protein stabilization

Proteins may be stabilized either by changing their structural characteristics (internally) or by changing the properties of solvent in contact with them (externally). Structural changes may increase the protein's unfolding free energy change (ΔG_{mut}), leading to its stabilization with a free energy change $\Delta\Delta G = \Delta G_{mut} - \Delta G_{nat}$. Since the difference between mesophilic and thermophilic proteins is small in terms of $\Delta\Delta G$ (5–7 kcal/mol), stabilization of mesophilic proteins to the level of thermophilic proteins is feasible (Querol et al., 1996). To estimate the degree of internal or external stabilization, the change in the protein's unfolding temperature (T_m) is often used as an indicator. A minimum increase of 1°C in T_m is considered as the threshold of thermostability increase (Querol et al., 1996).

4.1.1. Mechanisms of internal protein stabilization

As discussed in Section 2.1, native proteins are stabilized by many forces. Any structural change

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that increases any of the stabilizing forces without changing the overall protein conformation generally stabilizes proteins (Jaenicke, 1991). Protein thermal stability usually increases if amino acid substitution/modification results in increased internal and decreased external (or surface) hydrophobicity (Kristjánsson and Kinsella, 1991). Increasing internal hydrophobicity often correlates with increased packing efficiency and protein stability (Mozhaev and Martinek, 1984; Vieille and Zeikus, 1996). This positive correlation was demonstrated recently in a study on a series of hydrophobic mutants of human lysozyme (Takano et al., 1998). Protein stability also positively correlates with the content of hydrogen bond-forming amino acids and the ability of these amino acids to maintain these bonds, especially at critical locations (Kristjánsson and Kinsella, 1991). However, no direct correlation has been found between protein thermal stability and protein volume (Querol et al., 1996). Increasing charge density apparently destabilizes a protein (Sindelar et al., 1998).

Local interactions of amino acids are indispensable in native proteins. The interactions may come from residues close in sequence and contribute to the secondary structure propensities. Enhancement of native local interactions makes proteins more resistant to thermal and chemical denaturation, and introduction of non-native local interactions destabilizes proteins because non-native/denatured conformations are stabilized (Prieto et al., 1997). The α-helix can be stabilized by introducing residues of high helix propensity (such as Ala), which eventually stabilizes proteins (Querol et al., 1996; Vieille and Zeikus, 1996).

Thermostable proteins are less flexible than thermolabile ones (Daniel et al., 1996). Protein stability inversely correlates with its flexibility (Tang and Dill, 1998). The flexible regions in proteins are typically the labile areas (Vieille and Zeikus, 1996). Some inactivation processes, such as oxidation of Cys and sensitive Met, hydrolysis of peptide bonds at Asp residues, and deamidation of Asn and Gln residues, are more likely to occur in flexible (or solvent-accessible) areas of the protein (Powell, 1996; Querol et al., 1996). These areas should be considered first for structural modification/stabilization.

4.1.2. Mechanisms of external protein stabilization

There are two mechanisms of solvent-induced stabilization of proteins: (1) strengthening protein-stabilizing forces; and (2) destabilizing the denatured state. This is to say that any change in solvent property that enhances a native interaction or perturbs a non-native interaction increases stability of proteins (Wrabl and Shortle, 1996). Under non-native conditions, proteins may still be stabilized as long as the condition favors strengthening of non-covalent interactions, especially hydrophobic interactions. These non-native conditions, such as concentrated solutions of salt, polyols, or certain organic solvents can enhance hydrophobic interactions and stabilize proteins by altering the structure of water and reducing protein interaction with the solutes (Mozhaev and Martinek, 1984; Kristjánsson and Kinsella, 1991; Brange et al. 1997). Although an excipient(s) stabilizes a protein mostly by changing solvent property, some may interact favorably with charged sites on the protein surface and minimize electrostatic protein–protein interactions to achieve protein stabilization (Remmele et al., 1998).

4.1.3. Preferential Interaction—a major stabilization mechanism of excipients

The most tenable and widely-accepted mechanism of protein stabilization in aqueous solution is preferential interaction of proteins. Preferential interaction means that a protein prefers to interact with either water or an excipient (co-solute/co-solvent). In the presence of a stabilizing excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the protein domain (preferential exclusion). In this case, proportionally more water molecules and fewer excipient molecules are found at the surface of the protein than in the bulk (Arakawa et al., 1991, 1993; Timasheff, 1993, 1998; Lin and Timasheff, 1996).

Preferential interaction of a protein with an excipient has been defined as:

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \text{ or } (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$$

in which 1, 2, and 3 represent water, protein, and excipient, respectively, and μ is the chemical po-

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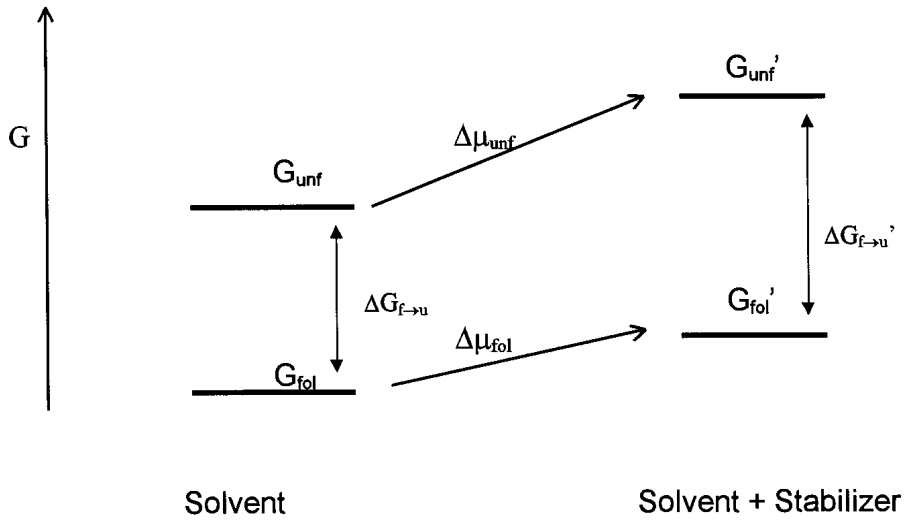


Fig. 1. Diagram of free energy change of a protein in the presence of a protein stabilizer

tential (Timasheff, 1992, 1993, 1998). The interaction can be expressed on a gram basis (g) or on a molar basis (m). Preferential interaction of a protein with either water or an excipient is the consequence of a difference in affinity of a protein for water or an excipient. If there is a preference for an excipient, the measured interaction is positive, preferential binding. In the opposite case, the measured interaction is negative, preferential hydration and the protein is stabilized. Sucrose is a commonly-used protein stabilizer, and the preferential interaction of rhIL-1ra and α-chymotrypsin in 1 M sucrose is, respectively, -0.164 g sucrose/g protein (Kendrick et al., 1997) and -7.6 mol sucrose/mol protein (Timasheff, 1993). In a recent study on stabilization of RNase by sucrose, Liu and Bolen (1995) have found that the protein side chains actually favor exposure to sucrose relative to water, facilitating protein unfolding, and that it is the highly unfavorable exposure of polypeptide backbone on unfolding that leads to protein stabilization by sucrose. In contrast, peptide backbone exposure is favorable in the presence of strong denaturants such as urea and GdnHCl because they preferentially bind to the backbone.

Stabilizing preferential interaction of a protein with an excipient is usually associated with an increase in chemical potential of the protein, which is thermodynamically unfavorable (Carpenter et al., 1990; Timasheff, 1993; Kendrick et al., 1997). This means that protein interaction with the excipient is unfavorable relative to water; hence, the protein is preferentially hydrated and the excipient is preferentially excluded from the protein surface. The positive change in chemical potential is marginal at low excipient concentrations and becomes more positive at higher concentrations. The increase in chemical potential is directly proportional to the surface area of the protein exposed to solvent. Since a denatured protein has a greater surface area than a native protein, preferential exclusion of the excipient from the surface of the denatured protein is more unfavorable than from that of the native protein. Therefore, protein stabilization arises from destabilization of the denatured state by the excipient, leading to a larger free energy change ($\Delta G_{f\to u}$). Fig. 1 shows the free energy changes associated with this type of protein stabilization; a stabilizer destabilizes the unfolded state to a greater degree than the native state.

Stabilization of proteins in solution by a variety of excipients appears to correlate positively with their capability of increasing the surface tension of water. Introduction of a protein molecule into water must form additional protein–water interface, and the interfacial tension is equal to the surface tension of water after proper correction

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for curvature of the protein surface (Kita et al., 1994). Substances lowering the surface tension of water accumulate at the protein surface; those raising it are kept away from the protein surface. It has been found that most stabilizers for lysozyme and BSA increase the surface tension of water, which also correlates with the increase in chemical potentials of the two proteins (Kita et al., 1994).

However, the surface tension effect of an excipient is not the sole factor involved in the protein–solvent interactions. Increasing the surface tension of water by an excipient does not necessarily mean automatic protein stabilization. Surface tension-derived stabilization can be overcome by weak binding of these excipients to proteins. For example, Arg hydrochloride (ArgHCl) increases the surface tension of water but destabilizes RNase A because ArgHCl binds to the protein, as indicated by a decreasing preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, with increasing ArgHCl concentration (Lin and Timasheff, 1996). Urea and $MgCl_2$ increase the surface energy of water but are preferentially bound to proteins at high concentrations (Kita et al., 1994). On the other hand, excipients decreasing surface tension of water may still stabilize a protein. Betaine and glycerol decrease the surface energy of water but induce protein preferential hydration (Kita et al., 1994). Four organic solvents, PEG, 2-methyl-2,4-pentanediol (MPD), DMSO, and TMAO, decrease the surface tension of water but induce preferential hydration of lysozyme and BSA (Kita et al., 1994). This has been attributed to the presence of two types of agents which are preferentially excluded: (1) those that are totally independent of the chemical nature of protein surface and excipient concentration; and (2) those that are not (Timasheff, 1993, 1998). The first-type agents achieve preferential exclusion either by increasing the surface free energy of water or by steric exclusion. The second-type agents rely on the solvophobic effect such as glycerol, PEG, 2-methyl-2,4-pentanediol (MPD), and may stabilize or destabilize a protein depending on its concentration and solvent conditions (Kita et al., 1994; Frye and Royer, 1997).

Alternative explanations for excipient-induced protein stabilization have been offered recently through studies on hydrogen exchange (HX) in proteins by NMR. Different types of protons have different rates of HX in a protein, including slow-exchanging amide protons mainly from the compact unfolded state and the fast and intermediate amide protons mainly from the native state (Wang et al., 1995). Sucrose at 1 M decreases the HX rate of slow-exchanging amide protons by decreasing the population of unfolded protein, suggesting that sucrose may favor protein folding (Wang et al., 1995). An increased rate of folding is also the primary reason for stabilization of staphylococcal nuclease by xylose (Frye and Royer, 1997).

The H-D exchange rate and solvent accessibility are considered a measurement of protein flexibility, which inversely correlates protein stability (Tang and Dill, 1998). It has been found that both the H-D exchange rate in IL-1ra and the reactivity of Cys (free thiol groups) with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NbdCl) are reduced by increasing sucrose concentrations, suggesting reduction of solvent accessibility and conformational mobility of rhIL-1ra by sucrose (Kendrick et al., 1997). The apparent hydrodynamic diameter of the protein also decreases gradually with increasing sucrose concentration, which indicates increased packing of the protein structure and decreased solvent accessibility.

4.2. Stabilization of proteins by excipients

The most common method for stabilizing liquid protein pharmaceuticals is the use of a formulation excipient(s), which should be at least soluble and nontoxic. These excipients are also referenced as chemical additives (Li et al., 1995c), co-solutes (Arakawa et al. 1993) or co-solvents (Timasheff, 1993, 1998; Lin and Timasheff, 1996). These excipients can also retard certain chemical degradations in proteins. Their stabilizing effects are usually concentration- and protein-dependent, although high concentrations of excipients may not be necessarily more effective, and in some cases, can have negative effects. For example, sorbitol reduces heat-induced aggregation of aFGF at

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55°C effectively at concentrations below 0.5 M but is less effective at higher concentrations (Tsai et al., 1993).

Protein-stabilizing excipients can be broadly divided at least into the following types: sugars and polyols, amino acids, amines, salts, polymers, and surfactants. Their stabilizing effects can be significantly different (Table 4). For example, the amounts of excipients required to reduce 50% of heat-induced aggregation of aFGF are 0.55 mM for sodium sulfate, 1.6 mM for His, 180 mM for Gly, 155 mM for trehalose, 250 mM for dextrose and 310 mM for sorbitol (Tsai et al., 1993).

4.2.1. Stabilizing buffer

Usually, a protein is stable only in a very narrow pH range, such as recombinant FVIII SQ, whose stable pH range is between 6.5 and 7 in 0.1 or 1 M NaCl solution (Fatouros et al., 1997a). Accurate control of the pH of a protein formulation is the first step toward protein stabilization. Unfortunately, no general rules have been reported for buffer selection. In a few cases, phosphate buffer has been shown to be detrimental. About 38% of hEGF at 0.5 μg/ml are deamidated in Tris–HCl at pH 7.0 on incubation at 60°C for 2 days, and the amounts of deamidated hEGF increase to 83, 63, 52, 51, and 49%, respectively, in buffers of PBS, sodium phosphate, sodium borate, sodium citrate, and sodium acetate (Son and Kwon, 1995). Similarly, at 37°C, rhKGF in 100 mM phosphate buffer (pH 7.0) has a half-life of 15 days, but the half-life increases to 25 and 88 days, respectively, in 100 and 500 mM sodium citrate buffers (Chen et al., 1994a). The better stability of rhKGF in citrate buffer may be due to the favorable interaction of citrate with positively charged residues in rhKGF as hypothesized in the stabilization of pig heart mitochondrial malate dehydrogenase (*phm*-MDH) (Jensen et al., 1996). In a different study, however, increasing citrate buffer concentrations increase the formation of bFGF particles at pH 5, and the citrate buffer also causes aggregation of bFGF at pH 3.7, whereas acetate buffer at pH 3.8 does not (Wang et al., 1996b). On the other hand, phosphate has been shown to stabilize certain proteins. Increasing phosphate buffer concentration from 4.6 to 9.2 mg/ml significantly decreases the rate of aFGF aggregation (Won et al., 1998). In the presence of 0.1 or 1 mM phosphate, the GdnHCl concentration for unfolding 50% alkaline phosphatase molecules is increased from 2.46 to 2.67 and 2.72 M, respectively (Hung and Chang, 1998). Phosphate at 0.05 M can also stabilize maize leaf phosphoenolpyruvate carboxylase (*ml*-PEPC) upon incubation at 40°C, although it is not as effective as citrate at the same concentration (Jensen et al., 1996).

Both buffer species and concentration may affect physical and chemical stability of a protein. Pikal et al. (1991) examined the effect of three phosphate buffer concentrations (0.11, 0.23 and 0.45 mg sodium phosphate/mg hGH) on the stability of hGH. While maximum aggregation occurs at 0.23 mg sodium phosphate/mg hGH, chemical degradations (oxidation and deamidation) are less significant at both 0.23 and 0.45 mg sodium phosphate/mg hGH. At a lower buffer concentration of 0.11 mg sodium phosphate/mg hGH, chemical degradation is relatively high. Therefore, a balanced buffer concentration should be selected to maximize protein stability. An ideal buffer should be able to inhibit both physical and chemical instabilities simultaneously.

4.2.2. Sugars and polyols

Sugars and polyols represent often-used nonspecific protein stabilizers. Their stabilizing effect is demonstrated and widely interpreted as the result of preferential exclusions, weak and nonspecific (Xie and Timasheff, 1997a,b; Timasheff, 1998). Among various sugars, sucrose and trehalose appear to be the most often-used stabilizers. Sucrose has been shown to effect concentration-dependent stabilization of rhDNase at pH 6.8 (Chan et al., 1996), and inhibition of IL-1ra dimer formation (Chang et al., 1996a) and GdnHCl-induced rhIFN-γ aggregation (Kendrick et al., 1998a). Similarly, trehalose is able to increase the T_m of RNase A (Lin and Timasheff, 1996), to inhibit heat-induced aggregation of aFGF (Tsai et al., 1993), and to stabilize rhDNase in a concentration-dependent manner (Chan et al., 1996).

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Table 4
Examples of protein stabilization by excipients in liquid state

Proteins	Formulation compositions	Study conditions	Remaining activity (or as stated)	References
Baker's yeast ADH	50 μg/ml in 20 mM potassium phosphate, pH 7.6	50°C for 10 min	50%	Ramos et al., 1997
	Control + 500 mM KCl	50°C for 10 min	~90%	Ramos et al., 1997
	Control + 500 mM trehalose	50°C for 10 min	~70%	Ramos et al., 1997
	Control + 500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
hEGF	0.5 μg/ml in 50 mM sodium phosphate, pH 8.0	60°C for 1 day	49%	Son and Kwon, 1995
	Control + 0.02% Triton X-100	60°C for 1 day	84%	Son and Kwon, 1995
	Control + 0.01% Tween 20	60°C for 1 day	75%	Son and Kwon, 1995
	Control + 0.05% fibronectin	60°C for 1 day	85%	Son and Kwon, 1995
	Control + 6 mM $ZnCl_2$	60°C for 1 day	76%	Son and Kwon, 1995
GDH	50 μg/ml in 20 mM potassium phosphate, pH 7.5	50°C for 10 min	0%	Ramos et al., 1997
	Control + 500mM KCl	50°C for 10 min	~90%	Ramos et al., 1997
	Control + 500 mM trehalose	50°C for 10 min	~2%	Ramos et al., 1997
	Control + 500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
rIL-2	0.2 μg/ml in PBS, pH 7.0	Moderate shaking at 4°C for 96 h	~30%	Wang and Johnston, 1993a
	+10% (w/w) Pluoronic F-127	Moderate shaking at 4°C for 96 h	100%	Wang and Johnston, 1993a
rhKGF	0.5 mg/ml in 140 mM NaCl and 10 mM sodium phosphate, pH 7.0	37°C	$T_{1/2}$ + 2 days (loss of soluble aggregates)	Chen et al., 1994a
	0.5 mg/ml in 100 mM sodium phosphate, pH 7.0	37°C	$T_{1/2}$ + 15 days	Chen et al., 1994a
	0.5 mg/ml in 100 sodium citrate buffer, pH 7.0	37°C	$T_{1/2}$ + 25 days	Chen et al., 1994a
	0.5 mg/ml in 500 sodium citrate, pH 7.0	37°C	$T_{1/2}$ + 88 days	Chen et al., 1994a
	0.5 mg/ml in 10 mM sodium phosphate, 140 mM NaCl, and 0.5% (w/v) heparin, pH 7.0	37°C	$T_{1/2}$ + 105 days	Chen et al., 1994a
Rabbit muscle LDH	50 μg/ml in 20 mM potassium phosphate, pH 7.6	50°C for 10 min	5%	Ramos et al., 1997
	Control + 500 mM KCl	50°C for 10 min	~20%	Ramos et al., 1997
	Control + 500 mM trehalose	50°C for 10 min	~25%	Ramos et al., 1997
	Control + 500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
Urease	0.33 mg/ml in phosphate buffer and 1 mM EDTA, pH 7.0	Moderate shaking at 4°C for 96 h	~40%	Wang and Johnston, 1993a
	Control + 10% (w/w) Pluoronic F-127	Moderate shaking at 4°C for 96 h	100%	Wang and Johnston, 1993a
Urease	0.33 mg/ml in phosphate buffer and 1 mM EDTA, pH 7.0	50°C for 96 h	AUC = 4868 (activity-time plot)	Wang and Johnston, 1993b
	Control + 1% Pluoronic F-127	50°C for 96 h	AUC = 6722	Wang and Johnston, 1993b

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In all these examples, the stabilizing effect of sugars depends on their concentrations. A concentration of 0.3 M (or 5%) sugar or polyols has been suggested to be the minimum to achieve significant protein stabilization (Arakawa et al. 1993). This seems true in many cases. As high as 1 M sucrose or 10% glycerol have been commonly used to protect the activity of proteins during their isolation (Timasheff, 1993). Other examples include stabilization of thrombin by 20% dextrose or sorbitol or 50% glycerol (Boctor and Mehta, 1992) and rhG-CSF by 5% mannitol (Herman et al., 1996), inhibition of bFGF aggregation by $\geq$ 30% sucrose (Wang et al., 1996b), IL-1ra dimer formation by 40% (w/v) sucrose (Chang et al., 1996a), and IgG aggregation by 33% sorbitol (Ganzález et al., 1995), and significant increase in the T_m of RNase A by 0.5–1 M sucrose or sorbitol (Liu and Sturtevant, 1996; McIntosh et al., 1998) and porcine pancreatic elastase by 20% sucrose, sorbitol, lactose, or mannitol (Chang et al., 1993).

Different sugars or polyols may stabilize a protein to a similar or different degree depending on the protein. It has been found that lactose, sucrose, trehalose, or mannitol stabilizes rhDNase at pH 6.8 to a similar degree based on their effect on the T_m (Chan et al., 1996). In a different case, while 0.55 M sucrose increases the T_m of RNase from 35.5 to 44.9°C, the same concentration of sorbitol only increases the T_m to 40.5°C (McIntosh et al., 1998). Similarly, different concentrations of excipients (155 mM trehalose, 250 mM dextrose, or 310 mM sorbitol) are required to reduce 50% of heat-induced aggregation of aFGF (Tsai et al., 1993). Sucrose at 10, 300, or 600 mg/ml is more effective than sorbitol or mannitol in the stabilization of FVIII SQ (Fatouros et al., 1997b).

Sugars and polyols can also protect proteins from chemical degradations. For example, Li et al. (1996a,b) studied the effect of sugars and polyols on the metal-catalyzed oxidation of human relaxin and found that sugars and polyols, such as glucose, mannitol, glycerol, and ethylene glycol, can significantly inhibit the oxidative degradation. The inhibitory effect was shown to be due to their complexation with metal ions instead of the commonly-accepted radical-scavenging mechanisms. Due to the weak complexation, as high as 50 mM mannitol is needed to have a significant protective effect.

Not all proteins can be stabilized by sugars or polyols. At 37°C, the half-life for the loss of soluble rhKGF in 10 mM phosphate buffer (pH 7.0) and 140 mM NaCl is 1.8 days and replacing NaCl with 9% (w/v) sucrose or 5% sorbitol does not improve its half-life (Chen et al., 1994a). Sorbitol does not show any effect against interfacial denaturation of pGH and is marginally effective to prevent precipitation by thermal and quanidine dilution denaturation (Charman et al., 1993). Mannitol at 5 mg/ml even destabilizes IL-1R by decreasing its T_m from 48.1 to 46.7°C (Remmele et al., 1998).

In the selection of sugars for protein stabilization, reducing sugars should be avoided whenever possible, as these sugars have the potential to react with amino groups in proteins via the Maillard reaction (see Section 3.2).

4.2.3. Surfactants

There are basically two types of surfactants—nonionic and ionic. These surfactants drop surface tension of protein solutions and decrease the driving force for protein adsorption and/or aggregation at hydrophobic surfaces.

Nonionic surfactants are generally preferred in protein stabilization. Low concentrations of nonionic surfactants are often sufficient to prevent or reduce protein surface adsorption and/or aggregation due to their relatively low critical micelle concentrations (CMC) (Bam et al. 1995). Complete or significant inhibition has been reported of surface adsorption loss of TGF-β_1 by Tween 80 at 0.01% (Gombotz et al., 1996), glass surface-induced rConIFN aggregation by Tween 20 or Tween 80 at 0.01% (Ip et al., 1995), shear (or shaking)-induced rhGH aggregation by Pluronic F88 or Tween 20 at 0.1 mg/ml (or at $\geq$ 4:1 Tween 20:rhGH molar ratio) (Maa and Hsu, 1997; Bam et al., 1998), shaking-induced hemoglobin aggregation by Tween 80 at 0.045% (Kerwin et al., 1998), vortex-induced hGH aggregation by Tween 80, Brij 35, or Pluoronic F68 at respective concentrations of 0.1%, 0.013%, and 0.1% (Katakam et al., 1995), refolding-induced rhGH aggregation by

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Tween 20, 40, and 80 at a Tween:rhGH molar ratio of 10 (Bam et al., 1996), shaking-induced loss of urease and rIL-2 activity by poloxamer 407 (Pluoronic F-127) at 10% (w/w) (Wang and Johnston, 1993a), and agitation-induced rhFXIII aggregation by Tween 20 at around 0.007% (w/v) (Kreilgaard et al., 1998). Tween 20 seems to be relatively more effective in a few cases. The rank order of minimizing rhG-CSF adsorption to PVC surface is Tween 20 > Tween 80 > Pluronic F127 > Pluronic F68 (Johnston, 1996). Tween 20 is also more effective than Tween 80 or Tween 40 in the inhibition of shaking-induced aggregation of rhGH (Bam et al., 1998). Not all proteins can be stabilized by surfactants and Tween 80 at 0.1% has no effect on the aggregation of IL-1β at 100 μg/ml at pHs from 3 to 7 (Gu et al., 1991).

Nonionic surfactants can also inhibit chemical degradations in proteins. hEGF degrades mainly by deamidation and loses 51% of its mitogenic activity in 50 mM sodium phosphate buffer (pH 8.0) during storage at 60°C for 1 day, and in the presence of 0.02% Triton X-100 or 0.01% Tween 20, the remaining activity is, respectively, 84 and 75% (Son and Kwon, 1995). At the same time, surfactants may be contaminated with alkyl peroxides, which can accelerate oxidation of proteins. rhCNTF has been found to dimerize by alkyl peroxides present in Tween 80 (Knepp et al., 1996). Inclusion of certain antioxidants, such as Cys, Met, or glutathione, can significantly retard the reaction. A correlation between the peroxide level in Tween 80 and the degree of oxidation in rhG-CSF has also been reported, and the peroxide-induced oxidation appears more severe than that induced by atmospheric oxygen present in the vial headspace (Herman et al., 1996). For this reason, only 0.004% Tween 80 is formulated in the liquid rhG-CSF formulation.

Ionic surfactants are usually not used in stabilizing proteins because they can bind to both polar and nonpolar groups in proteins and cause denaturation (Giancola et al., 1997). Anionic surfactants such as SDS generally bind more effectively than cationic detergents such as decyltrimethyl ammonium chloride. The binding of ionic detergents such as SDS to a protein is a function of the free detergent concentration and may become saturated beyond the critical micelle concentration (Volkin and Klibanov, 1989). Nevertheless, these surfactants have been reported to have stabilizing effect on proteins. It has been found that SDS stabilizes BSA up to a SDS/protein molar ratio of 10 (Giancola et al., 1997) and reduces aggregation of aFGF in a concentration-dependent manner (Won et al., 1998). SDS at either 0.1 or 1% effectively prevents aggregation of heat-denatured RNase at pH 7.8, possibly by increasing repulsion of the added negative charges on the protein (Tsai et al., 1998b). Anionic detergents have also been used to increase thermal stability of proteins in non-aqueous solvents by stoichiometric formation of complexes with proteins (hydrophobic ion pairing) (Manning et al., 1995). In addition, certain ionic (including nonionic) surfactants in a limited concentration range may help protein refolding. Rhodanese at 1 mg/ml denatures in a mixture of 6 M GdnHCl and 200 mM β-mercaptoethanol, and the denatured enzyme at 50 μg/ml can be reactivated to various extents in the presence of 1 mg/ml ionic (sodium cholate, cetyltrimethylammonium bromide, sodium lauryl sulfate), zwiterionic (*N*-octyl-, *N*-decyl, *N*-dodecyl-, *N*-tetradecyl- and *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate), and nonionic surfactants (Tween 20, Triton X-100, dodecyl β-D-maltoside) (Tandon and Horowitz, 1987). The activity recovery of the denatured enzyme is bell-shaped as a function of cetyltrimethylammonium bromide concentration in the range of 0–5 mg/ml, indicating different effects at different concentrations.

Nonionic surfactants also bind weakly to proteins. The binding stoichiometry between Tween 20 and rhGH has been determined to be 2.5–3.5 (Tween 20/rhGH) by electron paramagnetic resonance (EPR) (Bam et al., 1995). A recent study shows that complete blocking of shaking-induced rhGH aggregation can be achieved only at a Tween 20:rhGH molar ratio of $\geq$ 4:1 (Bam et al., 1998). This molar ratio is close to the binding stoichiometry, suggesting that the inhibition of rhGH aggregation is probably due to Tween 20 binding to and blocking the aggregation-prone hydrophobic sites on the protein surface. Since the Tween 20 concentration required for complete

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protection of the protein is much higher than the reported CMC of Tween 20 (59 μM), CMC does not appear to play a critical role. Therefore, the net effect of surfactants may depend on their concentrations relative to that of the protein (Katakam et al., 1995). In a different case, maximum protection of rhFXIII from agitation-induced aggregation is found at Tween 20 concentrations close to its CMC, independent of initial protein concentration (Kreilgaard et al., 1998). Therefore, additional mechanisms of protein protection by Tween 20 exist, which seem to arise from its competition with proteins for adsorption on denaturation interfaces.

Surfactant–protein interactions are hydrophobic in nature because proteins with more hydrophobicity bind more surfactants (Bam et al., 1995). Therefore, it has been suggested that surfactants should be avoided when formulating hydrophobic proteins (Dix et al., 1995). Since hydrophobic interaction increases with increasing temperature, a stronger hydrophobic protein–surfactant interaction is expected at high temperatures. This may explain why nonionic surfactants destabilize certain proteins, especially at higher temperatures or toward thermal denaturation. For example, poloxamer 407 at 1% significantly increases the stability of urease at 0.33 mg/ml in phosphate buffer (pH 7.0) at 37 or 50°C but decreases its stability at 75°C (Wang and Johnston, 1993b). This surfactant at 0.5% also decreases the stability of IL-2 at about 0.2 μg/ml in phosphate buffer (pH 7.0) at 50°C (Wang and Johnston, 1993b). rhCNTF, a hydrophobic and physically unstable protein, is destabilized by Tween 80 when subjected to thermal incubation (Dix et al., 1995). Either Pluoronic F68 at 0.1 mg/ml or Tween 80 at 1 mg/ml destabilizes IL-1R by decreasing its T_m (Remmele et al., 1998). Decreased T_m is also observed for rhGH at 3 mg/ml in the presence of Tween 80, Tween 40, or Tween 20 at a surfactant:rhGH molar ratio of 10:1 (Bam et al., 1998).

Poloxamers 407 and 188 (Pluronic F68) have been shown to inhibit thermally-induced aggregation/precipitation of recombinant hGH or methionyl pGH (Charman et al., 1995; Katakam and Banga, 1997). In comparison with Tweens, poloxamers may increase viscosity of a protein solution, restraining the motion of protein backbone to achieve thermal stabilization (Wang and Johnston, 1993a).

4.2.4. Salts

As discussed in Section 3.3, salts may stabilize, destabilize, or have no effect on protein stability depending on type and concentration of salt, nature of the ionic interactions, and the charged residues in a protein (Kohn et al., 1997). The net effect is a balance among non-specific (Debye-Hückel) electrostatic shielding, specific ion binding to a protein, and its effect on solvent properties.

The effect of salt at high concentrations correlates with the Hoffmeister lyotropic series both for anions and cations (Volkin and Klibanov, 1989; Timasheff, 1993, 1998):

$$(CH_3)_4N^+ > NH_4^+ > K^+, Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+} > GdnH^+$$

$$SO_4^{2-} > CH_3CO_2^- > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

in which anions and cations to the left of the series are the most stabilizing. By increasing ionic strength of a solution, these salts enhance hydrophobic interactions and reduce solubility of hydrophobic groups in proteins. In addition, they enhance accumulation of water molecules around proteins, leading to preferential hydration. The combination of these two effects makes proteins more compact and stable. Ions on the right are destabilizing by binding extensively to the charged groups or the dipoles (peptide bonds) of proteins.

NaCl, the often-used salt, has been found to play a critical role in stabilizing certain proteins. It increases both the denaturation temperature and enthalpy of BSA due to increased compactness of the protein structure resulting from an increase in ionic strength and possible reduction in long-range electrostatic repulsion between the net charges (Giancola et al., 1997). Recombinant factor VIII SQ (rFVIII SQ) at 125 IU/ml is not stable at low salt concentrations and precipitates at <0.1 M NaCl at pH 7 (Fatouros et al., 1997a). A higher amount of rFVIII SQ needs higher NaCl concentrations to prevent precipitation. Increasing NaCl concentration (up to 1 M) also linearly increases the T_m of RNase T1 from 59 to 63°C (Mayr and Schmid, 1993). Recently, NaCl has been found to be the most effective in improving

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the thermal stability of IL-1R among all the excipients tested, including ascorbic acid, sugars (mannitol, lactose, sucrose, glucose), polyols (PEGs, glycerol, ethanol), polymers (PVP 10k, dextran 40), salts ($CaCl_2$), amino acids (Gly, Lys, Cys, Ala, Arg) and surfactants (Pluoronic F68, Tween 80) (Remmele et al., 1998).

Another similar salt, KCl, is also a powerful protein stabilizer. It has been shown to protect the activity of yeast ADH and bovine liver glutamate dehydrogenase (GDH) against thermal inactivation more effectively than trehalose, a commonly-used nonspecific protein stabilizer (Ramos et al., 1997).

The separate contribution of anions and cations in salts to the stability of a protein can be significantly different or even in opposite direction (Timasheff, 1998). For example, GdnHCl at 3 M destabilizes RNase at pH 7.0 by decreasing its T_m from 60 to 25°C, but $(GdnH)_2SO_4$ at 3 M stabilizes the protein by increasing its T_m by 10°C due to the dominant contribution of stabilizing SO_4^{2-} (Timasheff, 1993). GdnHCl at low concentrations (up to 0.3 M) stabilizes RNase T1 against thermal and urea-induced unfolding, probably due to the significant contribution of the stabilizing effect of Cl^- (Mayr and Schmid, 1993). This also seems to be the case in the stabilization of *ml*-PEPC (Jensen et al., 1996),

4.2.5. Polyethylene glycols (PEGs)

PEGs are often used as protein cryoprotectants and precipitating/crystallizing agents in aqueous media at high concentrations. They are hydrophobic in nature and may interact with hydrophobic side chains in proteins, promoting unfolding, especially at high temperatures (Farruggia et al., 1997). Nevertheless, PEGs of different molecular weights have been demonstrated to stabilize certain proteins. For examples, PEG 300 at 0.5 or 2% inhibits the aggregation of rhKGF in 10 mM potassium phosphate buffer (pH 7.0) containing 5% mannitol at 45°C (Zhang et al., 1995). PEG 200, 400, 600, and 1000 stabilize lysozyme and BSA, even though they decrease the surface tension of water (Kita et al., 1994). PEG 4000 (up to 15%) inhibits the thermal aggregation of LMW-UK in a concentration-dependent manner (Vrkljan et al., 1994).

The stabilization mechanism of PEGs in solution has not been well established. Stabilization seems dependent on the protein and the size of PEGs. Liquid IL-1R is stabilized by PEGs, and the stabilization effect is PEG 300 > 1000 > 3350, possibly because PEG 300 is the least hydrophobic (Remmele et al., 1998). On the contrary, PEG 1000 and 4000 destabilize human albumin by decreasing its thermal transition temperature, while high-molecular-weight PEG 8000 and 10 000 stabilize the native state of the protein (negative preferential interaction with the protein) (Farruggia et al., 1997). Similarly, PEG 8000 at 1% significantly reduces the nebulization-induced aggregation and glass surface adsorption of rConIFN (Ip et al., 1995). Protein stabilization by high-molecular-weight PEGs is probably due to their steric hindrance of protein-protein interactions.

4.2.6. Polymers

Various kinds of polymers have been shown to stabilize proteins. Stabilization is generally due to one or more of these properties of polymers: surface activity, preferential exclusion, steric hindrance of protein–protein interactions, and increased viscosity limiting protein structural movement.

Serum albumin has been used often for inhibition of protein surface adsorption and general stabilization. However, its potential contamination with blood-borne pathogens limits its future applications. Among other polymers examined for protein stabilization, hydroxypropyl-β-cyclodextrin (HP-β-CD) appears to be one of the most valuable, partly because it is a good solubilizing agent and parenterally safe. HP-β-CD has been shown to prevent the thermal and interfacial denaturation and precipitation of pGH (Charman et al., 1993), to inhibit the aggregation of rhKGF (Zhang et al., 1995), and to stabilize IL-2 and bovine insulin (Brewster et al., 1991). Other polymers include dextran (51 and 18 kD) in increasing the T_m of thrombin (Boctor and Mehta, 1992), dextran (38 and 82 kD) in increasing the T_m of porcine pancreatic elastase (Chang, et al., 1993), PVP 10 k in increasing the T_m of IL-1R (Remmele et al., 1998), fibronectin (0.05%) in improving the

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mitogenic activity of hEGF in 50 mM sodium phosphate (pH 8.0) at 60°C (Son and Kwon, 1995), heparin (0.5%) in the inhibition of rhKGF aggregation in 10 mM phosphate buffer (pH 7.0) at 37°C (Chen et al., 1994a), 16-kD heparin in the concentration-dependent inhibition ($IC_{50} = 2$ μg/ml) of the heat-induced aggregation of aFGF in PBS at pH 7.2 (Volkin et al., 1993), gelatin type A and B (up to 1%) and hydroxyethyl (heta) starch (up to 20%) in the concentration-dependent inhibition of the thermal aggregation of LMW-UK (Vrkljan et al., 1994; Manning et al., 1995). A variety of polymers has been found effective in inhibiting the aggregation of rhKGF in 10 mM phosphate buffer (pH 7.0) containing 5% mannitol, including heparin, dextran sulfate, polyphosphoric acid, poly-L-glutamic acid, poly-L-lysine, etc (Zhang et al., 1995).

The effect of polymers on protein stability is strongly protein-dependent, and in certain cases destabilization may occur. While α-CD and γ-CD do not affect the stability of hen-egg lysozyme (HEL), some of its derivatives including M-β-CD, HE-β-CD, HP-β-CD, and HB-β-CD lower the protein's T_m (Brunchu et al., 1996). Type A gelatin at 0.1% destabilizes LMW-UK but increases its stability at higher concentrations (Vrkljan et al., 1994). PVP at 2, 5, or 10% increases the heat-induced aggregation of LMW-UK (Vrkljan et al., 1994). The failure of PVP to protect the protein could be attributed to its relatively hydrophobic nature as temperature increases. The increased hydrophobicity may promote preferential binding to proteins and facilitate protein unfolding because the denatured state has more hydrophobic binding sites than the native state.

Proteins can be stabilized by polymers through multiple electrostatic interactions. A number of sulfated polysaccharides, sulfated polymers, and poly-amino acids have been shown to stabilize aFGF against aggregation, including low-MW heparin (5 kD), sulodexide, dextran sulfate, fucoidan, pentosan polysulfate, polyvivyl sulfate, keratan sulfate, poly-Asp, and poly-Glu (Volkin et al., 1993; Won et al., 1998). Increasing the sulfation level of both heparin and sulfated β-cyclodextrin results in dramatic enhancement of their ability against thermal inactivation of aFGF. It is the sulfate ions that bind to specific sites in aFGF (in the vicinity of $Lys^{118}/Lys^{112}/Arg^{22}$ region) to achieve stabilization. Poly-Arg, a positively charged polymer, destabilizes aFGF. Detailed discussion can be found elsewhere on the structure, interaction, and specificity of aFGF polyanion binding sites (Volkin and Middaugh, 1996). By similar stabilization mechanisms, the aggregation of rhKGF, another member of the FGF family, is significantly inhibited by polyanions, including poly(acrylic acid), poly(-methacrylic acid), heparin, dextran sulfate (8 and 500 kD), and pentosan polysulfate (Chen et al., 1994b). In a recent study, as many as 13 model proteins have been stabilized to a different degree against thermal inactivation by forming multiple electrostatic complexes with selected soluble polyelectrolytes (such as DEAE-dextran) and polyhydroxyl compounds (such as ethylene glycol) (Gibson, 1996). However, the protection against aggregation of heat-denatured RNase by dextran sulfate at 0.1 or 1% is apparently due to the increased repulsion of the added negative charges on the protein (Tsai et al., 1998b).

Polymers may inhibit chemical degradations in proteins. For example, dextran can inhibit the metal-catalyzed oxidation of human relaxin (Li et al., 1996a,b). All the polyanions that protect aFGF against heat-induced aggregation also protect the protein at least partially against copper-catalyzed oxidation (Volkin et al., 1993). Among the polymers, heparin is the most effective. The protection could be due to both the dominant interaction of polymers with the protein and chelation of divalent metal cations.

Polymers used for protein stabilization should be stable enough against chemical and/or enzymatic degradations so that they can keep their stabilizing effect during storage. Unfortunately, proteins may be contaminated with trace amounts of enzymes, which may degrade a polymer stabilizer and result in loss of formulation quality or even protein stability. For example, hydroxyethylcellulose (HEC) has been formulated with recombinant bFGF. The viscosity of the formulation decreases with time due to the presence of trace amounts of a cellulase-like activity that originates from the *E. coli* lysate in the production of the protein (Shahrokh et al., 1995).

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4.2.7. Metal ions

Some metal ions (such as calcium, magnesium, and zinc) can be used as protein stabilizers as they bind to a protein and make the overall protein structure more rigid, compact, and stable (Kristjánsson and Kinsella, 1991). This is one of the mechanisms responsible for the high stability of thermophilic proteins (Mozhaev and Martinek, 1984). In addition to stabilization, metal ions may enhance the activity of a protein such as RNase H (Goedken and Marqusee, 1998). Among these metal ions, calcium is a well-known stabilizer for many enzymes at both high and low temperatures (Daniel et al., 1996).

Different metal ions may require different concentrations to achieve protein stabilization, and their specificity in protein stabilization varies. The effect of some divalent metal ions on rFVIII SQ has been investigated, including Ca^{+2}, Cu^{+2}, Mn^{+2}, Mg^{+2}, Zn^{+2}, Sr^{+2}, and Fe^{+2} at 1 and 10 mM (Fatouros et al., 1997a). At 1 mM, only Ca^{+2} and Sr^{+2} increase the stability relative to the control (0.1 mM Ca^{+2}) and Fe^{+2} sharply destabilizes rFVIII SQ. At 10 mM, Ca^{+2} and Sr^{+2} further increase the stability of rFVIII SQ up to about 50 mM. Cu^{+2}, Zn^{+2} and Fe^{+2} all sharply reduce the stability at 10 mM. Mn^{+2} and Mg^{+2} do not have any significant effect at both concentrations. In another study, the T_m of rhDNase increases with increasing Ca^{2+} ($CaCl_2$) concentrations and reaches a maximum at around 100 mM $CaCl_2$ (Chan et al., 1996). However, Mg^{2+}, Mn^{2+}, and Zn^{2+} destabilize the protein. The stabilizing effect of Ca^{2+} probably results from its binding to the protein and preventing the breaking of a disulfide bridge. Other examples of protein stabilization by metal ions include the inhibition of insulin fibrillation by calcium or zinc ions (Brange et al., 1997), the stabilization of 19 kD catalytic fragment of human fibroblast collagenase by calcium (Lowry et al., 1992), the stabilization of RNase H by Mn^{2+} (Goedken and Marqusee, 1998), the increase in T_m of porcine pancreatic elastase by 10 mM $CaCl_2$ (Chang, et al., 1993), the stability dependence of bovine pancreatic DNase on Ca^{2+} (Shire, 1996), and the increased hEGF mitogenic activity in the presence of 6 mM $ZnCl_2$ in 50 mM sodium phosphate buffer (pH 8.0) during storage at 60°C (Son and Kwon, 1995). Calcium or zinc stabilizes insulin probably by neutralizing negative charges in the center of insulin hexamer and enhancing native hydrophobic interaction. In a different case, $CaCl_2$, $MgCl_2$, and KSCN at concentrations below 0.5 M destabilize albumin and lysozyme by preferential binding to these proteins (Picó, 1996).

The effect of metal ions on protein stability can be significantly influenced by the negative counter ions. Picó (1996) studied the effect of sodium halides at 0.3 M on the stability of human serum albumin in 20 mM sodium phosphate (pH 7.4) and found that the negative ions increase the melting temperature and enthalpy of unfolding of albumin in the following order: $I^- > Br^- > Cl^- > F^-$.

4.2.8. Amino acids

Certain amino acids, either alone or in combination with other excipients, stabilize proteins most likely by preferential exclusion (Jensen et al., 1996). The aggregation of rhKGF in 10 mM potassium phosphate buffer (pH 7.0) containing 5% mannitol at 45°C can be inhibited to various degrees in the presence of the following amino acids: His, Gly, sodium aspartate, glutamate, and lysine hydrochloride (Zhang et al., 1995). Similarly, the stability of *ml*-PEPC can be significantly increased in the presence of 0.05 M sodium glycinate, sodium glutamate, sodium aspartate, or lysine hydrochloride upon incubation at 40°C (Jensen et al., 1996). Replacing 40 mM NaCl with 0.4% (w/v) L-Asp and 0.7% (w/v) L-Glu significantly increases the half-life of rhKGF from 1.8 to 2.6 and 2.4 days, respectively, for the loss of soluble protein at 37°C (Chen et al., 1994a). Gly at 1 M increases the T_m of RNase A from 65 to 68°C at pH 6.0 (Liu and Sturtevant, 1996) and at 2 M increases the $\Delta G_{f\rightarrow u}$ of cytochrome c by 17.3 kJ/mol (Foord and Leatherbarrow, 1998). Use of amino acids with propylene glycol improves the structural stability of rhCNTF (Dix et al., 1995). L-Lys and L-Arg increase the stability of IL-1R by increasing its T_m, although Gly and L-Ala decrease it (Remmele et al., 1998).

Certain chemical degradations in proteins can be reduced in the presence of certain amino acids.

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Met is an effective antioxidant. His at 2.5 mM can completely inhibit the oxidation-induced inactivation of papain by the ascorbate/Cu(II)/O_2 system, possibly through the formation of Cu-His complex (Kanazawa et al., 1994).

4.2.9. Miscellaneous compounds/approaches

The identification of certain cellular accumulants in thermophilic organisms upon temperature or salinity increase has led to the choice of some of these compounds as protein stabilizers. For example, potassium salt of cyclic diphosphoglycerate can stabilize the thermolabile GADPH at 90°C (Hensel and König, 1988). Another compound is 2-*O*-β-mannosylglycerate in the stabilization of several mesophilic proteins, including rabbit muscle LDH, baker's yeast ADH, and bovine liver GDH, which is more effective than trehalose (Ramos et al., 1997). Specifically, this compound at 500 mM keeps 90% of LDH activity while trehalose (or KCl) at the same concentration keeps about 30% activity after incubation of the protein at 50 μg/ml in phosphate buffer at 50°C for 10 min.

A few other compounds have been reported to increase the thermal stability of proteins. These include dipalmitoylphosphatidylglycerol (DPPG, an anionic lipid) for RNase and cytochrome c (Lo and Rahman, 1998), 2-bromo-2-chloro-1,1,1-trifluoroethane for serum albumin (Tanner et al., 1999), and some anionic phosphate species such as ATP, inorganic phosphates, and phosphorylated inositols for aFGF (Volkin et al., 1993). Perfluorodecalin, a non-aqueous solvent, has recently been used to stabilize plasma-derived factor IX and recombinant human α-IFN in the absence of moisture ($< 0.001\%$) (Knepp et al., 1998). Its stabilization mechanism is briefly discussed in Section 3.3.

Another potential way of stabilizing proteins is the use of so-called molecular chaperones. Folding of newly synthesized polypeptides in cells requires the assistance of molecular chaperone proteins because of the tendency of folding intermediates to aggregate at relatively high protein concentrations (Hendrick and Hartl, 1995; Lewis and Cowan, 1996; Ruddon and Bedows, 1997). For example, the GroEL minichaperone in bacteria has been found to facilitate protein folding by preventing protein aggregation and correcting protein misfolding (Golbik et al. 1998). α-Crytallin exhibits chaperone-like properties and inhibits the aggregation of denatured-reduced lysozyme (Raman et al., 1997). Recombinant human protein disulfide isomerase (rhPDI) enhances folding of C125A rhIL-2 by accelerating thiol-disulfide interchange (Du et al., 1998). Therefore, inclusion of a molecular chaperone in a protein formulation is foreseeable but seems practical only if the chaperone can be easily obtained and is safe and stable.

Reduction of dissolved oxygen in protein solutions should decrease oxygen-initiated oxidation. It has been found that replacing the air in container headspace with nitrogen reduces the loss of rFVIII SQ activity from 69 to 19% during storage at 7°C (Fatouros et al., 1997a). To prevent potential oxidation of proteins, oxygen dissolved in protein solutions can be simply and effectively reduced by a few low-temperature cycles of normal-low-normal pressure changes (Fransson et al., 1996).

Computer graphics can be used to facilitate design of more effective protein stabilizers based on proteins' steric structures. Such a molecule, benzene-1,4-disulfonic acid, has been designed to fill the center space of insulin hexamer and appears to stabilize the hexamer (Manallack et al., 1985).

4.3. Stabilization of proteins by structural modification

Although proteins are very delicate molecules, they can be made more stable by careful modification of their amino acids either chemically or genetically. Again, the key issue is preservation of protein activity during and after the modification.

4.3.1. Modification of amino acid and site-directed mutagenesis

Site-directed mutagenesis or chemical modification can be used to modify protein stability by mutating or blocking specific amino acids. Structural characteristics of labile proteins may be changed toward those of thermophilic proteins (see Section 2.3).

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After analyzing the effect of amino acid replacement on protein thermal stability, Querol et al. (1996) made the following recommendations for amino acid replacement: (1) maintaining or enhancing secondary structure propensity; (2) replacing preferentially solvent accessible residues; (3) replacing residues by introducing a negative charge at N-cap or a positive charge at the C-cap of the helix (same for β-stands); (4) substituting with residues of enhancing hydrogen bonding or van der Waals contacts; (5) favoring the formation of an additional disulfide bridge, metal binding site, or glycosylation site; and (6) substituting with Pro in loops.

Even with these clear-cut guidelines, it has not been always successful to increase protein stability simply by amino acid substitution because of the complexity of protein structure and the limited number of amino acids responsible for the extra protein stability (Daniel et al., 1996). For example, the higher thermostability of the novel bacillar ADH is apparently due to the replacement of only three amino acids ($Glu^{11} \rightarrow Lys$, $Lys^{14} \rightarrow Gln$, and $Pro^{242} \rightarrow Ala$) (Cannio et al., 1994). It is no surprise that amino acid substitution has been done often on a trial-and-error basis. Even after well-defined amino acid exchange(s), it is often difficult to predict the protein stability (Jaenicke, 1990, 1996; Lee and Vasmatzis, 1997). Recently, using tables of amino acid substitution and propensity, Topham et al. (1997) were able to correlate the predicted stability change after amino acid substitution with experimentally determined free energy difference ($\Delta\Delta G$) or difference in melting temperature between a mutant and the wild-type protein. A correlation coefficient of 0.80 is obtained for 83 staphylococcal nuclease and 68 barnase mutants. Also, mutations at the buried sites are more reliably predicted than those at the exposed or partially-buried positions, and better predictions are obtained with residues not engaged in hydrogen bonding interactions with neighboring residues than those that are.

Protein activity may change significantly after amino acid substitution. Both successful and unsuccessful examples exist in this regard. aFGF has three Cys residues (Cys-16, -83, -117), and all three single Ser mutants are as active as the wild type in the presence of heparin. In the absence of heparin, the triple mutant is the most active and stable (Volkin and Middaugh, 1996). However, substitution of Glu^{58} with Ala in the wild-type ribonuclease T1 not only destabilizes the protein by 0.8 kcal/mol ($\Delta\Delta G = -0.8$) but also removes the majority of the hydrolytic activity (Shirley et al., 1989). A more recent study on the stability of T4 lysozyme mutants shows that those mutants with increased protein stability have either abolished or reduced protein activity (Shoichet et al., 1995).

There are other concerns in modifying protein structure for increased stability. One of them is the immunogenicity of a protein, which may change significantly. Therefore, mutant proteins have to go through extensive toxicity studies if they are pharmaceutical candidates. Replacement of any hydrophilic amino acids with hydrophobic ones may change the solubility of a protein, presenting formulation challenges. Thus, any chemical or genetic modification of proteins should be carefully considered.

4.3.2. Glycosylation

Glycosylation can affect protein activity, antigenicity, solubility, proteolytic resistance, and stability (Liu, 1992). Different glycoforms frequently have different physical and chemical properties. Therefore, proteins can be properly glycosylated to increase their stability. Several stabilization mechanisms of protein glycosylation have been proposed. These include formation of hydrogen bonds with the polypeptide backbone or surface hydrophilic amino acids, and steric interaction with adjacent peptide residues (Baek and Vijayalakshmi, 1997; Runkel et al., 1998). Again, the key issue is preservation of protein activity after glycosylation.

There are many successful cases of protein stabilization by glycosylation. Glycosylation of RNase A dramatically increases its stability with its activity essentially unchanged (Baek and Vijayalakshmi, 1997). The native RNase A at 0.3 mg/ml in 10 mM phosphate buffer (pH 8.0) loses more than 90% of its original activity after incubation at 90°C for 15 min, while the glycosylated protein maintains about 75% of its activity under

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the same condition. Glycosylated α_1-antitrypsin has been found to be more resistant to urea-induced unfolding and thermal denaturation (an aggregation process) than non-glycosylated form (Kwon and Yu, 1997).

Insulin may form fibrils in solution, probably due to the presence of insulin monomer and its conformational change at a hydrophobic interface. To examine the effect of glycosylation on insulin fibrillation, several *p*-succinamidophenylglucopyranoside-insulin conjugate (SAPG-insulin) have been synthesized, including monosubstituted, disubstituted, and trisubstituted insulins. It has been found that the tendency to form insulin dimers/hexamers decreases and the physical stability increases as the number of SAPG moieties increases (Baudyš et al., 1995). This is seemingly due to increased hydrophilicity of the monomer surface and/or steric hindrance by SAPG moieties, preventing fibril formation. These derivatives also significantly increase insulin stability against shaking-induced fibrillation. While native insulin starts to form insoluble aggregates (fibrils) in about 0.5 day upon shaking at 37°C, it takes 18.8 days for $(SAPG)_3$-insulin to form any aggregates. At the same time, the bioactivity for most of these derivatives is close to the native insulin ($\pm 20\%$) except the Gly^{A1}-Lys^{B29}-substituted derivative, which shows about 65% activity of the native insulin.

4.3.3. Formation of disulfide bonds

Naturally-occurring disulfide bonds generally increase thermodynamic stability of proteins (Jaenicke, 1991; Darby and Creighton, 1997; Johnson et al., 1997). This seems to result from a reduction in the configurational entropy (ΔS_{conf}) of the unfolded state.

Crosslinking proteins (formation of external 'braces') with bi(poly)-functional agents such as glutaraldehyde and imidoesters can prevent/reduce unfolding of tertiary structure and dissociation of oligomeric proteins (Mozhaev and Martinek, 1984; Kristjánsson and Kinsella, 1991). This is true especially when a disulfide bond (or other crosslink) is created connecting two groups that are sequentially far apart but spatially close, since this creates physical constraint to unfolding (reducing entropy of the unfolded state) (Lee and Vasmatzis, 1997). For example, Matsumura et al. (1989) found that single or multiple disulfide-bonded mutants of T4 lysozyme significantly increase the T_m of the wild type. Similarly, Johnson et al. (1997) demonstrated that introduction of a single or multiple disulfide bonds increases the thermodynamic stability of barnase.

On the other hand, the added disulfide bonds may have potential stability problems particularly at a higher pH and temperature due to possible β-elimination-induced disulfide scrambling (see Section 3.2). β-Elimination of disulfide bonds has been observed for more than a dozen unrelated proteins at 100°C in the pH range of 4–8 (Volkin and Klibanov, 1987).

4.3.4. Pegylation

Conjugation of PEGs to proteins is referred to as 'PEGylation' (Katre, 1993). PEGs are mostly attached to Lys amino groups in proteins. Generally, PEG-modified proteins exhibit increased stability, improved solubility, decreased immunogenicity, increased circulation half lives, and low toxicity (Li et al., 1995b). For example, conjugation of PEG_{20000} to insulin (insulin-Gly_1-PEG_{20000} or insulin-Lys_{29}-PEG_{20000}) increases the protein's enzymatic stability in blood (Caliceti and Veronese, 1998). Conjugation of recombinant human megakaryocyte growth and development factor (rhMGDF) with PEG increases the storage stability of the protein in phosphate buffer at pH 7 (Guerra et al., 1998).

Pegylation may or may not change protein activity. The activity of recombinant consensus interferon (IFN-Con_1) does not change after pegylation (Jensen-Pippo et al., 1995). However, the *in vitro* bioactivity of interleukin-6 (IL-6) decreases linearly with increasing degree of PEG-modification, while the thrombopoietic activity of PEG-modified IL-6, in which 54% of the Lys amino groups are coupled with PEG, is more than 10 times higher (Tsutsumi et al., 1995).

PEGs are generally considered safe and have very low toxicity. However, adverse reactions such as seizure and anaphylactic shock have been seen in humans after topical or oral administration of PEG-containing formulations (Katre, 1993).

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4.4. Formulation of liquid protein pharmaceuticals

There are two major dosage forms for protein pharmaceuticals: liquid and freeze-dried solid. Generally, it is the protein stability that dictates selection of the final dosage form. Liquid formulation has advantages of simple processing, less manipulation, and easy application, whereas freeze-dried solid protein formulation is generally more stable, although lyophilization often causes significant protein instability. There are cases when proteins in a liquid formulation are as stable as or more stable than those in a solid state. For example, oxidation of Met[59] in hIGF-I is a major modification pathway, and its rate in solution is roughly the same as that in a freeze-dried formulation in air-filled vials at either 25 or 30°C (Fransson et al., 1996). Liquid mannitol formulation of IFN-γ is more stable at 5°C than a freeze-dried formulation of the same composition during storage (Perlman and Nguyen, 1992).

The science of protein formulation had been primarily empirical in the past (Middaugh, 1990). Although significant progress has been made in recent years, there is still no single pathway to follow in formulating proteins due to their structural diversities and complexities. There are several stages that require careful consideration and extensive experimentation in formulating a stable protein product. First, the route of drug administration should be addressed based on proposed indications. The remaining critical stages in protein formulation include: (1) purification, in which a protein is satisfactorily purified such that no harmful contaminants (such as proteases) exist and the process yields reproducibly stable active drug; (2) preformulation, in which physicochemical properties (such as protein p*I* and solubility), excipient compatibility, aggregation, and/or decomposition pathways are performed; and (3) final formulation, in which several key issues should be addressed, including choice of buffer, pH, stabilizers, and other excipients, stability-indicating assays, decomposition pathways, and optimal storage conditions.

4.4.1. Route of drug administration

Bolus intravenous injection generally requires an aqueous protein solution. This solution, usually not exceeding 10 ml, can be either a liquid formulation or reconstituted freeze-dried formulation. The tonicity of the solution is not a great concern since the volume is small. Other types of solutions, such as suspensions, emulsions, and cosolvent or oleagenous systems, may be used for intramuscular (up to 3 ml) or subcutaneous (up to 2 ml) injection. The buffer and pH in these systems should be carefully adjusted to alleviate irritation and provide maximum protein solubility and stability.

4.4.2. Preformulation

To develop a protein formulation successfully, the basic properties of a protein should be understood thoroughly. These include protein purity, p*I*, and solubility at different pHs. Other critical issues can then be addressed, including the effect of different buffer systems, pH, tonicity modifiers, stabilizers, freeze/thaw cycles, and protein adsorption. Before conducting any preformulation studies, the purity of a protein should be adequate; usually a purity level of 95% or better is recommended (Berry, 1996).

4.4.3. Composition of a liquid protein formulation

Multiple excipients are often required in a liquid protein formulation, although a single-excipient protein formulation may be sufficient, such as tPA, which is formulated in 0.5 M arginine–phosphate buffer (pH 7.3) (Overcashier et al., 1997). Both the type and level of excipients can significantly affect protein stability. These excipients include buffering agents, stabilizers, tonicity modifiers, and antimicrobial agents. Solubilizers should be used if a protein has limited solubility, such as rIFN-β-1b, which has a solubility of < 0.05 mg/ml at neutral pH and is solubilized by SDS or Tween 80 (Lin et al., 1996c). To ensure adequate solubility of a protein, the formulation pH should be at least 0.5 unit below or above its p*I* (Cleland et al., 1993). When multiple excipients are used, they should not interact with one another, and, more importantly, should not adversely affect protein stability. Thus, compatibility studies should be conducted.

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Almost without any exception, an optimum pH (or a range) is required for maximum protein stability, and this pH may shift depending on the presence of other excipients. The often-used buffering agents are phosphate for pHs between 6 and 8 and citrate for pHs below 6. If these two agents adversely affect protein stability, other buffering agents may be used such as acetate for rhG-CSF at pH 4.0 (Herman et al., 1996), Tris–HCl for hEGF at pH 7.0 (Son and Kwon, 1995), and succinate for IFN-γ at pH 5.0 (Lam et al., 1996).

In close relation to the selection of formulation excipients, a compatible container should be used. Type I borosilicate glass is usually the material of choice for containers due to its strong chemical resistance and low level of leachables. Closures should be carefully chosen based on their compatibility with protein formulation, resistance to formulation pH, excipients and sterilization, moisture/vapor transfer property, and resealability.

4.4.4. Rapid screening of protein formulations

Development of a liquid protein formulation often requires screening of many excipients and their combinations at different pHs, even under rigorous computer-aided formulation design. Therefore, use of an efficient method for screening these excipients is recommended to expedite excipient selection without undertaking time- and labor-extensive stability studies. A few screening methods are briefly described here, which should be used with caution as they may or may not lead to the identification of ideal formulations. These methods include: (1) comparison of protein unfolding temperature (T_m) in different formulations; (2) comparison of half denaturation concentration, C_{half}, in the presence of a denaturant; and (3) comparison of IR spectra of different protein formulations with a reference spectrum.

Although T_m does not have a defined relationship with the free energy of protein unfolding (an indicator of protein stability), it is widely accepted that any increase in T_m should lead to an increase in protein stability (Dill et al., 1989). Therefore, a change in T_m may indicate whether a particular excipient has any stabilizing or destabilizing effect. For example, an increase in the amount of IL-1R aggregates correlates roughly with a gradual decrease in T_m in the presence of one of the three preservatives: phenol, m-cresol, and benzyl alcohol (Remmele et al., 1998). On the other hand, a protein stabilizer may not always increase T_m. EDTA and two reducing agents, dithiothreitol and β-mercaptoethanol, can protect aFGF from thermally-induced aggregation, but at low concentrations these agents do not affect the protein's T_m (Tsai et al., 1993). The T_m of a protein in the presence of two or more excipients can be roughly estimated without experimentation by calculating the algebraic sum of T_ms obtained in the presence of individual excipient. T_m estimation by this method seems rather reliable whether the excipients have the same or opposite effects (Allison et al., 1996).

C_{half} is the concentration of a denaturant at which 50% of protein molecules are unfolded or denatured. It can be estimated from a protein denaturation curve as discussed in Section 3.4. The effect of a formulation excipient on protein stability is reflected in the change in C_{half}. The higher the C_{half},, the more stable the protein in the formulation. It should be noted that the time needed to unfold a protein by a denaturant may be very slow. Complete unfolding of the wide type Rop in 4 M GdnHCl takes 48 h (Munson et al., 1996).

IR has been used extensively to probe protein structural changes in liquid, frozen, and dried states (Dong et al. 1995b). The effect of excipients on protein stability can be examined by comparing the IR spectrum of a sample in the sensitive amide I region with that of a reference. Comparison can be based on the degree of spectral correlation (r) (Prestrelski et al., 1993a,b), or the extent of spectral area overlap (Heimburg and Marsh, 1993; Allison et al., 1996; Kendrick et al., 1996), although these parameters may not necessarily reflect changes in protein activity. Using the correlation coefficient (r), Prestrelski et al. (1995) successfully selected the formulation pH and stabilizers in designing a freeze-dried IL-2 formulation. Nevertheless, overlap comparison of area-normalized second-derivative or deconvo-

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luted spectra is seemingly more reliable and objective. To overcome the interference of water absorption, protein IR spectra have to be determined either in D_2O or at higher protein concentrations (> 10 mg/ml) as discussed in Section 3.4.

4.4.5. Stability studies

To accelerate formulation development, stability studies are often conducted under accelerated (stressed) conditions. These stressed conditions include high temperature, high humidity, intensive lighting, extreme pHs, increased air/water interfaces by vortexing or shaking, and repeated freeze/thaw cycles. These studies are usually short but very helpful in screening protein formulations. Parameters used in monitoring stability studies include protein activity, rate of protein degradation, product formation and aggregation/precipitation, solution color, pH and viscosity, sterility, and pyrogenicity.

Accelerated stability studies are often conducted at high temperatures. The key issue is whether and how well the data from accelerated stability studies can be extrapolated to those under real-time conditions. Extrapolation of stability data from high to lower temperature is usually limited to a temperature range over which the same degradation pathway is operative. Very often, protein stability results obtained at high temperatures do not reflect or predict what happens under real-time conditions. This is due to the complexity of multiple protein degradation pathways at different temperatures. For example, temperature-dependent degradation mechanisms have been reported for interleukin 1β (IL-1β) (Gu et al., 1991), and hGH (Pikal et al., 1991). Nonlinear Arrhenius relationships were observed for relaxin between 5 and 30°C (Nguyen and Shire, 1996) and for *phm*-MDH between 4 to 65°C in the presence of a variety of excipients, such as salts, salts of amino acids, and polyols (Jensen et al., 1997). The temperature nonlinearity may suggest at least two major degradation pathways in proteins.

Nevertheless, if the multiple degradation processes in proteins can be described separately, or the rate-limiting degradation step does not change with temperature, prediction of protein stability based on accelerated stability studies is very optimistic. For example, the following thermal denaturation model with two different reaction rates representing two isoenzymes of β-galactosidase has been used to describe the biphasic irreversible denaturation process, and linear Arrhenius plots are obtained for both k_1 and k_2 (Yoshioka et al., 1994b):

$$N_1 \overset{k_1}{\Rightarrow} N_2 \overset{k_2}{\Rightarrow} D.$$

Linear Arrhenius relationships have also been reported for a number of proteins in certain temperature ranges. Examples include the inactivation of *ml*-PEPC in the presence of 0.8 M sodium glutamate between 7.6 and 48°C (Jensen et al., 1997), thermal inactivation of recombinant hepatitis B surface antigen (HBsAg) between 60 and 90°C (Volkin et al., 1996), and second-order oxidation of hIGF-I between 7 and 37°C (Fransson et al., 1996).

5. Conclusions

Native proteins are properly folded, which involves many forces, including hydrophobic interactions, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding, intrinsic propensities, and van der Waals forces. Among these forces, hydrophobic interactions seem to be the dominant. Proteins are generally not very stable, as stabilization energy of the native state is mostly between 5 and 20 kcal/mol, which is equivalent to that of a few hydrogen bonds. Most mesophilic proteins such as those from human beings can be unfolded/denatured easily at temperatures between 50 to 80°C.

A common phenomenon of protein instability is formation of protein aggregates, which can be soluble or insoluble, chemical or physical, and reversible or irreversible. There are many factors affecting protein stability. These include at least temperature, pH, ionic strength, metal ions, surface adsorption, shearing, shaking, additives, solvents, protein concentration, purity, morphism, pressure, and freeze-thawing/drying. Chemical transformations that lead to protein instability include at least deamidation, oxidation, hydroly-

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sis, isomerization, succinimidation, disulfide bond formation and breakage, non-disulfide crosslinking, and deglycosylation. Today, many analytical techniques are available to monitor protein instability. Even with the sophistication of present instrumentation, no single technique can satisfactorily provide sufficient information about a protein. Thus, a combination of analytical methods needs to be used to characterize a protein and to monitor protein instability.

To develop a liquid protein pharmaceutical, the basic properties of a protein need to be examined first. These include protein purity, p*I*, and solubility and stability at different pHs and in different buffer systems. With these data, protein formulation issues can then be addressed. These include the indication, route of drug administration, selection of proper formulation buffer and excipients, and finally, stability studies. To facilitate rapid development of a protein formulation, a formulation screening method may be used with caution, such as the determination of T_m, $C_{half,}$ or degree of IR spectral similarities. Alternatively, accelerated stability studies may be conducted under high-temperature conditions, but stability results obtained at high temperatures may not reflect or predict what happens under real-time conditions. For proteins, real-time stability studies are necessary for selection of the final formulation.

In the development of a protein formulation, the most challenging task is the stabilization of a protein to achieve an acceptable shelf life. Due to complication of structural modifications, proteins are commonly stabilized by excipients. The often-used protein stabilizers include sugars, polyols, surfactants, salts, PEGs, polymers, metal ions, and amino acids. Unfortunately, there is no single pathway to follow in selection of a suitable stabilizer(s), partly due to the lack of a clear and definitive understanding of protein-cosolute interactions and proteins' multiple inactivation mechanisms. Depending on the protein, these traditional protein stabilizers may increase protein stability only to a limited level. Therefore, new types of protein stabilizers need to be further explored. One obvious direction is to identify additional cellular components in hyperthermophilic organisms or cellular accumulants under various stressed conditions, which may have the potential to stabilize proteins.

In summary, the most formidable challenge in formulating a liquid protein pharmaceutical is to preserve the biological activity of the protein for an acceptable shelf life. Unfortunately, there is no single pathway to follow in formulating such a product. Usually, proteins have to be evaluated on a case-by-case basis. Much more effort is still needed to understand the basic behavior of proteins, their instability factors and mechanisms, and their stabilization mechanisms in a broader and clearer perspective. This further understanding will definitely depend on further advancement of sophisticated analytical instrumentation. Therefore, research activities directed toward a general solution to protein instability will continue for at least a few decades (Richards, 1997).

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Phase I Study of Anticolon Cancer Humanized Antibody A33[1]

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ABSTRACT

Purpose: **Humanized A33 (huA33; IgG1) monoclonal antibody detects a determinant expressed by 95% of colorectal cancers and can activate immune cytolytic mechanisms. The present study was designed to (*a*) define the toxicities and maximum tolerated dose of huA33 and (*b*) determine huA33 immunogenicity.**

Experimental Design: **Patients (n = 11) with advanced chemotherapy-resistant colorectal cancer received 4-week cycles of huA33 at 10, 25, or 50 mg/m^2/week. Serum samples were analyzed using biosensor technology for evidence of human antihuman antibody (HAHA) response.**

Results: **Eight of 11 patients developed a HAHA response. Significant toxicity was limited to four patients who developed high HAHA titers. In two of these cases, infusion-related reactions such as fevers, rigors, facial flushing, and changes in blood pressure were observed, whereas in the other two cases, toxicity consisted of skin rash, fever, or myalgia. Of three patients who remained HAHA negative, one achieved a radiographic partial response, with reduction of serum carcinoembryonic antigen from 80 to 3 ng/ml. Four patients had radiographic evidence of stable disease (2, 4, 6, and 12 months), with significant reductions (>25%) in serum carcinoembryonic antigen levels in two cases.**

Conclusions: **The complementarity-determining region-grafted huA33 antibody is immunogenic in the majority of colon cancer patients (73%). HAHA activity can be measured reproducibly and quantitatively by BIACORE analysis. Whereas the huA33 construct tested here may be too immunogenic for further clinical development, the antitumor effects observed in the absence of antibody-mediated toxicity and in this heavily pretreated patient population warrant clinical testing of other IgG1 humanized versions of A33 antibody.**

INTRODUCTION

The original mAb[3] A33 is a murine IgG2a that has undergone extensive preclinical analysis (1–8) and a series of pilot Phase I localization-radioimmunotherapy studies from 1991–1995 (9–11). Immunohistochemical testing of a large panel of normal and malignant tissues has shown A33 antigen expression to be restricted to the epithelia of the lower gastrointestinal tract and to carcinoma lesions that originate from the rectal and colonic mucosa (2). Despite limited normal tissue reactivity, this antigenic system was selected as a target for clinical studies because the A33 antigen is expressed homogeneously and at high levels in 95% of cases of metastatic colorectal cancers (2).

Although much has been learned regarding the biochemical, immunological, and molecular biology of the A33 antigen, the function of this molecule remains unknown. The A33 antigen is a M_r 43,000 glycoprotein that has been sequenced; its cDNA has been cloned (12–15), and posttranslational modifications have been characterized (15). Biochemical and fluorescence microscopy experiments have shown that a portion of cell-bound mAb A33 is internalized into cytoplasmic vesicles (1). In general, antibodies targeting cell surface antigens, which are internalized into pathways that lead to the lysosomal vesicles, have been found to undergo rapid catabolism (16, 17). In contrast, cell-bound mAb A33 represents a stable pool of antibody in equilibrium between the cell surface and macropinosomal or endosomal compartments (1). The clinical observation that radioiodinated mAb A33 is retained in tumor tissues for up to 6 weeks has led to the hypothesis that this retention is a consequence of the unique cell trafficking pathways of this antibody, which only slowly enters the lysosomal compartment (1, 10).

In our initial clinical imaging and biopsy-based biodistribution studies using the mouse A33 antibody, we observed specific targeting to antigen-positive tumor tissues in 95% of patients (9–11). Whereas the only other tissue that concentrated the radioisotope was bowel, clearance from the gastrointestinal tract was much more rapid than that from tumor tissue, so that at the end of the imaging period, only the tumors were visualized (11). These localization characteristics led us to a Phase I ^{131}I-labeled mAb A33 therapy trial that demonstrated bone marrow as the dose-limiting organ toxicity (9). As the colon expresses A33 antigen, gastrointestinal toxicity was monitored

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[3] The abbreviations used are: mAb, monoclonal antibody; huA33, humanized A33; CDR, complementarity determining region; HAHA, human antihuman antibody; MTD, maximum tolerated dose; CEA, carcinoembryonic antigen; MSKCC, Memorial Sloan-Kettering Cancer Center; CT, computed tomography; RU, response unit(s); LDH, lactate dehydrogenase.

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closely and found to be minimal at the MTD for the bone marrow (80 mCi/m^2). Antitumor effects were observed in 5 of 23 assessable patients, despite the fact that only a single dose could be administered due to the development of an antimouse Ig response (human antimouse antibody) in all patients (9). Based on the unique internalization pathway of mAb A33 and its retention in tumor cells, we also examined whether a low-energy electron-emitting radionuclide, such as ^{125}I, might have therapeutic advantages over high-energy β emitters such as ^{131}I (1, 10). This concept was supported by therapy experiments in a nude mouse model, which demonstrated complete ablation of tumors with nontoxic doses of ^{125}I-mAb A33 (8). This clinical study confirmed the longer retention of ^{125}I-mAb A33 in tumor sites when compared with the uptake by the colon, and external imaging of the ^{125}I was possible even 6 weeks after antibody administration (10). Whereas the radiotherapeutic doses tested did not approach the MTD of bone marrow or other normal tissues, plasma levels of ^{125}I-mAb A33 reached cytotoxic levels for colon cancer cells based on *in vitro* assays. Minor antitumor effects were observed in 4 of 22 patients (10). Of note was the observation that additional major responses were observed in a subgroup of patients who were treated with a specific chemotherapy regimen (10) after treatment with ^{125}I-mAb A33. This finding is consistent with results in a mouse model indicating synergistic antitumor effects between radiolabeled A33 antibody and chemotherapy (3).

These preliminary studies with mAb A33 have defined the targeting capability of this antigenic system as well as its limitations. However, full characterization of the therapeutic potential of the mouse-derived A33 antibody could not be accomplished due to the inability to effectively retreat patients with an immunogenic foreign protein. Thus, a fully humanized CDR-grafted IgG1 was developed (18, 19) and evaluated in the current report. This IgG1 humanized antibody has the added advantage of being able to direct cell-mediated immune lysis of human colon cancer cell lines *in vitro*. As a consequence of this cytotoxic capability, normal antigen-positive tissue toxicity was monitored closely in this study.

MATERIALS AND METHODS

Production and Purification of huA33 Antibody. huA33, a fully humanized IgG1 mAb, was derived from murine A33 antibody by CDR grafting (18). The antibody was expressed in NSO cells, and culture supernatant was produced in bioreactors by Celltech Limited (Berkshire, United Kingdom) and concentrated. huA33 was purified from NSO supernatant at the New York Branch of the Ludwig Institute for Cancer Research at MSKCC, using a three-step chromatography process: Q-Sepharose anion-exchange; protein A affinity; and S-Sepharose cation exchange chromatography. Virus inactivation was achieved by exposure of the protein A elute to pH 3.0 at room temperature for 30 min. The antibody was formulated in PBS [0.05 M sodium phosphate and 0.15 M sodium chloride (pH 7.0)], sterile filtered, and stored at –70°C. The purified huA33 did not form aggregates, did not contain detectable levels of protein A, and retained the original binding specificity of the murine A33 antibody (20).

Patient Selection and Eligibility. Histology slides from all patients were reviewed by the Department of Pathology at MSKCC and confirmed to be colorectal carcinoma. Eligibility criteria were as follows: patients had to be at least 18 years of age; have unresectable stage IV disease; and have tumors refractory to standard chemotherapy and measurable or evaluable by conventional imaging. Patients had to be off prior therapy for at least 4 weeks, be ambulatory with a Karnofsky performance status of 70 or greater, and have an estimated survival of at least 6 weeks. Laboratory requirements included serum creatinine $<$ 2 mg/100 ml, serum bilirubin $<$ 2 mg/100 ml, granulocyte count $>$ 1,500/mm^3, platelet count $>$ 100,000/mm^3, and prothrombin time $<$ 1.3$\times$ control. All patients had to be capable of giving written informed consent according to institutional and federal guidelines. Patients with the following criteria were excluded from the study: clinically significant cardiac disease (New York Heart Association class III/IV); infections requiring antibiotics; illnesses requiring the use of steroids or other anti-inflammatory reagents; clinical evidence of central nervous system tumor involvement; history of prior administration of mouse-derived antibodies or protein constructs or human anti-mouse antibody serum reactivity; and positive pregnancy test or lactation.

Study Design/Administration of huA33. The study protocol was reviewed and approved by the Institutional Review Board at MSKCC (Protocol 94-100), and the study was conducted under a United States Food and Drug Administration Investigational New Drug Application (BB-IND #6084). The study was designed with two major objectives: (*a*) to define the toxicities and MTD of huA33 administered in weekly doses; and (*b*) to determine whether huA33 is immunogenic and characterize the anti-idiotypic response and toxicities associated with the development of anti-huA33 Ig (human antihuman Ig antibody, HAHA).

Patients were monitored with weekly complete blood counts, weekly tests for occult blood in stools, and liver function and renal function tests every 4 weeks. CT scan evaluation of response was performed at least every 9 weeks. We had previously shown that upon retreatment, the mouse A33 antibody was cleared rapidly and prevented from targeting colon cancer in the presence of human antimouse antibody (9–11). Thus, in addition to directly monitoring HAHA levels using biosensor technology (BIACORE), we also measured A33 antigen-reactive huA33 in serum (by ELISA) as another indicator of anti-huA33 activity after treatment. Because clinical toxicities directly related to the effects of huA33 binding to cell surface A33 antigen would be masked by the presence of neutralizing antibodies (HAHA), it was critical to detect the presence of even low titers of anti-huA33 activity. Blood samples were collected every week, before and after antibody infusion, to monitor HAHA activity. Thus, the observed toxicities could be correlated to the presence or absence of HAHA throughout the treatment course. Doses (10–50 mg/m^2/week) and schedule (weekly doses) were selected based on the amount of clinical grade huA33 available and the preliminary clearance rates of radiolabeled huA33 antibody.

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria. Dose-limiting toxicity was defined as follows: any grade 2 or greater allergic reaction; any

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Table 1 Patient characteristics and HAHA response

Patient no.	Age (yrs)	Sex	Site[a]	huA33 dose (mg/m^2)	Serum CEA (ng/ml)	LDH (units/dl)	HAHA[b]
1	75	M	R	10	11	225	Negative
2	53	M	A	10	342	186	I
3	76	F	S	10	80	301	Negative
4	71	F	A	10	3110	765	I
5	52	F	D	10	156	194	II
6	70	M	A	25	186	280	I
7	56	F	D	25	2237	357	Negative
8	59	M	A	25	219	167	I
9	73	F	S	50	22	290	II
10	48	F	R	50	333	224	II
11	71	F	A	50	992	440	I

[a] Site of primary disease: A, ascending colon; D, descending colon; R, rectum; S, sigmoid colon.
[b] I, type I HAHA response; II, type II HAHA response.

grade 4 nonhematological toxicity; any grade 4 WBC toxicity lasting >1 week (2 consecutive weeks); or any grade 4 platelet count toxicity requiring platelet transfusion. To be dose-limiting, an adverse event had to have been judged by the principal investigator to be possibly related to the investigational agents. The MTD was defined as the dose level immediately below the dose level inducing dose-limiting toxicity in two patients (of three to six patients tested). Standard criteria for response to treatment were based on radiological examination before and after treatment cycles (2 cycles or 9 weeks), and response was rated as complete (disappearance of all lesions), partial (>50% decrease in the sum of the products of the perpendicular diameters), stable (<50% decrease or <25% increase in the sum of the products of the perpendicular diameters), or progression (>25% increase or new lesions). Serum CEA levels were monitored every 5 weeks.

The antibody was diluted in 100–150 ml of physiological saline containing 5% human serum albumin and infused through a line containing normal saline at a rate of approximately 1 mg/min. All patients were treated in the outpatient facility, and all infusion-related toxicities had to be resolved before discharge. Patients who could not be treated as outpatients were removed from the study.

Immune Response to huA33 and Pharmacokinetics. Induction of an antibody response to huA33 (HAHA) was monitored by surface plasmon resonance technology using a BIACORE 2000 instrument (21). This methodology allows direct measurement of specific antihuman Ig antibodies in sera of patients, which bind to huA33 in a label-free manner in real time. huA33 and control antibodies were immobilized by amine coupling to CM5 biosensor microchips. Control antibodies included murine anti-A33 mAbs, A33 (IgG2a), and 100.310 (IgG2a; Ref. 11). SK10B, a humanized IgG1 of the same allotype as huA33, and a humanized IgG4 antibody, SK10C, were provided by Celltech. Goat antihuman IgG, goat antihuman IgM, and goat antimouse IgG were obtained from Sigma Chemical Co. (St. Louis, MO). mAbs against human IgG, IgE, and IgM were from Zymed Laboratories, Inc. (San Francisco, CA). Antibodies against human IgG1, IgG2, IgG3, and IgG4 were from Southern Biotechnologies (Birmingham, AL). Diluted patient serum (1:100) was passed over the chip, and alterations in the refractive index were recorded as the relative RUs (21). Controls included pretreatment of serum samples with protein G-Sepharose to remove IgG and blocking with huA33 or control antibodies before BIACORE analysis (21). huA33 serum levels were assayed by ELISA, using recombinant A33 antigen-coated plates incubated with serial dilutions of serum samples. Bound huA33 was quantitated with alkaline phosphatase-coupled goat antihuman IgG. Attophos (JBL, San Luis Obispo, CA) was used as a substrate, and fluorescence was measured in a Cytofluor 2350 reader (Millipore, Bedford, MA).

RESULTS

Patient Characteristics

Twelve patients were registered into this study. Characteristics of the 11 evaluable patients are listed in Table 1. All patients had tumors resistant to conventional chemotherapy consisting of a fluorouracil-based regimen and received a mean of 3.3 chemotherapeutic agents before protocol entry. All patients had stage IV disease; two patients had known metastatic colorectal cancer limited to one site (lung), two patients had two sites of disease (pelvic/lung and liver/lung), and seven patients had three or more sites of disease. Review of tissue sections in the Department of Pathology, MSKCC confirmed that all patients had colorectal cancer. Because the antigen is not detectable after formalin fixation using mAb A33, tumor typing for A33 antigen expression was not performed. Because the A33 antigen is expressed in >95% of colorectal cancers, requiring additional fresh-frozen biopsy specimens specifically for A33 antigen typing was not justified.

All patients who received at least one full dose of huA33 were considered evaluable for toxicity ($n = 11$). One patient with underlying hypertension and coronary artery disease could not tolerate the saline/human serum albumin infusion and received <10% of her first dose; she was removed from the study and excluded from the analysis.

Toxicity

Induction of Anti-huA33 Activity in Serum. Blood samples collected before and after each huA33 treatment were monitored for anti-huA33 reactivity. Two patterns of reactivity (type I and type II) emerged from this serological analysis, and these data have been reported in detail elsewhere (21). Patients

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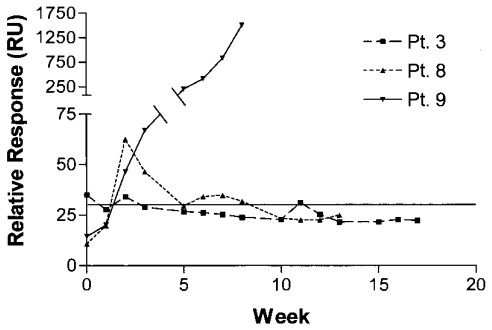


Fig. 1 Examples of the three patterns of HAHA activity are plotted as relative RU over time. Patient 9 had a type II pattern with increasing high anti-huA33 activity. Patient 8 had a transient low-level type I pattern and became tolerant to the huA33. Patient 3 had no significant induction of anti-huA33 activity.

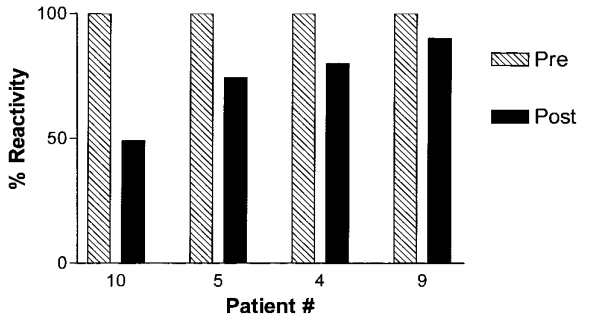


Fig. 2 HAHA activity before (*Pre*) and after (*Post*) infusion of huA33 in four patients with high HAHA levels (type I, patient 4; type II, patients 5, 9, and 10) expressed as the percentage of HAHA activity remaining after huA33 administration, where the pretreatment value is normalized to 100%. In each case, the administered dose was not sufficient to overcome or neutralize the anti-huA33 activity. These posttreatment serum samples had little or no measurable huA33 activity by testing on A33 antigen-coated plates (data not shown).

with a type II response developed anti-huA33 antibodies with progressively increasing titers. This pattern has been the general experience with HAHA activities. Patients with a type I response to huA33 developed a limited anti-huA33 response usually by week 2 or 3 and had stable levels of activity, that resolved, in most cases, by week 7 or 8 of treatment (tolerance). Induction of reactivity was scored as type II [persistently increasing titers (n = 3); patients 5, 9, and 10], type I [stable or transient levels of anti-huA33 activity (n = 5); patients 2, 4, 6, 8, and 11], or no detectable reactivity (n = 3; patients 1, 3, and 7; Table 1; Fig. 1). Patient 4 had modestly high titers of anti-huA33 activity, but the titers stabilized, and she was therefore designated as a type I pattern. Serum reactivity was shown to be specific for huA33-variable regions or CDRs by preincubation of serum samples with either huA33, mouse mAb A33, or control humanized or mouse antibodies. No IgG1 constant region (allotypic) reactivity was demonstrated. There did not appear to be a clear correlation between induction of anti-huA33 and antibody dose level because HAHA occurred in all dose levels; however, the highest titer was in a patient at the highest antibody dose level (patient 9). The duration of treatment did not appear to be a factor because patients developed type II HAHA titers as early as the 4th week of treatment (patient 9) or only after 5 months (patient 10), whereas one patient was treated for almost 4 years without evidence of HAHA (patient 3). Low HAHA titers of the type I pattern appeared earlier than those of type II and were detectable at week 2 in some cases (patients 2, 6, and 8).

Serum samples from four patients (patients 4, 5, 9, and 10) with strong anti-huA33 serum titers, type I or type II patterns, were shown to have clinically significant huA33 binding reactivities by two assays. In the first assay (BIACORE), anti-huA33 activity was measured in percentage response units of post- *versus* pretreatment serum (Fig. 2). In one case, HAHA was shown to decrease only modestly, even after an infusion of a 100-mg dose (50 mg/m^2 dose level) of huA33 (Fig. 2, patient 9). Thus, there were sufficiently high levels of anti-huA33 activity in the blood so that an administration of 100 mg of huA33 could not completely neutralize the HAHA activity. The second assay measured serum huA33 antibody binding to solid phase A33 antigen from blood samples collected after each antibody infusion. The results indicate that once the patients developed detectable (either type II or type I or high or low titer response) anti-huA33 activity by biosensor methodology (n = 8), they cleared or neutralized virtually all or a substantial portion of serum huA33 binding activity to antigen (data not shown).

Clinical toxicity correlates of these laboratory results indicated that two of four patients (patients 9 and 10) with high anti-huA33 activity had acute infusion-related toxicities such as fever, rigors, facial flushing, nausea with vomiting, and changes in blood pressure (grade 1–2) resulting in discontinuation of treatment (Table 2). In contrast, toxicities in the other patient with a type II pattern of HAHA and in the five patients with a type I pattern of HAHA activity consisted of persistent erythematous rashes, myalgias, and fevers in two of these patients (patients 4 and 5) and no symptoms in the remainder (patients 2, 6, 8, and 11). The four patients (patients 4, 5, 9, and 10) with symptoms had the absolute highest titers by BIACORE analysis, and these symptoms only occurred when BIACORE measurements of HAHA exceeded 100 response units, suggesting that symptoms correlate quantitatively with BIACORE results.

Further characterization of the serum anti-huA33 activity of these patients indicated that HAHA consisted of an IgG response, without evidence for significant IgM activity and no detectable IgE anti-huA33 antibodies. This was the case for both type I and type II patterns. Of note is that the response detected at 2 weeks consisted of IgG antibodies, which was consistent with a memory response rather than a primary immunization. An anti-HAHA response or an idiotope network antibody 3 response, reactive with A33 antigen, could not be detected. The four patients with the highest HAHA levels did not have IgE anti-huA33 or eosinophilia. One patient without HAHA activity received only 4 weeks of antibody (1 cycle). Thus, of the 10 patients receiving more than 4 weeks of treatment, 8 patients (80%) developed HAHA, and 4 patients (40%) had clinical

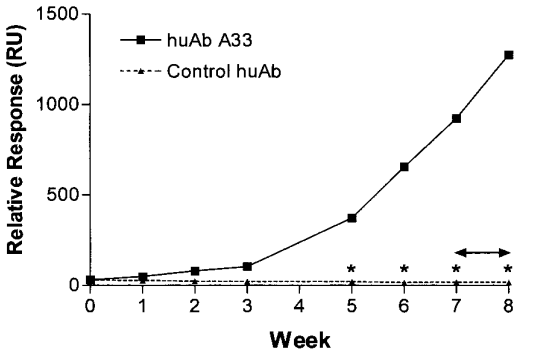
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Table 2 Toxicities[a]

HAHA type I-related toxicity
Rash (n = 1)
Myalgia (n = 1)
Fever (n = 1)
HAHA type II-related toxicities
Fevers (n = 3)
Rigors (n = 2)
Facial flushing (n = 2)
Nausea (n = 2)
Emesis (n = 2)
Blood pressure changes (n = 2)
Fever (n = 1)
Myalgia (n = 1)
Hyperurecemia (n = 1, grade 4)
Renal insufficiency (n = 1, grade 4)
huA33 toxicity unrelated to HAHA
Rhinorrhea, periorbital fullness (n = 8)
Headache (n = 4)
Cough (n = 2)
Eosinophilia (n = 2, grade 3 in one case)
Abdominal pains (n = 2)
Diarrhea (n = 2)
Fever (n = 1)

[a] Grade 3 or 4 toxicities are indicated.



Lab Values	0	1	2	3	4	5	6	7	8
UA (mg/dl)	6.6			6.5		8.9			22.6
LDH (U/ml)	290.0								3210.0
CEA (ng/ml)	21.7			13.1					42.0
CREAT (mg/dl)	1.4			1.3		1.6			2.0

Fig. 3 huA33-specific HAHA activities measured in RU increased each week of treatment (patient 9). By week 5, the patient developed infusion-related fever, chills, nausea, and vomiting (*), which responded to Demerol and acetaminophen. On week 8, the patient complained of arthralgias (arrow), and upon treatment she developed a grade 1 decrease in blood pressure, and her treatments were discontinued. Corresponding laboratory values are consistent with a cell lysis syndrome with elevated uric acid and a 10-fold increase in LDH.

symptoms. Although tolerance can be achieved with continued administration of huA33 in patients with low levels of HAHA, in practical terms, antibody treatment during a type I response may have no benefit for the patient because antibody-antigen binding activity is blocked. The fine specificity analysis (epitope mapping) of the induced anti-A33 activity is described in Ref. 21.

Adverse Reactions to huA33. Eight patients (patients 1, 2, 3, 5, 6, 7, 9, and 10) reported one or more of the following symptoms (mild rhinorrhea, cough, periorbital fullness, or headache) occurring by the end of the infusion or within a few hours thereafter. These symptoms, which commonly started with the first infusion and always resolved by the next day, occurred in patients who never developed HAHA or before the development of HAHA and thus are attributable to the effects of huA33 alone (Table 2).

Two patients (patients 3 and 7), one each from the first and second dose levels, developed eosinophilia after 24 and 20 months of treatment, respectively. Eosinophil counts ranged from 15–25% (normal up to 7%) of WBC counts, whereas other blood elements remained unchanged or within normal limits. At the time the eosinophilia was first noted, patient 3 also had intermittent episodes of diarrhea (grade 2), and patient 7 developed a pruritic erythematous rash with a minor urticarial component (grade 2). Colonoscopy of patient 3 did not reveal any gross pathology, and a blind biopsy demonstrated only normal mucosa. Eosinophilia resolved spontaneously in patient 3 when treatment was withheld for 5 weeks. A skin biopsy of a rash site of patient 7 showed an intense perivascular eosinophilic infiltration. Her antibody treatments were withheld, and she received a short course of prednisone (15 mg/day) with resolution of her rash and blood eosinophilia 4 weeks later (grade 3). Neither patient 3 nor patient 7 had evidence of HAHA during the time when eosinophilia was present, and IgE anti-huA33 activity was specifically excluded.

At the third dose level, patient 9 had no adverse events until, beginning with the fifth treatment, she developed infusion-related symptoms consisting of nausea (grade 2), emesis (grade 1), hypotension (grade 1), and fever and rigors (grade 2), which were controlled with Demerol and Tylenol. At her eighth treatment, she also complained of joint pain, and her treatments were discontinued. She was found to have hyperuricemia (22.6 mg/dl) and a 10-fold increase over her baseline serum LDH (Fig. 3). Except for resolution of obstructive edema, there was no evidence of tumor response on physical exam, and her adverse infusion-related reactions correlated with the development of increasing anti-huA33 serum levels. The patient's serum CEA levels initially decreased (week 4); however, the laboratory findings suggesting lysis of tumor cells (hyperuricemia) occurred during the subsequent 4-week period during significant HAHA activity (weeks 5–9) when there was an absence of detectable free huA33 antibody in the blood. The rapid increase of serum uric acid level to 22.6 mg/dl is suggestive of a cell lysis process; however, there is no evidence indicating whether the cells undergoing lysis were A33 antigen-positive tumor cells or normal cells or whether the process was nonspecific due to the deposition of immune complexes. The presence of circulating anti-huA33 IgG-IgG1 complexes and a serum uric acid of 22.6 mg/dl resulted in progressive renal insufficiency. This patient did not wish any further medical intervention, and she was maintained with supportive care, having no radiological examinations or biopsy to document the status of her disease.

Patient 10 had a history of radiation enteritis with recurrent episodes of nausea, emesis, abdominal pains, and ileus. These symptoms, which had subsided before study entry, recurred 3–4 days after huA33 treatments. Although these symptoms preexisted, they appeared to be induced in a synchronized fashion

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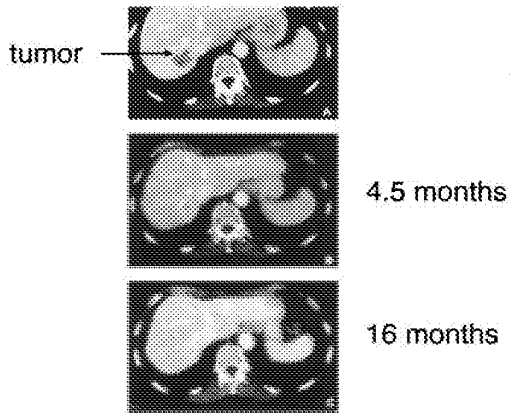


Fig. 4 Representative sections through a liver metastasis in a pretreatment CT scan and a follow-up scans at 4.5 and 16 months. Patient 3 achieved a partial response of liver and lung metastases lasting 16 months, at which time small pulmonary metastases were visible, whereas the liver remained free of disease.

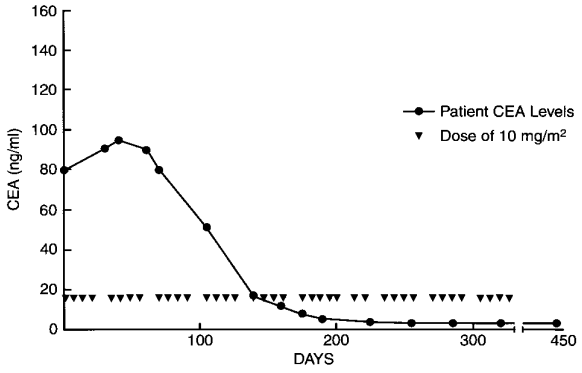


Fig. 5 A decline in serial serum CEA levels of patient 3 was apparent only after 3 months of treatment with huA33.

after antibody treatments, and thus the antibody treatments may have contributed to an underlying radiation-induced functional bowel disorder. A decrease of her dose from 50 to 10 mg/m^2 did not affect the frequency of her symptoms. The patient underwent a surgical procedure in which the segment of involved bowel was resected. Pathology revealed changes consistent with radiation enteritis, without evidence for antibody effects. However, given the antibody's known targeting to normal bowel and the temporal relationship to the antibody infusions, bowel dysfunction was felt to be reactivated by the huA33 treatments in a fashion similar to chemotherapy induced radiation recall reactions.

Patients had stools monitored for occult blood, and no episodes of antibody-induced bowel bleeding were documented. Diarrhea was a common preexisting condition, but no grade 3 or 4 toxicity was encountered in the huA33 doses examined here, although two patients (patients 9 and 10) at the highest antibody dose level had a low-grade increase in frequency of bowel movements and abdominal pains related to huA33 treatment.

Patient accrual was discontinued before establishing a MTD unrelated to HAHA-associated toxicities, due to the immunogenicity of huA33 (73% of patients) and the inability to give repeated treatments and because all three patients at the highest dose level developed HAHA. Because HAHA activity was not clearly huA33 dose dependent, and HAHA responses occur at all huA33 dose levels, a MTD could not be assigned to HAHA toxicities. Nevertheless, the most severe HAHA-associated toxicities occurred in the two patients (patients 9 and 10) treated at the highest dose level. These data emphasize the potential toxicity of HAHA-related IgG1 immune complexes.

Responses. All patients had measurable disease and were evaluated for response by CT scan every two cycles (9 weeks), except for patients 1 and 9 (CT scan done on week 5 only). Patient 3 had a partial response of lung and liver metastases for 16 months (Fig. 4). huA33 treatments were discontinued after 1 year, at which point her serum CEA had been normal for 6 months (Fig. 5), and all lesions had disappeared except for a single residual defect in the liver. While off treatment, her pulmonary metastases recurred, and she was restarted on huA33. She had stable disease for another 32 months, eventually progressing in the lymph nodes.

An additional four patients (patients 5, 6, 7, and 10) had evidence of stable disease by CT scan (week 9), with two patients (patients 7 and 10) having reduced serum CEA levels (>25%). Patients 5, 6, 7, and 10 had stable disease for 4, 2, 12, and 6 months, respectively. Patient 7 had stable disease for 12 months when she underwent a surgical debulking procedure. The stable disease observed on CT scan and the modest serum CEA declines were observed only before development of HAHA (patients 5, 6, and 10) or in the absence of HAHA (patient 7). Stabilization of disease was not seen in the presence of HAHA. Four patients had progression of disease on CT scan at week 9.

DISCUSSION

The present study indicates that huA33, at the doses examined, induces little toxicity by direct effects of the antibody targeting to antigen in normal tissue. However, the neutralization of huA33 by HAHA may block the complete manifestation of toxicity. All but three patients developed HAHA, and one of these three patients was treated for only 4 weeks. Therefore, a complete evaluation of huA33 as an immunotherapeutic agent must await construction of a nonimmunogenic form of this antibody. Based on the clinical targeting data available for the A33 antibody, we expect that, in the absence of immunogenicity, dose escalation would be limited by bowel toxicity (9–11). Despite the limitations of the current study, it provides preliminary evidence of antitumor activity in a group of heavily pretreated patients at doses below the level where significant bowel toxicity would be observed. One patient had a major response of liver and lung disease, and four patients had stabilization of disease by CT scan. The patient with the major response was one of three patients who did not develop HAHA. The patient with the longest-lasting stable disease was also one of the three who did not develop HAHA. One patient with stable disease and decreasing serum CEA levels subsequently devel-

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oped type II HAHA and was removed from the study. No radiological evidence of response or declining serum CEA levels were noted while patients had HAHA. Thus, in addition to blocking tumor targeting of huA33, HAHA is also responsible for abrogating the antitumor activity of the antibody.

The mechanism by which huA33 exerts its antitumor activity is believed to be via activation of immunological effector functions. huA33 has no direct growth-inhibitory or cytotoxic effect on colon cancer cell lines *in vitro*, and the function of the antigen is unknown (3–5). Human IgG1 antibodies in general have the potential to direct cellular and complement-mediated cytotoxicity, and it was for this reason that we selected IgG1 as the isotype of huA33 for clinic therapy studies (22). As we and others have reported previously (23), critical factors for immune-directed tumor lysis include antibody isotype, cell surface antigen quantity, and density. Quantitative measurements of mAb A33 binding to cell surface A33 antigen on colon cancer cell lines predicted the immune-mediated lytic capability of the humanized version (1). Without tissue biopsies from regressing tumors, it is difficult to document that this mechanism is active in patients. Experience with humanized IgG1 antibodies as mediators of immune effector function in patients with solid tumors has been limited thus far. However, the inherent potential of IgG1 antibodies for activation of immune-mediated effects, such as antibody-dependent cell-mediated cytotoxicity, human complement lysis, opsonization, and induction of targeted inflammation, may render IgG1 isotype treatment a new potent antitumor therapeutic modality.

Studies of the IgG1 anti-p185HER2 antibody (Herceptin) clearly show antitumor activity in selected breast cancer patients whose tumors express high levels of antigen (24). Whether clinical antitumor activity is due to a growth factor-inhibitory effect, Herceptin's ability to mediate immune-cellular cytotoxicity, or a combination of these two mechanisms is not clear (25). When Herceptin was first engineered, consideration was given to capture both of these activities (cell growth inhibition and antibody-dependent cell-mediated cytotoxicity; Ref. 26); however, from a mechanistic point of view, it is not possible at present to separate these effects in the clinic. Similar considerations apply to the anti-epidermal growth factor receptor antibodies. However, in this case, clinical studies are proceeding, evaluating antibodies with (C225 and IgG1) or without (ABX-EGF and IgG2) immune capabilities or drugs that specifically interrupt the signaling pathways (27, 28). Results in animal studies suggest that immune mechanisms are not required for antitumor activity (29), and clinical studies that are under way will provide important information with regard to sorting out the relative contributions of each antitumor mechanism. Thus, we will be able to distinguish whether the immune effects of antibody add to the antitumor activity of growth factor signal blockade. Another antigenic system, the Lewis Y epitope found on glycoproteins and glycolipids, is expressed at high levels in a large number of solid tumor types, and new humanized anti-Lewis Y IgG1 antibodies are strong mediators of immune-directed lysis (30, 31). Preliminary clinical reports of the Phase I trial of a humanized IgG1 antibody to the 17.1A antigen (anti-EGP40) indicated that toxicity in antigen-expressing pancreatic ducts and bowel may be dose-limiting (32). This is in striking contrast to the experience with the widely used mouse antibody to the 17.1A antigen, demonstrating the potency of the human IgG1 constructs when compared with mouse antibody (32, 33). Even among the human IgG isotypes, there may be subtle differences in effector functions using IgG1 or IgG3 or combinations of these isotypes, and these differences need to be explored (34).

The two patterns of HAHA activity described here for huA33 have been confirmed by analysis of a larger population of patients treated with this antibody (21). Of note is that in the current study, three patients had a type II pattern of HAHA response. These patients had a classical response to an immunogen (huA33), with increasing titers as exposure to antigen continued. Considering the potential toxicity of continued huA33 treatments in patients with type II HAHA responses, this rate of immunogenicity [3 of 11 (27%)] is too high for the general use of this antibody. In addition, five patients had the more unusual HAHA response, a type I pattern, developing a limited response that in some cases resolved (tolerance). Because these HAHA activities occurred early (as soon as week 2), these are not consistent with primary immunological responses but rather secondary responses and we speculate that the huA33 contains a determinant that mimics a common immunogen. Thus, the unexpected high rate of HAHA activity is due to a chance cross-reactivity between one or more epitopes in the variable portion of the huA33 and a common immunogen. To our knowledge, huA33 may be the only such example of an antibody inducing a type I immunogenicity pattern, but this needs further examination. Type I HAHA activities may be missed unless sensitive assays are used, and it may be an important mechanism of resistance to antibody-based therapies regardless of the antitumor mechanism involved. Expectations that the humanization process would be successful for each new reagent was perhaps unrealistic, given the complexity of the engineering required to maintain specific antigen binding. The current study illustrates that in some cases, clinically significant immunogenicity can be induced despite the humanization process.

Due to the binding of huA33 to normal bowel, gastrointestinal symptoms were monitored closely. No occult bleeding was detected. Diarrhea was more difficult to evaluate. Due to the effects of their prior treatments, 6 of 11 patients required antidiarrheal medication at some point in the 30-day period before starting the protocol. However, two of the three patients (patients 9 and 10) at the highest dose level developed increased frequency of bowel movements associated with grade 2 abdominal pains, clearly related to the antibody administration. Of note was that this toxicity occurred only 24–48 h after antibody treatment. Another patient (patient 3) developed significant diarrhea (grade 2) after 1 year of treatments, but only in conjunction with the onset of eosinophilia. When the huA33 treatments were temporarily discontinued, eosinophilia and diarrhea resolved.

Three unusual clinical observations were made during this study that may be of more general relevance regarding immunotherapy with humanized IgG1 antibodies. First, the renal toxicity induced in patient 9, in the setting of continued treatment with huA33 and rising HAHA activity, no doubt led to increasing levels of circulating immune complexes. Because both the immune complexes and hyperuricemia induced in this patient are toxic to the kidneys, the reversal of renal dysfunction

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was particularly difficult in the presence of these two factors. Second, the hyperuricemia of patient 9 appeared to be part of a cell lysis syndrome because it occurred with a 10-fold increase of LDH and minor changes in phosphate and calcium. Hyperkalemia was not a prominent feature because the patient was on furosemide. This patient had a large volume of tumor, and although some rapid tumor lysis may have occurred, the patient remained with a significant amount of active disease. This partial cell lysis may be a manifestation of heterogeneity of antigen expression within the tumor leading to rapid lysis of a subset of colon cancer cells. Alternatively, the cell lysis may have involved normal A33 antigen-expressing cells or nonspecific toxicity due to immune complexes. The third observation regarding reactivation of symptoms in patient 10, with a pathologic diagnosis of radiation enteritis, indicates that human IgG1 binding to normal tissue in the area of prior radiotherapy may reinduce or exacerbate tissue radiotoxicity. The interaction between chemotherapy and radiotherapy is well known. Radiation recall reactions have the potential to cause severe tissue damage. In the case described, retreatment with antibody reinduced a defined radiation-related bowel dysfunction. The corollary to this observation is that there might be additive antitumor effects from combining radiotherapy with the immune effector functions of human IgG1 antibodies.

We had reported previously (10) that a subset of patients with tumors refractory to chemotherapy who were treated with a specific drug regimen after a single dose of radiolabeled mAb A33 had significant responses. We investigated these effects in a mouse model and found that there is synergy between the antitumor activity of 5-fluorouracil and ^{131}I-mAb A33 (3). Similar observations have now been made with some chemotherapeutic agents when they are administered after completing treatment with huA33 without a radiolabel.[4] The significance of these observations will be better defined in subsequent studies designed to investigate the interaction between immunotherapy and chemotherapy (35). The molecular mechanisms critical for the enhancement of immune-directed lysis of tumor cells by chemotherapeutic regimens have been studied since the original observations focused on lipid synthesis (36, 37). Whereas some chemotherapeutic agents have been reported to show synergy with complement and antibody-dependent cellular toxicity, development of relevant assays to identify the most promising drug-antibody combinations and to define the molecular mechanisms critical for this interaction remains of primary importance. Studies have demonstrated that Herceptin has additive or synergistic antitumor effects when used in combination with chemotherapy (38). In this case, the dual activities of Herceptin (cytostatic effects and immune effector function) may both be critical for the enhanced antitumor responses observed when antibody is used in conjunction with chemotherapy in patients (25, 39). Studies with rituximab have also supported the role for an enhancing effect of chemotherapy on immune-mediated killing of tumor cells (40).

Two patients (patients 3 and 9) with evidence of antitumor effects shared a characteristic, which may indicate that a certain level of autoimmune reactivity may be required for antibody-based therapy to be fully effective in the clinic. Both patients had evidence of preexisting autoimmunity. Patient 3 had a diagnosis of polymyalgia rheumatica, and patient 9 had myositis with elevated creatine phosphokinase serum levels and muscle weakness. The evidence of immune-mediated normal tissue dysfunction in these patients may indicate preexisting dominance of activation Fc receptors (41). Identifying biological factors that modulate antibody-directed immune killing in patients is critical to increase the response rate of IgG1 therapeutic antibodies.

[4] N. E. Kemeny, personal communication.

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Phase I Study of Anticolon Cancer Humanized Antibody A33

Sydney Welt, Gerd Ritter, Clarence Williams, Jr., et al.

Clin Cancer Res 2003;9:1338-1346.

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COMMUNICATION

Framework Residues 71 and 93 of the Chimeric B72.3 Antibody are Major Determinants of the Conformation of Heavy-chain Hypervariable Loops

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Structural analysis derived from the crystallographic study of the chimeric B72.3 antibody illustrated that some heavy-chain framework residues having atomic interactions with heavy-chain CDR residues may directly affect the conformation of CDR loops. For example, an alanine residue at H71 provides room for packing CDR2/CDR1 and lysine residues at H73 and H93 contribute a salt-bridge to aspartic acid at H55 in CDR2 and a hydrogen bond to the carbonyl group at H96 in CDR3, respectively. We have analysed the contribution of these framework residues to the TAG72-binding affinity. We altered these framework residues by site-directed mutagenesis, and determined the affinity of these mutant chimeric antibodies for the TAG72 antigen by solid phase radioimmunoassay. We found that a single amino acid substitution of alanine by phenylalanine at H71 or lysine by isoleucine at H93, significantly reduced the binding affinity for the TAG72 antigen by 12 and 20-fold, respectively, whereas the substitution of lysine by alanine at H73 reduced the binding affinity only two-fold. Our results indicate that heavy-chain framework residues alanine at H71 and lysine at H93 of the chimeric B72.3 antibody are the major determinants of the conformation of heavy-chain CDR2/CDR1 and CDR3 loops, whereas the salt-bridge between lysine at H73 and aspartic acid at H55 is less important. The hydrogen bond between two framework residues, glutamine at H5 and serine at H25 does not affect any CDR conformation. Our results will thus be of importance especially when the humanized B72.3 antibody is constructed by grafting the CDR loops to a human framework. The important framework region interactions must be maintained in the final humanized antibody.

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Keywords: FR residues; site-directed mutagenesis; CDR conformation; binding affinity; TAG72 antigen

The determination of the three-dimensional structure of antibody fragments by X-ray crystallography led to the realization that polypeptide chains of immunoglobulin molecules are folded into globular domains with four in the heavy (H) chain and two in the light (L) chain. These domains are connected by extended peptide segments like pearls on a string (Amzel & Poljak, 1979). All domains exhibit a similar polypeptide folding pattern characterized by two β-sheets. Sequence comparisons of heavy and light-chain variable (V) domains reveal that each domain has three complementarity-determining regions (CDR) flanked by four framework regions (FR) of less variable sequences (Kabat *et al.*, 1987). The antigen combining-site is formed by the juxtaposition of six CDRs appearing as loops at one end of the β-sheet, three from V_H and another three from V_L. These CDR loops present a surface that interacts with the antigen. It has been reported that mutations in CDR can significantly alter both antibody specificity (Rudikoff *et al.*, 1982; Ohno *et al.*, 1985; Kussie *et al.*, 1994) and antigen-binding affinity (Roberts *et al.*, 1987; Allen *et al.*, 1988; Sharon, 1990). The FRs comprise the conserved

Abbreviations used: H, heavy; L, light; CDR, complementarity-determining region; FR, framework region; V, variable.

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β-sheet framework and are responsible for the main-chain conformation of the CDRs and for the interchain interactions that bring domains together (Chothia *et al.*, 1985). It has also been demonstrated that some framework residues may greatly affect the conformation of CDR loops (Kettleborough *et al.*, 1991; Foote & Winter, 1992; Lavoie *et al.*, 1992).

B72.3 is a murine monoclonal antibody with specificity for the mucin-like tumour-associated glycoprotein TAG72 (Thor *et al.*, 1986). The chimeric cB72.3-1-3 V_H gene was previously cloned (Xiang *et al.*, 1990). The amino acid sequence is numbered (Figure 1) according to Kabat's method (Kabat *et al.*, 1987). Recently, crystallization of the chimeric B72.3 Fab' fragment has been reported (Brady *et al.*, 1991) and its crystal structure has been analysed (Brady *et al.*, 1992). The data illustrated that some atomic interactions of FR/CDR residues may directly affect the conformation of heavy-chain CDR loops (Figure 2). For example, the side-chain of alanine at H71 in FR3 provides room for packing CDR2/CDR1 (Figure 2A), whereas lysine residues at H73 and H93 in FR3 contribute a salt-bridge to aspartic acid at H55 in CDR2 (Figure 2B) and a hydrogen bond to the carbonyl group at H96 in CDR3 (Figure 2C), respectively.

Site-directed mutagenesis and affinity constants

In order to confirm these observations, we performed site-directed mutagenesis at four FR positions (H25, H71, H73 and H93) with six oligonucleotides (oligo 17 to 22) resulting in six mutant chimeric antibodies cB72.3m17-22, respectively. These four mutation sites in the context of the entire Fv are shown in Figure 3. The affinity constants of these six mutant antibodies for the TAG72 antigen determined in a solid-phase radioimmunoassay (Xiang *et al.*, 1990) were compared to that of the original cB72.3-1-3 antibody. The cB72.3m17 antibody with only one amino acid substitution of alanine at H71 by phenylalanine showed a reduced binding reactivity for the TAG72 antigen. Its affinity constant K_a for the TAG72 antigen is only $0.57 \times 10^8\ M^{-1}$, which is 12-fold lower than that of the original cB72.3-1-3 antibody ($6.80 \times 10^8\ M^{-1}$) (Table 1). The cB72.3m18 antibody with one amino acid substitution of lysine at H73 by arginine with a similar basic side-chain showed the same TAG72-binding reactivity as the original cB72.3-1-3 antibody, suggesting that the salt-bridge between H73 and H55 was maintained. However, the cB72.3m19 antibody with the amino acid substitution of lysine by alanine at H73 showed slightly decreased binding reactivity for the TAG72 antigen. Its affinity constant K_a is $3.38 \times 10^8\ M^{-1}$, which is about twofold less compared to that of the cB72.3-1-3 antibody. Interestingly, the amino acid substitution of lysine at H93 by the hydrophobic isoleucine significantly decreased the TAG72-binding reactivity of cB72.3m21 (K_a, $0.33 \times 10^8\ M^{-1}$), which is 20-fold less compared to that of the cB72.3-1-3 antibody. On the other hand, the TAG72-binding reactivity of cB72.3m20 with the amino acid substitution of lysine at H93 by arginine with a similar basic side-chain was almost the same as the original cB72.3-1-3 antibody; the corresponding affinity constants for the TAG72 antigen were approximately $6 \times 10^8\ M^{-1}$ (Table 1). The cB72.3m22 antibody with the amino acid substitution of serine at H25 by alanine showed no difference in its TAG72-binding reactivity compared to the cB72.3-1-3 antibody. Overall, these results indicate that two framework residues, namely H71 and H93, are major determinants of the conformation of CDR loops of the chimeric B72.3 antibody. Furthermore, the results show that the salt-bridge formed between H55 and H73 is less important in the conformation of the CDR2 loop, and that the hydrogen bond formed between the glutamine residue at H5 and the serine side-chain at H25 does not affect any CDR loop conformation at all.

In the analysis of immunoglobulins of known structure, Tramontano *et al.* (1990) defined residues

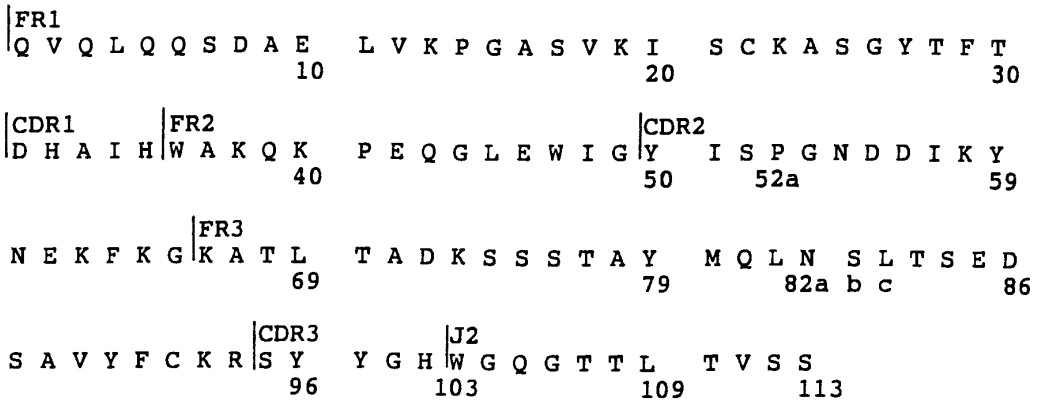


Figure 1. Amino acid sequences of the cB72.3-1-3 V_H region. The one-letter amino acid code is used. Amino acids are numbered sequentially according to Kabat's method. Demarcated are framework regions (FR), complementarity-determining regions (CDR) and joining segment (J).

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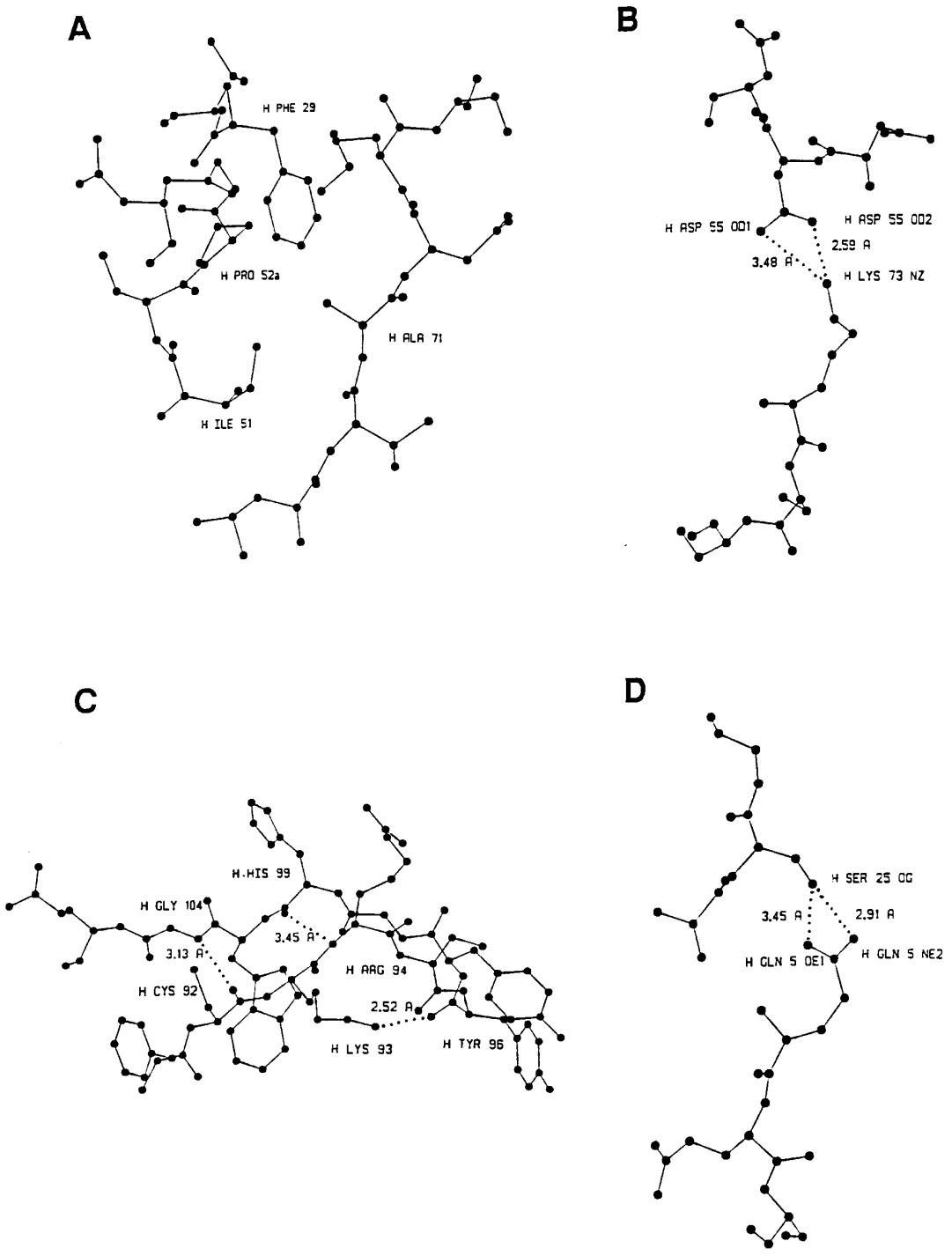


Figure 2. Diagrams of some FR residues with atomic interaction with CDR residues from the co-ordinates of crystallographic analysis of chimeric B72.3 Fab' deposited in the Brookhaven Data Bank. A, Alanine at H71 in FR3 provides room into which packs CDR2 residues isoleucine of H51 and proline of H52a, and the FR1 residue phenylalanine at H29. B, The salt-bridge formed between lysine of H73 in FR3 and aspartic acid of H55 in CDR2. C, The hydrogen-bond formed between the side-chain of lysine at H93 in FR3 and the main-chain carbonyl group at H96 in CDR3. D, The hydrogen-bond formed between serine of H25 and glutamine of H5 in FR1. The α-carbon trace and the amino acid side-chain conformation are marked respectively. Putative hydrogen bonds and salt-bridge are shown as broken lines.

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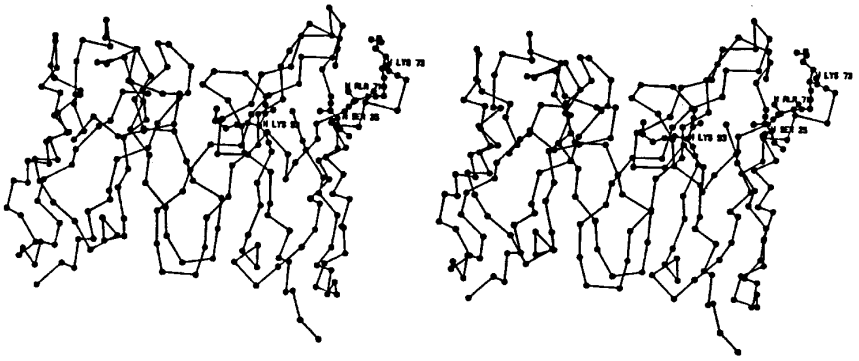


Figure 3. Stereo diagram of the α-carbon trace of the Fv region with mutation sites displayed.

Table 1. Affinity constants of mutant cB72.3m antibodies

Antibody	Amino acid substitution	Affinity constant (K_a)
cB72.3-1-3	—	6.80
cB72.3m17	Alanine by phenylalanine (H71)	0.57
cB72.3m18	Lysine by arginine (H73)	6.42
cB72.3m19	Lysine by alanine (H73)	3.38
cB72.3m20	Lysine by arginine (H93)	6.24
cB72.3m21	Lysine by isoleucine (H93)	0.33
cB72.3m22	Serine by alanine (H25)	6.61

Six oligonucleotides (oligo 17 to 22) complementary to different areas of the V_H region of the chimeric cB72.3-1-3 antibody were designed and synthesized for site-directed mutagenesis. Since alanine at H71 provides room into which packs CDR2 residues isoleucine at H51 and proline at H52a as well as the FRl residue phenylalanine at H29 (Figure 2A), oligo 17 (5' gggca aggcc acact gactt ttgac aaatc ctcca gcac 3') complementary to FR3 of the V_H region was synthesized for substitution of alanine at H71 by phenylalanine. Due to the large size of the side-chain in this amino acid substitution at H71, the CDR1/CDR2 conformation is altered. Since lysine at H73 forms a salt-bridge to aspartic acid at H55 in CDR2 (Figure 2B), oligo 18 (5' ctgac tgcag acaga tcctc cagc 3') and 19 (5' ccaca ctgac tgcag acgca tcctc cagca ctgc 3') were synthesized for substitution of lysine at H73 by arginine with a similar basic side-chain to examine if the salt-bridge between H73 and H55 would be maintained; substitution by alanine was also examined to see if the loss of the salt-bridge would affect its binding affinity for the TAG72 antigen. Lysine at H93 forms a hydrogen bond to the carbonyl group at H96 in CDR3 such that the CDR3 loop is bent and lies at the base of the pocket formed by the CDR loops (Figure 2C). Oligo 20 (5' gtgta tttct gtaga agatc gtac 3') and 21 (5' gtgta tttct gtata agatc gtac 3') were synthesized for substitution of lysine at H93 by arginine with a similar basic side-chain for maintenance of this hydrogen-bond, and by isoleucine for inducing the loss of this hydrogen-bond. For completeness and for comparison purposes, we also selected to alter the hydrogen-bond formation between two FR residues, serine at H25 and glutamine at H5 (Figure 2D) to examine if FR/FR interaction might affect CDR loop conformation. Amino acid substitution of serine by alanine at H25 was made by using oligo 22 (5' tcctg caagg cttct ggcta cacc 3'). All of these amino acid substitutions were conducted by site-directed mutagenesis using oligo 17 to 22 primers and verified by the dideoxy nucleotide-sequencing method (Sanger *et al.*, 1977). Expression vectors mpSV2neo-EP1-$V_{m17\text{-}22}C_{\tau 1}$ used for production of mutant chimeric antibodies cB72.3m17-22 were derived from the mpSV2neo-EP1-$C_{\tau 1}$ (Xiang *et al.*, 1990). The mutant $V_{m17\text{-}22}$ region fragments (*Ecor*I/*Sal*I) containing an *Eco*RI site at the 5' end and the RNA splice junction consensus sequence (5' caggtaagt 3') at the 3' end were purified from M13mp19-$V_{m17\text{-}22}$ and ligated into the mpSV2neo-EP1-$C_{\tau 1}$ (*Eco*RI/*Sal*I-digested) to form six expression vectors including mpSV2neo-EP1-$V_{m17}C_{\tau 1}$, mpSV2neo-EP1-$V_{m18}C_{\tau 1}$, mpSV2neo-EP1-$V_{m19}C_{\tau 1}$, mpSV2neo-EP1-$Vm_{20}C_{\tau 1}$, mpSV2neo-EP1-$V_{m21}C_{\tau 1}$ and mpSV2neo-EP1-$V_{m22}C_{\tau 1}$, respectively. The DNA of these expression vectors was purified in quantity by standard methods (Maniatis *et al.*, 1982), and then transfected into the heavy-chain-loss mutant cell line B72.3Mut(K) (Xiang *et al.*, 1990), which expresses only the cytoplasmic K light-chain, by electroporation (Potter *et al.*, 1984). The transfected cells were selected for growth in the presence of geneticin at 2.0 mg/ml. The growth transfectants for each mutant antibody showing binding reactivities in a human τ chain capture ELISA (Xiang *et al.*, 1990) were selected and sub-cloned. Mutant antibodies were then purified from their culture supernates by protein A affinity chromatography (Xiang *et al.*, 1990). Protein concentrations were determined using a BioRad protein assay kit (BioRad, CA, USA) according to the method described in the manual. Affinity constants (K_a, $\times 10^8$ per mol per litre) were determined from Scatchard plots of the binding of different mutant chimeric antibodies cB72.3m17-22 to the TAG772 antigen in a solid-phase radioimmunoassay as described (Xiang & Chen, 1992). Bovine mucin (Sigma Chemical Co., MO, USA), which contains a large amount of the TAG72 epitope (Xiang *et al.*, 1990), was used as the TAG72 antigen source in this study. ELISA, enzyme-linked immunosorbent assay.

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from H52a to H55 as the H2 region; this region links two strands of the β-hairpin, V_H CDR2. They further divided this H2 region into three groups, namely, the three, four and six-residue H2 regions (Tramontano *et al.*, 1990). Examination of the interactions of the CDR2 loop with the rest of V_H domains shows that the determinants of conformation are not entirely within the sequence of the loop itself, but involve packing of the loop against the rest of the structure. Tramontano *et al.* have also illustrated that the framework residue 71 is a major determinant of the conformation of V_H CDR2 (Tramontano *et al.*, 1990). The B72.3 heavy-chain belongs to the family with a four-residue H2 loop. Residues at H52a/H53 and H29 pack against the residue at H71; the latter is alanine with a small side-chain that provides a cavity for bringing the CDR1 and CDR2 loops close together. In order to examine the importance of alanine at H71 in CDR loop conformation, alanine at H71 was substituted by phenylalanine. A single amino acid substitution of alanine by phenylalanine significantly reduced the binding affinity of the mutant cB72.3m17 antibody for the TAG72 antigen by 12-fold; this is possibly due to a major conformational change of the CDR1/CDR2 loops. Therefore, our study provides an additional evidence that the FR residue alanine at H71 is a major determinant of the conformation of CDR loops.

The B72.3 heavy-chain CDR3 loop is short and comprises only five residues, which adopts a sharp hairpin turn (Figure 2C). The amino acid residue at H93 in FR3 is usually an alanine which packs at the H/L chain interface, but is a lysine in the B72.3 antibody, and makes a charged hydrogen-bond to the carbonyl group of tyrosine at H96 in CDR3. From an examination of the Fab' crystal structure, we found that the hydrogen-bond formed between lysine at H93 and tyrosine at H96 is the only significant atomic interaction between the side-chain of lysine at H93 and the rest of other V_H residues (data not shown). This atomic interaction gives the CDR3 loop an unusual conformation. The CDR3 loop forms part of the bottom of the antigen combining-site as well as a side of the pocket. The amino acid substitution of lysine at H93 by hydrophobic isoleucine which lacks the capability of hydrogen-bond formation, significantly decreased the binding affinity of mutant cB72.3m21 antibody for the TAG72 antigen by 20-fold, indicating the framework residue H93 is a major determinant of the conformation of the CDR3 loop.

Recently, a number of mouse/human chimeric B72.3 antibodies have been constructed by recombinant DNA technology in order to reduce the human anti-mouse antibody response (Whittle *et al.*, 1987; Xiang *et al.*, 1992). However, clinical studies suggested that an anti-idiotypic immune response to the variable region was still present (Khazaeli *et al.*, 1991). In order to minimize this response, the approach of genetically engineering a humanized antibody was taken, in which mouse CDR loops were grafted directly onto a human framework (Jones *et al.*, 1986). In this communication we report experimental proof of the involvement of FR residues (H71 and H93) in the conformation of B72.3 CDR loops. Therefore, our results will be of great importance when the humanized B72.3 antibody is constructed. The important framework region interactions must be maintained in the final humanized antibody.

Acknowledgements

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UNITED STATES
SECURITIES AND EXCHANGE COMMISSION
WASHINGTON, D.C. 20549

FORM 8-K

CURRENT REPORT
PURSUANT TO SECTION 13 OR 15(D) OF
THE SECURITIES EXCHANGE ACT OF 1934

Date of report (Date of earliest event reported): September 5, 2019

ALEXION PHARMACEUTICALS, INC.

(Exact name of registrant as specified in its charter)

Delaware	000-27756	13-3648318
(State or other jurisdiction of incorporation or organization)	**(Commission File Number)**	**(I.R.S. Employer Identification No.)**

121 Seaport Boulevard, Boston, Massachusetts 02210

(Address of Principal Executive Offices) (Zip Code)

Registrant's telephone number, including area code: (475) 230-2596

Check the appropriate box below if the Form 8-K filing is intended to simultaneously satisfy the filing obligation of the registrant under any of the following provisions (*see* General Instruction A.2. below):

☐ Written communications pursuant to Rule 425 under the Securities Act (17 CFR 230.425)

☐ Soliciting material pursuant to Rule 14a-12 under the Exchange Act (17 CFR 240.14a-12)

☐ Pre-commencement communications pursuant to Rule 14d-2(b) under the Exchange Act (17 CFR 240.14d-2(b))

☐ Pre-commencement communications pursuant to Rule 13e-4(c) under the Exchange Act (17 CFR 240.13e-4(c))

Indicate by check mark whether the registrant is an emerging growth company as defined in Rule 405 of the Securities Act of 1933 (§230.405 of this chapter) or Rule 12b-2 of the Securities Exchange Act of 1934 (§240.12b-2 of this chapter).

☐ Emerging Growth Company

If an emerging growth company, indicate by check mark if the registrant has elected not to use the extended transition period for complying with any new or revised financial accounting standards provided pursuant to Section 13(a) of the Exchange Act. ☐

Item 8.01 Other Events

On September 5, 2019, the European Patent Office determined, following examination proceedings, not to grant Alexion Pharmaceuticals, Inc.'s (the "Company") European patent applications 3124029 and 3167888 which relate to the pharmaceutical compositions of SOLIRIS® (eculizumab) and composition of matter of SOLIRIS® (eculizumab), respectively. The Company will evaluate its option to appeal the European Patent Office's decision with respect to these patent applications following receipt and review of the written decision.

Signature

Pursuant to the requirements of the Securities Exchange Act of 1934, the registrant has duly caused this report to be signed on its behalf by the undersigned hereunto duly authorized.

Date: September 5, 2019

ALEXION PHARMACEUTICALS, INC.

By: /s/ Doug Barry
Name: Doug Barry
Title: Vice President, Corporate Law

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 11, 2019
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN5
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard Bell *et al.*

Application No.: 15/642,096

Confirmation No.: 7606

Filed: July 5, 2017

Art Unit: 1644

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Examiner: J. L. Rogers

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

In compliance with 37 C.F.R. § 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached PTO/SB/08. In accordance with 37 C.F.R. § 1.98(a)(2)(i)-(iv), Applicant submits herewith copies of foreign patents and non-patent literature references, but has not included copies of U.S. patents and/or U.S. patent applications.

It is respectfully requested that the documents listed on the PTO/SB/08 be expressly considered by the Examiner during the prosecution of this application, and that the documents be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

The filing of this Information Disclosure Statement is not to be interpreted as a representation that the cited documents are material, that an exhaustive search has been conducted, or that no other relevant information exists. Nor shall the citation of any documents herein be

construed *per se* as a representation that such document is prior art. Moreover, Applicant understands the Examiner will make an independent evaluation of the cited documents.

This Information Disclosure Statement is filed after the mailing date of a first Office Action on the merits, but before the mailing date of any of a Final Action under 37 C.F.R. § 1.113, a Notice of Allowance under 37 C.F.R. § 1.311 or an action that otherwise closes prosecution in this application (37 C.F.R. § 1.97(c)).

Please charge our Deposit Account No. 50-1196 in the amount of $240.00 covering the fee set forth in 37 C.F.R. § 1.17(p). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed to our Deposit Account No. 50-1196, under Order No. AXJ-114BUSCN5.

Dated: December 11, 2019

Respectfully submitted,

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/642,096	Filing Date 07/05/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

	(Column 1)	(Column 2)			
FOR	NUMBER FILED	NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	12/11/2019	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 8	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

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This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) AXJ-114BUSCN5

In re Application of: Leonard BELL *et al.*

Application No.: 15/642,096-Conf. #7606

Filed: July 5, 2017

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

The applicant, Alexion Pharmaceuticals, Inc., owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of **prior patent** No. (see attached) as the term of said **prior patent** is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

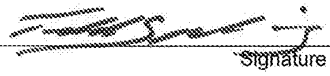
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I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. [ ] The undersigned is an attorney or agent of record. Reg. No. _____



Signature — 12/13/19 Date

Todd Spalding
Typed or printed name

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Title — Telephone Number

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Dated: December 11, 2019 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

SUPPLEMENTAL SHEET FOR TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) AXJ-114BUSCN5

Prior patent Nos. applicable to this terminal disclaimer (referenced on first page):

9,732,149
9,718,880
9,725,504
9,409,980
9,415,102
9,556,263
9,352,035
9,925,262
10,239,937

PTO/AIA/96 (08-12)
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Alexion Pharmaceuticals, Inc.

Application No./Patent No.: 15/642,096 Filed/Issue Date: July 5, 2017

Titled: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Alexion Pharmaceuticals, Inc. , a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. [X] The assignee of the entire right, title, and interest.

2. [ ] An assignee of less than the entire right, title, and interest (check applicable box):
   - [ ] The extent (by percentage) of its ownership interest is _____ %). Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.
   - [ ] There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. [ ] The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. [ ] The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. [ ] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____ , Frame _____ , or for which a copy thereof is attached.

B. [X] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Mark J. Evans To: Alexion Pharmaceuticals, Inc.
   The document was recorded in the United States Patent and Trademark Office at Reel 042921 , Frame 0158 , or for which a copy thereof is attached.

2. From: Leonard Bell To: Alexion Pharmaceuticals, Inc.
   The document was recorded in the United States Patent and Trademark Office at Reel 042921 , Frame 0063 , or for which a copy thereof is attached.

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: Russell P. Rother To: Alexion Pharmaceuticals, Inc.
The document was recorded in the United States Patent and Trademark Office at
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Reel _____ , Frame _____ , or for which a copy thereof is attached.

5. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
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The document was recorded in the United States Patent and Trademark Office at
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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature	Date: 12/13/19
Todd Spalding	Vice President, Head of Intellectual Property
Printed or Typed Name	Title or Registration Number

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 11, 2019 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 11, 2019
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN5
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard BELL *et al.*

Application No.: 15/642,096

Confirmation No.: 7606

Filed: July 5, 2017

Art Unit: 1644

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Examiner: James L. Rogers

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. § 1.111

Dear Sir:

In response to the Office Action dated June 11, 2019, please amend the above-identified U.S. patent application as follows:

Listing of Claims begin on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

LISTING OF CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

1-51. (Canceled)

52. **(Previously presented)** A method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH) comprising administering to the patient a pharmaceutical composition comprising an antibody that binds C5, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, and wherein the composition comprises a single-unit dosage form comprising 300 mg of the antibody in 30 mL of a sterile, preservative-free solution.

53. **(Previously presented)** The method of claim 52, wherein the patient has received at least four transfusions in the twelve months prior to administration of the antibody.

54. **(Previously presented)** The method of claim 53, wherein the patient has a platelet count greater than or equal to 100,000 per microliter prior to administration of the antibody.

55. **(Previously presented)** The method of claim 54, wherein the patient is treated with the antibody for at least 26 weeks.

56. **(Previously presented)** A method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH) comprising administering to the patient a pharmaceutical composition comprising an antibody that binds C5, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, wherein the composition comprises a single-unit dosage form comprising 300 mg of the antibody in 30 mL of a sterile, preservative-free solution, and wherein the patient exhibits decreased lactate dehydrogenase levels.

57. **(Previously presented)** The method of claim 56, wherein the patient has received at least four transfusions in the twelve months prior to administration of the antibody.

58. **(Previously presented)** The method of claim 57, wherein the patient has a platelet count greater than or equal to 100,000 per microliter prior to administration of the antibody.

59. **(Previously presented)** The method of claim 58, wherein the patient is treated with the antibody for at least 26 weeks.

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REMARKS

Applicants acknowledges with appreciation the in-person interview with the Examiner and his supervisor on November 12, 2019, during which the following rejections were discussed.

Claims 52-59 are pending. *No new matter has been added.*

Information Disclosure Statement

Applicants acknowledge with appreciation the Examiner's indication that the Information Disclosure Statements submitted on August 22, 2017, March 28, 2018, April 27, 2018, and April 29, 2019 have been considered. Applicants submit herewith a Supplemental Information Disclosure Statement to make of record additional information for the Examiner's consideration.

Rejection of Claim 52-59 under 35 U.S.C. § 103(a)

Claim 52 is rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen *et al.* (*N Engl J Med* 2004; 350: 552-9), in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660), and Bowdish *et al.* (US 2003/0232972).

In addition, claims 52-59 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen *et al.*, in view of Evans *et al.*, Wang, and Bowdish *et al.*, as applied to claim 52, and further in view of Bell *et al.* (US 2005/0191298).

A. Rejection of Claim 52

Claim 52 is directed to a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH) comprising administering to the patient a pharmaceutical composition comprising an antibody that binds C5, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, and wherein the composition comprises a single-unit dosage form comprising 300 mg of the antibody in 30 mL of a sterile, preservative-free solution.

The Examiner relies on Hillmen *et al.* for teaching a method of treating a paroxysmal nocturnal hemoglobinuria (PNH) patient by administering an effective amount of eculizumab. The Examiner relies on Evans *et al.* for teaching effective doses of eculizumab and that it is

within the skill of the art to determine the optimal dose. While the Examiner acknowledges that neither Hillmen *et al.* nor Evans *et al.* teach "a 300 mg single unit dosage as recited in the claims", the Examiner asserts that it would have been obvious to modify the teachings of Hillmen *et al.* and Evans *et al.* to arrive at a 300 mg dosage.

Moreover, the Examiner alleges that one of ordinary skill in the art would have had a reasonable expectation of success in making a 10 mg /ml solution of the antibody in a sterile, preservative free solution in view of Wang, which the Examiner alleges teaches that eculizumab is highly soluble, including up to 30 mg per ml and can be formulated in a sterile, preservative free solution. While the Examiner acknowledges that neither Hillmen *et al.*, Evans *et al.*, nor Wang teach an antibody having the presently claimed heavy chain consisting of SEQ ID NO:2 and light chain consisting of SEQ ID NO:4, the Examiner nonetheless concludes that:

> [i]t would have been obvious to one of ordinary skill in the art at the time the invention was made to substitute Bowdish's TPO mimic amino acid sequence in Bowdish's H chain published SEQ ID NO:67 which is 96% identical with currently claimed H chain SEQ ID NO:2 with Evan's CDR3 5G1.1 eculizumab amino acid sequence, thereby arriving at a H chain sequence having 100% sequence identity to currently claimed H chain SEQ ID NO:2 and to have combined that H chain sequence with Bowdish's L chain SEQ ID NO: 68 that has 100% sequence identity to currently claimed L chain SEQ ID NO:4.

Applicant respectfully traverses the rejection. To establish a *prima facie case of obviousness*, it must be demonstrated that the presently claimed invention constitutes nothing more than a combination of known elements ***yielding a predictable result***, and that one of ordinary skill in the art would have had a ***clear reason to combine the elements***, as well as the technical ability to do so, based on the teachings and motivation provided by the prior art (see *KSR International Co. v. Teleflex Inc. et al*.127 S. Ct. 1727 (2007) ("*KSR International Co.*") and *Ecolab, Inc. v. FMC Corp.*, 569 F. 3d 1335 (Fed. Cir. 2009) ("*Ecolab, Inc.*")). The cited references fail to render the presently claimed invention obvious, at least because ***they provide no motivation, let alone a reasonable expectation of success***, in arriving at the claimed invention. Moreover, the secondary indicia of non-obviousness presented by Applicant herein (discussed below) provide clear, factually supported evidence, that the invention was non-obvious prior to the relevant filing date (see, *e.g.*, *Transocean Offshore Deepwater Drilling, Inc.*

v. Maersk Drilling USA, Inc., 699 F.3d 1340, 1349 (Fed. Cir. 2012)), noting objective evidence of non-obviousness "may often be the most probative and cogent evidence in the record" and "may often establish that an invention appearing to have been obvious in light of the prior art was not").

1. **The Relevant Literature Prior to March 15, 2007 Repeatedly and Consistently Described "Eculizumab" as Having an IgG4 Heavy Chain Constant Region**

Prior to March 15, 2007, the priority date of the present application, the unique amino acid sequence of the antibody in Soliris® (eculizumab) recited in the pending claims was not publicly known or disclosed in the prior art. In particular, while a person of ordinary skill in the art as of that date would have known that Alexion had designed and clinically tested an antibody named "eculizumab," the person of ordinary skill in the art would not have known that "eculizumab" had the uniquely-engineered amino acid sequence claimed in the present application. That is because the literature as of March 15, 2007 – shown by at least eight publications listed in Table 1 below – consistently identified "eculizumab" as the antibody described in the "Thomas" publication (Thomas TC, *et al.*, Mol. Immunol., 1996;33:1389-1401), which has a naturally-occurring "IgG4" heavy chain constant region. Accordingly, a person of ordinary skill in the art as of March 15, 2007 would have had no doubt that "eculizumab" was Thomas's IgG4-isotype humanized antibody, because the pertinent literature consistently and unambiguously said so:

Table 1: References to "Eculizumab" as Thomas's Humanized IgG4 Antibody

Exhibit	Statement Identifying "Eculizumab" as the IgG4 Humanized Antibody of Thomas
Hillmen 2004 (Hillmen, P., *et al.*, "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 350:552-559 (2004)) at 553 – Phase II clinical trial for treatment of PNH	"***Eculizumab is*** a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components." (Citing ***Thomas***, Ref. No. 15)

Exhibit	Statement Identifying "Eculizumab" as the IgG4 Humanized Antibody of Thomas
Hill 2005 (Hill, A., *et al.,* "Sustained response and long-term safety of eculizumab in paroxysmal nocturnal Hemoglobinuria," Blood 106: 2559-2565 (2005)) at 2559 – 52-week extension of Hillmen 2004 Phase II clinical trial	"***Eculizumab is*** a humanized monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage." (Citing ***Thomas***, Ref. No. 9)
Hillmen 2006 (Hillmen, P., *et al*., "The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 355:1233-1243 (2006)) at 1234 – pivotal Phase III clinical trial for treatment of PNH,	"***Eculizumab (Soliris, Alexion Pharmaceuticals) is*** a humanized monoclonal antibody directed against the terminal complement protein C5." (Citing ***Thomas***, Ref. No. 13)
Hill 2007 (Hill, *et al*., Erythropoietin treatment during complement inhibition with eculizumab in a patient with paroxysmal nocturnal hemoglobinuria, THE HEMATOLOGY JOURNAL, 31-33, 2007**)** – post-Phase III case report for PNH patient	"***Eculizumab is*** a novel humanized monoclonal antibody directed against the complement protein C5." (Citing ***Thomas***, Ref. No. 6)
Bell (Bell, L. and Rother, R., "Method of Treating Hemolytic Disease," U.S. Patent Application Publication No. 2005/0191298 Al (filed February 3,2005; published September 1, 2005)) at [0052] –Phase II clinical studies described in Hillmen 2004 and Hill 2005	States that "[m]ethods for the preparation of h5G1.1-mAb" are described in ***Thomas***, and that "[t]he antibody h5G1.1-mAb is currently undergoing clinical trials under the name ***eculizumab***."
Kaplan (Kaplan, M., "Eculizumab (Alexion)," Curr. Opin. Investig. Drugs 3: 1017-1023 (2002)) at 1018	States that "***Eculizumab*** (5G1.1), under development by Alexion Pharmaceuticals Inc. is a humanized C5 inhibitory monoclonal antibody (mAb)," and cites ***Thomas*** for the synthesis and complement-blocking activity of "intact humanized 5G1.1 antibody" or "humanized 5G1.1"

Exhibit	Statement Identifying "Eculizumab" as the IgG4 Humanized Antibody of Thomas
Brekke (Brekke, 0. and Sandlie, I., "Therapeutic antibodies for human diseases at the dawn of the twenty-first century," Nature Reviews Drug Discovery 2: 52-62 (2003)) at 56	"***Eculizumab*** (5G1.1; Alexion Pharmaceuticals) ***is*** a humanized monoclonal antibody that prevents the cleavage of human complement component C5 . . . ." (citing ***Kaplan***, Ref. No. 31, which in turn cites to ***Thomas***)
Pierangeli (Pierangeli, S., *et al.*, "Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia," Arthritis & Rheumatism 52: 2120-2124 (2005)) at 2123	Stating that "***eculizumab*** has been shown to prevent C5 activation in humans and to have beneficial effects in patients with [PNH] . . . ." (citing to ***Hillmen 2004***, Ref No. 18, which in turn cites to ***Thomas***)

Looking at this literature, a person of ordinary skill in the art as of March 15, 2007 would have believed that Thomas's IgG4 antibody was the ***only*** full-length humanized antibody shown to bind C5 and prevent its cleavage, that had been tested for safety and efficacy in treating PNH, and that had been submitted to the FDA for marketing approval.[1] In Thomas, a person of ordinary skill in the art would have seen the extensive work in rationally designing an "intact," full-length anti-C5 humanized monoclonal antibody using an IgG4 isotype. In the published literature that followed Thomas, a person of ordinary skill in the art as of March 15, 2007 would have seen how researchers had fully established "eculizumab" – consistently identified as Thomas's IgG4 antibody – to be safe and effective in treating PNH, applying for FDA and European approval for that antibody under the trade name Soliris®.

Contrary to the Examiner's assertion, while Hillmen *et al.* discusses administering "eculizumab" for treating PNH, ***nowhere*** does this reference teach the structure of the antibody, let alone the uniquely-engineered heavy chain constant region amino acid sequence of SOLIRIS® (eculizumab) reflected in SEQ ID NO: 2. Instead, as noted above in Table 1, Hillmen *et al.* references Thomas to further describe "eculizumab," which in turn taught an antibody with an

[1] Thomas also does not disclose the specific light chain amino acid sequence recited in SEQ ID NO: 4 of the present claims. In particular, the light chain variable region sequence provided by Thomas differs from SEQ ID NO: 4 at amino acid position 38, flanking light chain CDR1.

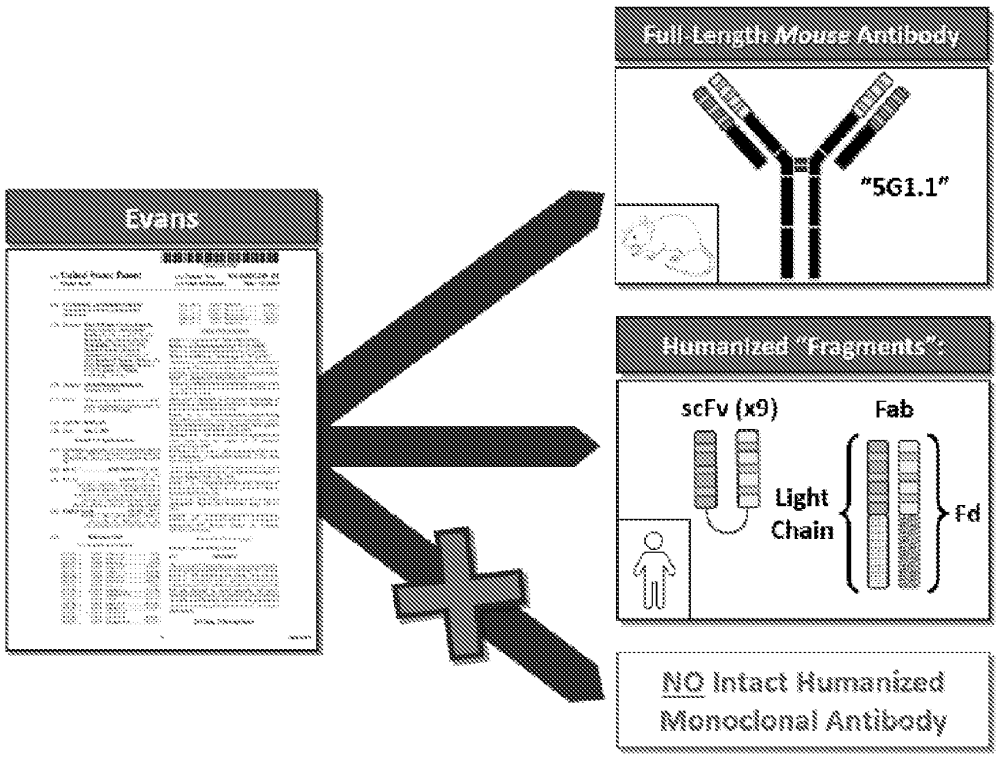
IgG4 heavy chain constant region having a very different amino acid sequence from the heavy chain constant region reflected in SEQ ID NO: 2. Accordingly, a person of ordinary skill in the art as of March 15, 2007 reading Hillmen *et al.* would have envisioned that the "eculizumab" antibody shown in that study to be safe and effective for treating PNH was the ***IgG4*** isotype antibody described in Thomas. Hillmen *et al.* clearly would have led a person of ordinary skill in the art in a direction divergent from the path that was taken by the present invention *i.e.*, towards the IgG4 antibody of Thomas, as opposed to the very different, uniquely-engineered antibody encompassed by the pending claims (see *Millennium Pharm., Inc. v. Sandoz Inc.*, 862 F.3d 1356, 1366 (Fed. Cir. 2017)).

2. Evans *et al.* Does Not Disclose Any Full-Length Humanized Antibodies

Evans *et al.* fails to make up for the deficiencies of Hillmen. In contrast to Thomas and the literature regarding "eculizumab" that followed, a person of ordinary skill in the art would have understood that Evans *et al.* did ***not*** disclose "eculizumab." Rather, a person of ordinary skill in the art would have understood that Evans *et al.* describes an earlier stage of research that predated the design of the intact, full-length humanized antibody "eculizumab" – because Evans *et al.* does not disclose any full-length humanized antibodies. At most, a person of ordinary skill in the art would have read Evans *et al.* as a precursor to the research that resulted in "eculizumab." In fact, the only full-length antibody described in Evans *et al.* is the "5G1.1" mouse antibody, which Evans *et al.* obtained from the "5G1.1" hybridoma (Evans *et al.*, 19:47-49 and Figs. 18-19). Evans *et al.* further describes the researchers' characterization of the "5G1.1" mouse antibody, including its binding affinity, in vitro activity blocking complement in hemolytic assays, and the sequencing and cloning of the variable regions of the "5G1.1 mouse antibody" (Evans *et al.*, Examples 7-10). But Evans *et al.* provides no such information for a full-length humanized antibody derived from the "5G1.1" mouse antibody – which a person of ordinary skill in the art would have understood would have a different amino acid sequence and different clinical properties from the mouse antibody, as attested to by Arturo Casadevall, an independent expert consultant, a Bloomberg Distinguished Professor of Molecular Microbiology & Immunology and Infectious Diseases at the Johns Hopkins Bloomberg School of Public

Health, and the Alfred and Jill Sommer Professor and Chair of the W. Harry Feinstone Department of Molecular Microbiology and Immunology at the Johns Hopkins Bloomberg School of Public Health (see ¶126 of the Declaration of Arturo Casadevall; Appendix A).

To the extent Evans *et al.* describes "humanization" work based on the "5G1.1" mouse antibody, it was the development of recombinant "fragments" – scFv or Fab – that did not contain an intact heavy chain constant region, let alone the uniquely-engineered heavy chain constant region reflected in SEQ ID NO: 2 of the present application (see Example 11 of Evans *et al.* and ¶126 of the Declaration of Arturo Casadevall; Appendix A). For example, Evans *et al.* describes nine different humanized "scFv" fragments, which are recombinantly-produced molecules containing two variable regions connected by a linker, with no constant region (Evans *et al.*, 43:6-14 and 43:62-45:4), and humanized "Fab" fragments that also lack the "Fc" portion (regions CH2 and CH3) of an intact antibody (Evans *et al.* 43:21-61 and ¶126 of the Declaration of Arturo Casadevall; Appendix A). Notably, the humanized Fab fragments of Evans *et al.* have different heavy chain sequences ("Fd," Evans SEQ ID NOs: 11 and 12) from the non-prior art SEQ ID NO: 2 of the present application, including in the "CH1" constant region (¶12 of the Declaration of Arturo Casadevall; Appendix A). The following figure illustrates how a POSA would have understood what Evans *et al.* did disclose – a mouse antibody and humanized "fragments" – and what Evans *et al.* did <u>not</u> disclose, *i.e.*, an intact humanized monoclonal antibody:



In sum, nothing in Evans *et al.*, suggests or discloses constructing the uniquely-engineered heavy chain constant region set forth in SEQ ID NO: 2, and one of ordinary skill in the art as of March 15, 2007 would not have been motivated to make the heavy chain constant region set forth in SEQ ID NO:2, since they would have understood from the literature that the IgG4 isotype antibody of Thomas was clinically successful. Accordingly, like Hillmen *et al.*, Evans *et al.* would not have led one of ordinary skill in the art to make an antibody with the specific sequence encompassed by the pending claims.

3. <u>A Person Skilled in the Art Would Have Had No Reason to Look to Bowdish *et al.*, and, Even Looking to Bowdish, a Person Skilled in the Art Would Not Have Combined Various Portions of Various Sequences to Arrive at the Claimed Sequence</u>

Bowdish *et al.* concerns using an antibody – ***any*** antibody – as a "scaffold" to house a peptide (*e.g.*, the hormone thrombopoietin ("TPO")), thereby providing greater stability and longer half-life for the peptide *in vivo*. The assays reported in Bowdish *et al.* have nothing to do with C5 binding or blocking complement-mediated lysis. Rather, Bowdish *et al.* assessed the binding of TPO-mimetic peptide (presented on an antibody scaffold) with a TPO receptor (the "cMpl receptor") – a biological interaction that is not part of the complement pathway and has nothing to do with cleavage of C5. Accordingly, one of ordinary skill in the art seeking a safe and effective anti-C5 antibody composition for the treatment PNH would <u>not</u> have looked to art, *e.g.*, Bowdish *et al.*, that teaches about something entirely different (see, *e.g.*, *Broadcom Corp. v. Emulex Corp.*, 732 F.3d 1325, 1334 (Fed. Cir. 2013)).

Importantly, like Evans *et al.*, Bowdish *et al.* fails to even mention "eculizumab." There is no reason why a person of ordinary skill in the art as of March 15, 2007 would have set aside the literature's clear teaching regarding the structure of the therapeutically successful "eculizumab" antibody and would have instead looked to Bowdish *et al.*, which addresses unrelated problems. None of the prior art references – including Hillmen *et al.*, Evans *et al.*, and Bell *et al.* – cited or pointed to Bowdish *et al.* in any manner.

Indeed, the term "5G1.1" in Bowdish *et al.*, would <u>not</u> have supplied any definitive information about the structure or sequence of "eculizumab." As discussed above, the term "5G1.1" had been used in the literature to broadly refer to many different constructs such as, *e.g.*, the original mouse ("murine") antibody identified by Alexion, and a wide variety of "humanized" constructs (also termed "h5G1.1"), including at least nine different variable region fragments ("scFv") described in Evans *et al.*, as well as Thomas's humanized IgG4 construct that the literature as of March 15, 2007 described as "eculizumab". Thus, neither Bowdish *et al.*'s reference to using "5G1.1" as a scaffold for a TPO-mimetic peptide, nor Bowdish *et al.*'s citation to Evans *et al.*, would have explained to a person of ordinary skill in the art how Bowdish *et al.*'s

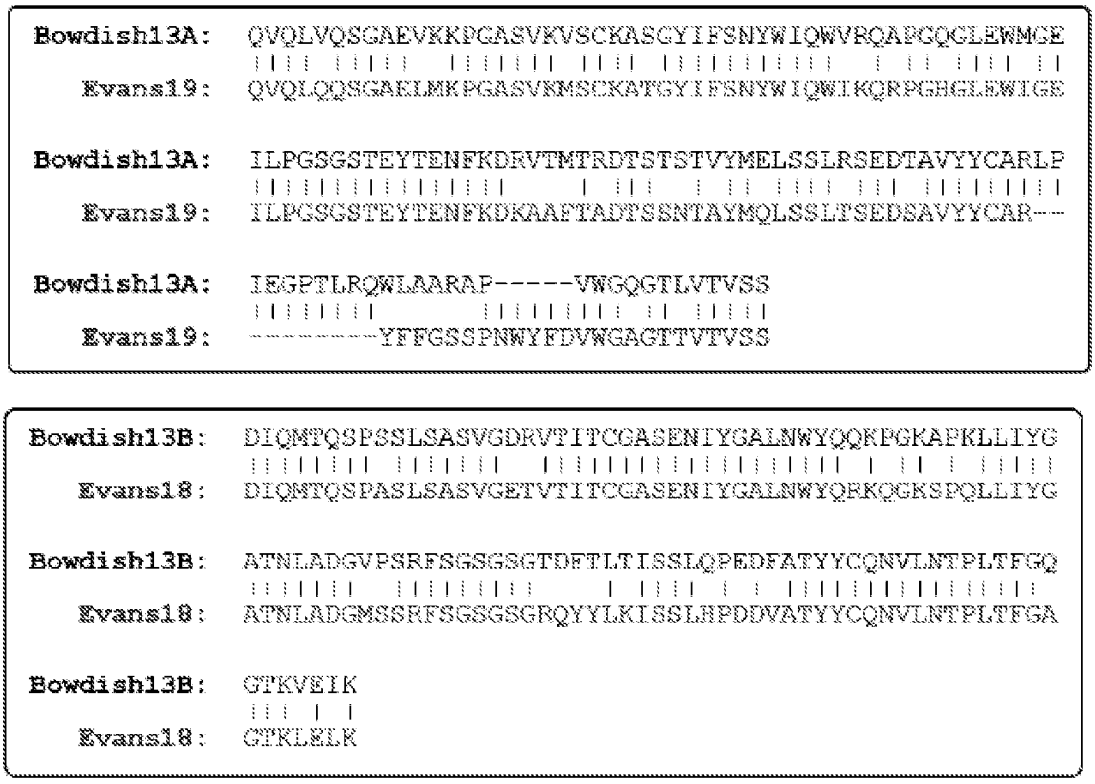
TPO-mimetic construct relates to the structure of "eculizumab," let alone the complete unique sequence recited in the present claims.

Moreover, the only reference in Bowdish *et al.* to Evans *et al.* states: "Construction of 5G1.1 is described in U.S. Application. Ser. No. 08/487,283, incorporated by reference herein." However, Bowdish's vague statement referencing the '283 application for "[c]onstruction of 5G1.1," without further explanation, is not effective to incorporate any specific material from Evans into Bowdish *et al*., because it fails the requirement that "the host document . . . identify with ***detailed particularity*** what specific material it incorporates and ***clearly indicate*** where that material is found in various documents." *Advanced Display Sys., Inc. v. Kent State Univ.*, 212 F.3d 1272, 1282 (Fed. Cir. 2000). Even if a person of ordinary skill in the art had attempted to make sense of Bowdish e*t al.*'s reference to "[c]onstruction of 5G1.1 as described in [the '283 application]," it would not have led them to a clear and unequivocal disclosure of the presently claimed invention. A person of ordinary skill in the art reading Bowdish *et al.*, would have understood that its incorporation was ***limited*** to the portion of the Evans *et al.* describing the "[c]onstruction of 5G.1."

In turn, a person of ordinary skill in the art looking to Evans *et al.* for "[c]onstruction of 5G1.1" would have been directed to Evans's description of a ***mouse*** antibody – "the 5G1.1 antibody" or "the 5G1.1 mAb", obtained from the original "5G1.1" hybridoma (see Evans *et al.* Example 7-10 and Figs. 18-19). For example, Evans *et al.* explains that "[a] particularly preferred antibody of the invention is ***the 5G1.1 antibody*** (5G1.1, ***produced by the 5G.1.1 hybridoma***, ATCC designation HB-11625)," and identifies Figs. 18 and 19 as disclosing the light chain and heavy chain variable regions of "***the antibody 5G1.1***." In particular, nothing in Bowdish *et al.*'s reference to the "5G1.1" mouse antibody of Evans *et al.* would have indicated to a person of ordinary skill in the art that the "scaffold" antibody (whose sequence was not disclosed in Bowdish *et al.*) that was used to generate Bowdish *et al.*'s TPO-mimetic compound was the specific antibody consisting of SEQ ID NOs: 2 and 4, as recited in the pending claims.

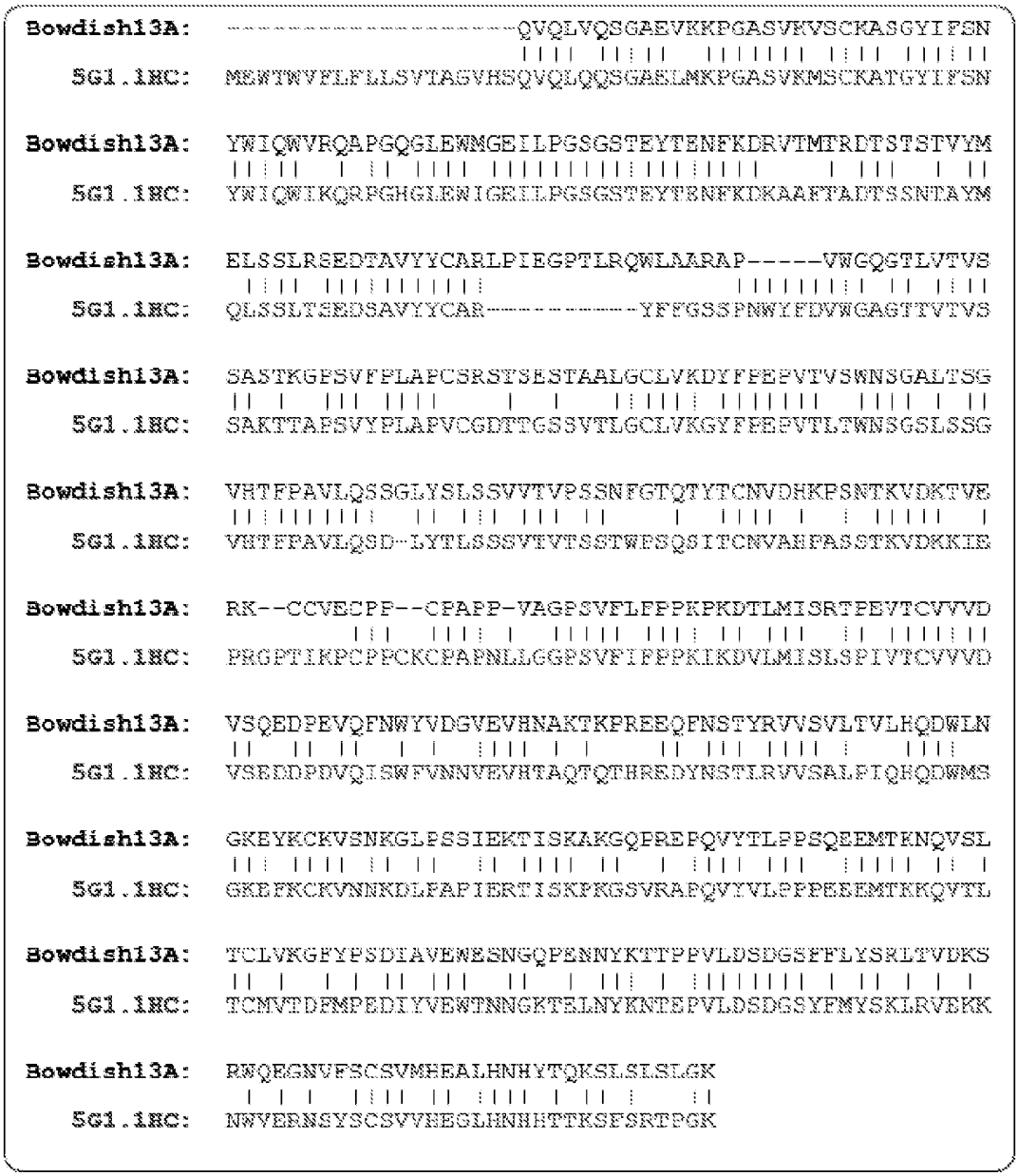
Moreover, as the following figures illustrate, a comparison of the sequences for Evans's "5G1.1" mouse antibody variable regions (Figs. 18-19) or the whole "5G1.1" mouse antibody with the sequences for Bowdish's TPO-mimetic compound (Figs. 13A and 13B) would have

revealed a mismatch in amino acids beyond those that Bowdish *et al.* identified as the TPO-mimetic peptide insert (see ¶¶214-215 of the Declaration of Arturo Casadevall; Appendix A):



Top: Bowdish Figure 13A (SEQ ID NO: 67) compared to Evans Fig. 19

Bottom: Bowdish Fig. 13B compared to Evans Fig. 18



Bowdish Figure 13A (SEQ ID NO: 67) compared to heavy chain of 5G1.1 antibody from HB-11625

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In view of the mismatch between the sequences of Bowdish *et al.* and the incorporated sequences of Evans *et al.*, a person of ordinary skill in the art as of March 15, 2007 would have found Bowdish *et al.*'s reference to Evans *et al.*, to be at most, ambiguous, and certainly would not have understood Bowdish *et al.* to clearly and unequivocally disclose an antibody consisting of SEQ ID NOS: 2 and 4 recited in the pending claims. In particular, nothing in Bowdish *et al.*'s reference to the "5G1.1" mouse antibody of Evans *et al.* would have indicated to a person of ordinary skill in the art that the "scaffold" antibody (whose sequence was not disclosed in Bowdish *et al.*) used to generate Bowdish *et al.*'s TPO-mimetic compound was the specific antibody consisting of SEQ ID NOs: 2 and 4 of the present application (see ¶¶162-163, 190 of the Declaration of Arturo Casadevall, Appendix A).

In sum, prior to March 15, 2007, no single document disclosed the entire amino acid sequence in eculizumab. Nor was eculizumab available to the public for testing or analysis prior to that date. If a person of ordinary skill in the art were searching for the sequence of "eculizumab" as described in the art, the literature as of March 15, 2007 identified an amino acid sequence and corresponding structure that is very different from what the present application claims. In particular, publications describing the safety, efficacy, and clinically relevant biological activity of "eculizumab" consistently directed one of ordinary skill in the art to the 1996 Thomas publication for the structure and design of the antibody, which in turn described a humanized antibody constructed with a naturally-occurring ***IgG4*** heavy chain constant region. The antibody encompassed by the pending claims has a very different, uniquely-engineered, non-naturally occurring constant region that was nowhere described in Thomas or the prior art literature showing the safety and efficacy of "eculizumab."

4. <u>The Rejection Improperly Uses Hindsight Knowledge to Piece Together the Claimed Invention</u>

Moreover, the Examiner's reliance on Bowdish *et al.* uses hindsight knowledge to reconstruct the sequences claimed in the present applications from bits and pieces of unrelated, prior art that teaches in an entirely different direction (*e.g.*, TPO-mimetics). Such hindsight-

driven analysis is always an improper basis for alleging obviousness. *See, e.g.*, *Graham v. John Deere Co.*, 383 U.S. 1, 36 (1966) (warning against "slipping into use of hindsight" and "the temptation to read into the prior art the teachings of the invention in issue"); *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398, 421 (2017) (warning against "the distortion caused by hindsight bias" and "arguments reliant upon *ex post* reasoning").

In particular, the Examiner uses hindsight to cobble together the sequence recited in the pending claims from Bowdish *et al.* (for portions of the claimed sequence, including its non-natural heavy chain constant region) and Evans *et al.* (for the variable region of the claimed sequence). However, the Examiner fails to show why one of ordinary skill in the art ***without*** hindsight would have selected Bowdish *et al.* and combined it with Evans *et al.* to obtain the antibody encompassed by the pending claims, or how they could have reasonably expected success in doing so. Specifically, the Examiner improperly uses the present application as a ***reference point*** for reconstructing the amino acid sequence of the claimed antibody ***in hindsight***, from sequences selectively combined from Bowdish *et al.* and Evans. *et al.* However, a person of ordinary skill in the art could not have made any of these comparisons, because a person of ordinary skill in the art would not have had access to the sequence of the present application. But "working backwards from [a] compound, with the benefit of hindsight, once one is aware of it does not render it obvious." *Amerigen Pharm. Ltd. v. UCB Pharma GmbH*, 913 F.3d 1076, 1089 (Fed. Cir. 2019).

For example, in addition to the disclosure of a humanized scFv compounds, Evans *et al.*, also discloses many other sequences, including:

- Figures 18 and 19 (i.e., the variable regions of the mouse antibody that Bowdish *et al.*, references (with respect to the '283 application) for "[c]onstruction of 5G1.1" (see ¶¶214-15 of the Declaration of Arturo Casadevall; Appendix A);
- The "Fd" molecules of Evans et al., SEQ ID NOs: 11, and 12, which a person of ordinary skill in the art would have understood were more "complete" fragments of a humanized antibody than an scFv, and provide sequences in the constant region (and for SEQ ID NO: 11, the variable region) that do not align with the presently

claimed sequences (see ¶¶ 129 and 158 of the Declaration of Arturo Casadevall; Appendix A); and

- The eight other humanized "scFv" molecules of Evans *et al.* (*see* ¶157 of the Declaration of Arturo Casadevall; Appendix A).

Even assuming the disclosures of Bowdish *et al.* and Evans *et al.* were fully combined, a person of ordinary skill in the art without hindsight would not have been directed to the complete amino acid sequence recited in the pending claims. Among other things, a person of ordinary skill in the art would not have reasonably assumed that "heavy chain CDR3" referenced in Bowdish *et al.* was the same as the heavy chain CDR3 described in Evans *et al.* for humanized scFv compounds, which are *not* full-length structures like the TPO-mimetic compound of Bowdish *et al.* (see ¶¶216-220 of the Declaration of Arturo Casadevall, enclosed herewith as Appendix A). Even if a person of ordinary skill in the art reading Bowdish *et al.* were to consider Evans *et al.* for its disclosure of heavy chain CDR3 sequences, Evans *et al.* allows for multiple options, and nothing in Bowdish *et al.* or Evans *et al.* indicates which, if any, were used in the "scaffold" antibody used to produce Bowdish *et al.*'s TPO- mimetic peptide (see ¶¶216-220 of the Declaration of Arturo Casadevall, enclosed herewith as Appendix A).

Critically, ***nowhere*** did Bowdish *et al.* even reference the word "eculizumab." Nor did Bowdish *et al.* address the problem of the instant application – development of a monoclonal antibody that prevents C5 cleavage and is safe and effective for treating PNH. In fact, the Examiner disregards the art's ***actual*** teachings about the structure and sequence of "eculizumab" in favor of a theory that improperly picks and chooses from unrelated art in hindsight.

5. <u>Wang Does Not Disclose or Teach the Remaining Claim Limitations, and a Person Skilled in the Art Would Not Have Reasonably Expected the Claimed Composition to be Successful in Treating PNH</u>

Finally, the Examiner relies on Wang for teaching that eculizumab is highly soluble, including at up to 30 mg per ml. Simply because an antibody may be soluble at a given concentration in a particular medium does not mean that it would be suitable in a pharmaceutical composition at that concentration. As explained in the enclosed Declaration by Dr. Trout (Appendix B), who is a Professor of Chemical Engineering at the Massachusetts Institute of

Technology (MIT) and the Director of the Society, Engineering and Ethics (SEE) Program at MIT, antibody formulation was an unpredictable field prior to March 15, 2007 and one of ordinary skill in the art could not have reasonably expected that any particular uncharacterized antibody would be capable of development into *any* pharmaceutical composition suitable for human therapeutic use, or treatment of PNH in particular, as required by the pending clams (see ¶39-54, 69 of the Declaration by Dr. Trout; Appendix B). Specifically, as attested to be Dr. Trout, "a person of ordinary skill in the art as of March 15, 2007 would have known that, even '[a]mong all the commercial antibody products," only "about half are stable enough to be formulated in a liquid form' (which a solution is)" (¶71 of the Declaration by Dr. Trout; Appendix B).

Moreover, contrary to the Examiner's suggestion, Wang does not disclose "a single-unit dosage form comprising 300 mg of the antibody in 30 mL of a sterile, preservative-free solution", as presently claimed (see ¶80 of the Declaration by Dr. Trout; Appendix B). Specifically, Wang does not specifically teach eculizumab formulations of between 1 and 30 mg/ml, but rather discloses wide ranges, including 1-30 mg/ml, 40-200 mg/ml and 1-200 mg/ml that hypothetically might be used for nebulization of various unrelated "anti-C5" antibodies and derivative compounds (see Wang ¶¶[0067], [0130], [0170]-[0172], Fig. 10; and ¶¶82-83 of the Declaration by Dr. Trout; Appendix B). Regarding stability that a person skilled in the art would understand is needed for a pharmaceutical composition, Wang only contains a highly general, hypothetical statement that compositions of undefined anti-C5 antibodies "may be stable" over a wide concentration range of 1-200 mg/ml (Wang ¶ [0067]). A person skilled in the art would ***not*** have taken this vague, general statement from Wang as fact with respect to every specific antibody and concentration point, as attested in ¶84 of the Declaration by Dr. Trout (Appendix B). Accordingly, one or ordinary skill in the art, without the benefit of hindsight, would not have arrived at the dosage unit, concentration and volume recited in the pending claims (see, *e.g.*, *KSR Int'l Co. V. Teleflex, Inc.*, 550 U.S. 398 (2007), wherein the Supreme Court emphasized that "[a] factfinder should be aware of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex post reasoning").

B. Rejection of Claims 52-59

Claims 52-59 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen *et al.*, in view of Evans *et al.*, Wang, and Bowdish *et al.*, as applied to claim 52, and further in view of Bell *et al.* The Examiner relies on Hillmen *et al.*, in view of Evans *et al.*, Wang, and Bowdish *et al.* for the reasons discussed above. The Examiner relies on Bell *et al.* for teaching treatment of PNH "using an anti-C5 antibody such as h5G1.1 mAb eculizumab", that LDH is a known indicator of efficacy, and that treatment can continue for 64 weeks.

As discussed above, none of Hillmen *et al.*, in view of Evans *et al.*, Wang, and Bowdish *et al.*, either alone or in combination, render the present claims obvious. Bell *et al.* fails to cure the deficiencies of these references. Bell, like Hillmen *et al.*, would have reinforced one of ordinary skill in the art's understanding as of March 15, 2007 that "eculizumab" was the IgG4 isotype antibody of Thomas, because Bell cited Thomas as describing "[m]ethods for the preparation of h5G1.1-mAb . . . under the tradename eculizumab"[2], and described positive Phase II clinical results for "eculizumab" in PNH patients. Bell thus would have further taught away from other antibody constructs that lacked such published reports of clinical safety and efficacy, such as the uniquely-engineered antibody defined by the claimed full length heavy and light chain sequences (*i.e.*, SEQ ID NOs: 2 and 4). Accordingly, given the consistent teaching as of March 15, 2007 that "eculizumab" was the IgG4 antibody of Thomas, one of ordinary skill in the art would not have found it obvious to use the very different, uniquely-engineered antibody encompassed by the pending claims, without the benefit of hindsight (see, *e.g.*, *KSR Int'l Co. V. Teleflex, Inc.*, 550 U.S. 398 (2007).

Moreover, one of ordinary skill in the art without hindsight also would not have reasonably expected success in combining the Hillmen *et al.*, Evans *et al.*, Wang, Bowdish *et al.*, and Bell *et al.* references. Bell, like Hillmen, taught one of ordinary skill in the art as of March 15, 2007 that "eculizumab" had the ***IgG4*** constant region of Thomas; and nothing in Evans *et al.*, Wang, or Bowdish *et al.* would have given one of ordinary skill in the art a reasonable

[2] While paragraph [0052] of Bell also cited Evans for the disclosure of antibody fragments, one of ordinary skill in the art would have understood that Bell's statements regarding the complete antibody "eculizumab" must refer to Thomas, not Evans, because Evans provided only antibody fragment sequences and no full-length humanized antibody sequence.

expectation that an antibody with a different, non-naturally occurring constant region – with no published reports indicating that such a constant region had even been tested in humans – would be safe and effective to block C5 cleavage and treat conditions such as PNH. One of ordinary skill in the art also would not have reasonably expected that substituting such a uniquely-engineered constant region would allow the antibody to retain the anti-C5 activity and therapeutic efficacy of "eculizumab" as reported in the literature. To the contrary, it was well-known as of 2007 that changes to the amino acid sequence of a monoclonal antibody (including in the heavy chain constant region) could significantly alter antibody properties including affinity, specificity, and immunogenicity, which could translate into unwanted clinical differences *in vivo*.

In sum, the cited references, alone or in combination, fail to render the presently claimed invention obvious, at least because they provide no motivation in arriving at the claimed method, let alone a reasonable expectation of success (see "*KSR International Co.*").

C. Secondary Considerations

It is well-established that objective indicia of non-obviousness (*e.g.*, evidence of copying, long-felt but unsolved needs, industry accolades, and commercial success) play a ***critical role*** in the analysis, and are "not just a cumulative or confirmatory part of the obvious calculus but constitute independent evidence" (see *Ortho-McNeil Pharm., Inc. v. Mylan Labs, Inc.*, 520 F.3d 1358, 1365 (Fed Cir. 2008)). In fact, objective evidence of non-obviousness "may often be the most probative and cogent evidence in the record" and "may often establish that an invention appearing to have been obvious in light of the prior art was not" (see *Transocean Offshore Deepwater Drilling, Inc. v. Maersk Drilling USA, Inc.*, 699 F.3d 1340, 1349 (Fed. Cir. 2012)). Indeed, the Federal Circuit has clearly emphasized that "consideration of the objective indicia is part of the whole obviousness analysis, not just an afterthought," and "are crucial in avoiding the trap of hindsight when reviewing, what otherwise seems like, a combination of known elements" (see *Leo Pharmaceutical Products, Ltd. v. Rea,* 726 F.3d 1346, 1357,1358 (Fed. Cir. 2013)).

There are two requirements for evidence of non-obviousness to be relevant. First, the evidence of non-obviousness must be commensurate in scope with the claimed invention (see *In*

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re Kao, 639 F.3d 1057, 1068 (Fed. Cir. 2011) citing *In re Tiffin*, 58 CCPA 1420, 448 F.2d 791, 792 (1971) and *In re Hiniker*, 150 F.3d 1362, 1369 (Fed. Cir. 1998)). Second, to be accorded substantial weight, there is fundamental requirement that there be a nexus between the merits of the claimed invention and the evidence of secondary considerations (see M.P.E.P. § 716.01(b), *In re GPAC Inc.*, 57 F.3d 1573, 1580 (Fed. Cir. 1995), and *In re Kao* citing *Wyers v. Master Lock Co.*, 616 F.3d 1231, 1246 (Fed. Cir. 2010)). Moreover, there is a "presumption of a nexus" when a product is "coextensive" with a patent claim (see *Teva Pharms. USA, Inc. v. Sandoz, Inc.*, 723 F.3d 1363, 1372 (Fed. Cir. 2013). Specifically, the Federal Circuit has held that "if the marketed product embodies the claimed features, and is coextensive with them, then a nexus is presumed and the burden shifts to the party asserting obviousness to present evidence to rebut the presumed nexus." (see *Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1130 (Fed. Cir. 2000).

In the present case, the strong evidence of secondary considerations (*e.g.*, copying, long-felt unresolved need, industry accolades, and commercial success) weigh significantly in favor of non-obviousness, as discussed in further detail below and in the enclosed Declaration Pursuant to 37 C.F.R. § 1.132 by Dr. Deepak Jain. Importantly, the factually supported evidence of non-obviousness presented herein is both commensurate with the scope of the claims *and* results from what is both claimed and novel. As discussed below, the presently claimed complete, full length, uniquely-engineered heavy chain sequence (which was <u>not</u> known in the prior art before the March 15, 2007 priority date of the present application), imparts significant therapeutic advantages which, in turn, directly relate to the merits of the invention (*i.e.*, the commercial success of the marketed product, Soliris® (eculizumab). Therefore, the marketed product embodies the claimed features and is co-extensive with them, such that the required nexus between the merits of the claimed invention and the evidence of secondary indicia has been firmly established.

i. <u>Long-Felt Need</u>

Soliris® (eculizumab) has satisfied a long-felt, but previously unmet need, for a safe and effective means for treating the rare, debilitating, and potentially fatal blood disease, PNH, which

has been referred to as the "most vicious acquired thrombophilic state known to medicine" (see, Fatimah Al-Ani *et al*., *Ther. Clin. Risk Manag*. 2016; 12: 1161–1170; copy enclosed herewith as Appendix C). The unique anti-C5 therapeutic antibody, Soliris® (eculizumab), defined by the complete structure recited in the present claims (*i.e.,* SEQ ID NOs: 2 and 4) is not only the first FDA-approved anti-C5 antibody, but also the first FDA-approved treatment for PNH. In fact, in 2006, the FDA granted Soliris® (eculizumab) "Priority Review"[3] status (*i.e*., an FDA program to expedite the review process for drugs that are expected to have a particularly great impact on the treatment of a disease), demonstrating just how groundbreaking and transformative the FDA believed it could be for PNH patients.

Prior to FDA approval of Soliris® (eculizumab), patients with PNH had to suffer from debilitating symptoms, such as anemia, fatigue, thrombosis, hemoglobinuria, dyspnea, dysphagia, erectile dysfunction, hypertension and kidney problems for 10 to 15 years, with extremely limited treatment options (see https://www.healio.com/hematology-oncology/news/print/hemonc-today/%7B01f97d7b-36b4-4462-aab4-02918370caa1%7D/eculizumabs-triumph-over-pnh; enclosed herewith as Appendix E). In the absence of treatment, PNH resulted in a 5-year mortality rate of up to ~35% (see Appendix T). The "FDA approval of the orphan drug eculizumab in March 2007 marked a distinct change in how physicians were able to treat patients with paroxysmal nocturnal hemoglobinuria" (see Appendix I). Specifically, as attested by Dr. Peter Hillmen, "[e]culizumab has dramatically changed PNH…[u]ntil eculizumab arrived there was no specific treatment for PNH apart from transfusion and occasionally bone marrow transplant" (see Appendix E).

Dr. Robert A. Brodsky similarly praised Soliris® (eculizumab) as a groundbreaking first-in-class treatment:

> "We had very little to offer patients," Robert A. Brodsky, MD, director of the division of hematology and Clara Lucas Lynn Chair in Medicine at The John Hopkins University, said in an interview. "I recently had a patient with PNH

[3] "A *Priority Review* designation will direct overall attention and resources to the evaluation of applications for drugs that, if approved, would be significant improvements in the safety or effectiveness of the treatment, diagnosis, or prevention of serious conditions when compared to standard applications." (FDA website, https://www.fda.gov/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review; enclosed herewith as Appendix D).

> present in absolute crisis. Several years ago the only thing we would have had to offer him was narcotics and maybe fluids. Now that eculizumab is approved, all I have to do is call down to the pharmacy and tell them that we need to start the patient on this drug." (see Appendix F).

In his own publication, Brodsky touts Soliris® (eculizumab) as being "highly effective in treating PNH" and states that "it has changed the natural history of the disease" (see Brodsky, *Blood*. 2014 Oct 30; 124(18): 2804–2811; enclosed herewith as Appendix F). Brodsky further states:

> [t]he efficacy and safety of eculizumab has been demonstrated in 2 multinational phase 3 trials and a multinational extension study. The drug is highly effective in stopping intravascular hemolysis, eliminating or decreasing the need for red [blood] cell transfusions, improving quality of life, and reducing the risk of thrombosis, the leading cause of mortality from PNH. It has also been shown to improve renal function and to reduce prothrombotic and proinflammatory markers in PNH patients.

The literature is replete with similar testimonials and accolades (see, *e.g.*, Fatimah Al-Ani *et al.*, stating that "[t]he advent of eculizumab has resulted in a dramatic improvement of the natural history of PNH patients with improvement in virtually every aspect of these patients' lives").

Importantly, Soliris® (eculizumab) has not only transformed the standard of care for PNH patients, but also significantly improved individual patient lives. Specifically, the cumulative survival rate increased from 66.8% before Soliris® (eculizumab) to 95.5% with Soliris® (eculizumab) (see Appendices U-W). Moreover, as evidenced by the enclosed testimonials from PNH patients (available at https://alexion.com/patients/patient-stories), treatment with Soliris® (eculizumab) has dramatically improved their quality of life and eliminated painful and debilitating symptoms that once plagued their lives and made day-to-day life challenging and unbearable (see Appendices G-J).

For example, prior to treatment with eculizumab, "Bill", who was diagnosed with PNH in 1985, required hospitalizations and blood transfusions:

> In addition to severe fatigue, Bill experienced stomach pain, kidney pain, dark urine, and dysphagia (difficulty swallowing). His symptoms made it difficult to eat and sleep. Soon, Bill became dependent on regular blood transfusions, which made day-to-day life all the more challenging…After years of managing his

> symptoms as best he could with blood transfusions—and periodic hospitalizations—Bill learned about Soliris® (eculizumab), a then investigational therapy that was undergoing clinical trials. He successfully enrolled in one of the trials in 2005, and soon began to see improvements in his symptoms. Specifically, ***Bill was able to stop receiving blood transfusions*** (emphasis added; see Appendix G)

Similarly, when "Joe" was diagnosed with PNH, "he felt like his dreams were nearly shattered[,]…was worried that he would not have a future with his fiancée and was told he would never be able to compete in contact sports again." Post-treatment with eculizumab, however, Joe "married his college sweetheart, welcomed the birth of two daughters, and is back in his routine" (see Appendix H).

"Victor" was equally devastated when he was diagnosed with PNH and "felt as if he had crawled into a cave":

> He was getting blood transfusions monthly, had little energy and was told he might only have 10 more years to live. The most difficult thing about having PNH was missing out on activities with friends and family, and the toll it took on him mentally. He became depressed. 'People were doing things without me and I felt like I wasn't a part of normal society,' he says. 'I didn't want to talk to people.'

However, after being treated with eculizumab and receiving appropriate support, Victor's life and outlook "radically changed" (see Appendix I).

When "Ruthie" was diagnosed with PNH in 1999, "she and her husband were scared to learn that PNH was a life-threatening illness that at the time had no approved treatments":

> Ruthie first learned about Soliris® (eculizumab) while it was in clinical trials, and began treatment shortly after it was approved by the U.S. FDA in 2007. Today, Ruthie continues to receive treatment, and has the energy to be an engaged and involved mother to three active kids. As her husband David notes, 'In the beginning, we didn't know how long Ruthie would be around, but today I'm excited for our children and for our future' (see Appendix J).

Pursuant to M.P.E.P. § 716.04, the relevance of long-felt need and the failure of others to the issue of obviousness depends on several factors.

> First, the need must have been a persistent one that was recognized by those of ordinary skill in the art. *In re Gershon*, 372 F.2d 535, 539, 152 USPQ 602, 605 (CCPA 1967)…Second, the long-felt need must not have been satisfied by

> another before the invention by applicant. *Newell Companies v. Kenney Mfg.* Co., 864 F.2d 757, 768, 9 USPQ2d 1417, 1426 (Fed. Cir. 1988)…Third, the invention must in fact satisfy the long-felt need. *In re Cavanagh*, 436 F.2d 491, 168 USPQ 466 (CCPA 1971).

In the present case, it is clear that all three prongs have been met. Specifically, prior to eculizumab, there was a persistent need in the art for an effective treatment option, as physicians "had very little to offer patients" aside from palliative care (see Appendix E). This long-felt need was not satisfied until Soliris® (eculizumab) was introduced. The introduction of Soliris® (eculizumab) dramatically improved patient lives and satisfied a long-felt need for a safe and effective means for treating the rare, debilitating, and potentially fatal blood disease, PNH, where prior existing treatment options had been limited and ineffective (see Appendices E-J). Importantly, it is the complete structure (*i.e.,* particular full length heavy and light chain sequences - SEQ ID NOs: 2 and 4) recited in the present claims that imparts Soliris® (eculizumab) with the therapeutically advantageous features that have made it the "gold" standard treatment and forever changed the standard of care for PNH patients. Accordingly, there is a direct nexus between the claimed invention and its ability to satisfy a long-felt, but previously unmet need.

ii. Copying

There are at least four instances of commercial copying (*i.e.*, biosimilar development) of Soliris® (eculizumab):

1. Amgen is developing a biosimilar eculizumab ("ABP 959") for use in treating paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), and generalized myasthenia gravis (gMG) (see https://www.amgenbiosimilars.com/products/our-pipeline/; enclosed herewith as Appendix K).
2. Samsung Bioepis is developing a biosimilar eculizumab ("SB12") and is currently recruiting for a clinical trial to compare "SB12 (Proposed Eculizumab Biosimilar) to Soliris in subjects with Paroxysmal Nocturnal Haemoglobinuria" (see

https://www.samsungbioepis.com/en/product/product02.do; enclosed herewith as Appendix L) and https://www.clinicaltrials.gov/ct2/show/NCT04058158?cond=sb12&rank=1; enclosed herewith as Appendix M).

3. Generium is developing a biosimilar eculizumab ("Elizaria®"), which launched in April 2019 in Russia (see http://www.generium.ru/en/products/elizaria/; enclosed herewith as Appendix N).
4. Biocad is developing a biosimilar of eculizumab ("BCD-148"), which was to be tested in a clinical trial alongside eculizumab in healthy volunteer study (see https://clinicaltrials.gov/ProvidedDocs/03/NCT04027803/Prot_000.pdf; enclosed herewith as Appendix O).

These companies are copying Soliris® (eculizumab) and developing biosimilars because the antibody as a whole (*i.e.*, the antibody's complete sequence) renders it effective and advantageous in treating PNH, as claimed. Accordingly, there is a direct nexus between the claimed invention and copying by others.

iii. Industry Accolades

In 2008, Soliris® (eculizumab) received the Prix Galien USA Award for Best Biotechnology Product. The Prix Galien Award recognizes the technical, scientific and clinical research skills necessary to develop innovative medicines and "is considered the industry's equivalent of the Nobel Prize, the highest accolade for biomedical research and innovation" (see http://register.prix-galien-usa.com/registration/index; Appendix P). The Prix Galien awards committee is made up of 11 individuals, including seven Nobel Laureates, founders of major biotechnology companies and editors of world-renowned biology journals. The candidates for the 2008 awards were selected from drugs approved during the past five years.

Soliris® received this prestigious award because it is a groundbreaking, first-in-line treatment that is effective in treating PNH patients. Again, it is the complete structure (*i.e.*, particular full length heavy and light chain sequences - SEQ ID NOs: 2 and 4) recited in the present claims that imparts Soliris® (eculizumab) with the features that has made it so exceptionally therapeutically effective and worthy of the Prix Galien USA Award. Accordingly,

there is a direct nexus between the claimed invention and its award-winning recognition.

iv. Commercial Success

Soliris® (eculizumab) is unquestionably a commercial success, having generated substantial sales in the relevant market. Soliris® (eculizumab) has been one of the twenty top selling biopharmaceutical products in recent history (see Table 3 of Gary Walsh, *Nature Biotechnology*, volume 36, pages 1136–1145 (2018); enclosed herewith as Appendix Q). As attested in the enclosed Declaration by Daniel Bazarko, the Senior Vice President and Chief Accounting Officer at Alexion (Appendix R), and as evidenced by the enclosed excerpts from Alexion's Forms 10-K for FY 2007-2019 (Appendix S), the annual net product sales for Soliris® have grown consistently since launch, including total U.S. sales of $1 billion over each of the past three years, continuing to grow to over $1.588 billion in 2018 (a 28.6% increase from 2017). Moreover, as also shown in Mr. Bazarko's Declaration, more than $3.4 billion of Soliris' net product sales since its launch in 2007 is attributed to sales for PNH treatment. This astounding commercial success has a direct nexus to the methods of treating patients with Soliris® (eculizumab) (including PNH patients) by administering a unique anti-C5 antibody defined by a novel full-length sequence, which was not known or disclosed prior to the March 15, 2007 priority date of the present application. Specifically, the claimed complete sequence imparts Soliris® (eculizumab) with (*i.e.*, is coextensive with) efficacious and advantageous functional features (*e.g.*, binds to C5 with sufficient affinity and specificity to prevent cleavage of C5, while not activating complement and having no effector function) that have made it a groundbreaking blockbuster product. Accordingly, there is a direct nexus between the claimed invention and its commercial desirability.

D. Conclusion

In sum, the cited references, alone or in combination, fail to render the presently claimed invention obvious because they provide no motivation to arrive at the claimed invention, let alone a reasonable expectation of success (see "*KSR International Co.*"). Moreover, the factually supported evidence of secondary considerations weighs *significantly* in favor of non-obviousness

(see *In re Kao*, 639 F.3d 1057, 1068 (Fed. Cir. 2011). Importantly, the evidence of copying, long-felt unresolved need, industry accolades, and commercial success, associated with the use of eculizumab for treatment of PNH has a direct nexus to the novel features of the claimed invention (see *In re GPAC Inc.*, 57 F.3d 1573, 1580 (Fed. Cir. 1995)). Therefore, the provided evidence of secondary indicia must be considered in evaluating non-obviousness of the invention.

In view of the above, the presently claimed methods are patentable and Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

Non-Statutory Double Patenting

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claim 1 of US Patent 9,732,149, in view of Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-3 of US Patent 9,718,880, in view of Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-10 of US Patent 9,725,504, in view of Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-17 of US Patent 9,409,980, in view of Evans *et al.* (US 6,355,245 B1), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-15 of US Patent 9,415,102, in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-14 of US Patent 9,556,263, in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

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Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-12 of US Patent 9,352,035, in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

Claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-13 of US Patent 9,925,262, in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

Finally, claims 52-59 are rejected on the ground of nonstatutory obviousness- type double patenting as being unpatentable over claims 1-33 of US Patent 10,239,937, in view of Evans *et al.* (US 6,355,245), Wang (US 2005/0271660) and Bell *et al.* (US 2005/0191298).

While in no way acquiescing to the rejection, Applicants respectfully submit herewith a terminal disclaimer over US Patent 9,732,149, US Patent 9,718,880, US Patent 9,725,504, US Patent No: 9,409,980, US Patent No: 9,415,102, US Patent No: 9,556,263, US Patent No: 9,352,035, US Patent 9,925,262, and US Patent 10,239,937.

Samsung Bioepis Exhibit No. 1002
Page 14864

SUMMARY OF APPENDICES

A	Declaration by Arturo Casadevall
B	Declaration by Dr. Trout
C	Fatimah Al-Ani *et al*., *Ther. Clin. Risk Manag*. 2016; 12: 1161–1170
D	FDA Priority Review (https://www.fda.gov/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review)
E	https://www.healio.com/hematology-oncology/news/print/hemonc-today/%7B01f97d7b-36b4-4462-aab4-02918370caa1%7D/eculizumabs-triumph-over-pnh
F	Brodsky, *Blood*. 2014 Oct 30; 124(18): 2804–2811
G	Patient "Bill" (https://alexion.com/patients/patient-stories)
H	Patient "Joe" (https://alexion.com/patients/patient-stories)
I	Patient "Victor" (https://alexion.com/patients/patient-stories)
J	Patient "Ruthie" (https://alexion.com/patients/patient-stories)
K	Amgen (https://www.amgenbiosimilars.com/products/our-pipeline/)
L	Samsung Bioepis (https://www.samsungbioepis.com/en/product/product02.do)
M	Samsung Bioepis (https://www.clinicaltrials.gov/ct2/show/NCT04058158?cond=sb12&rank=1)
N	Generium (http://www.generium.ru/en/products/elizaria/)
O	Biocad (https://clinicaltrials.gov/ProvidedDocs/03/NCT04027803/Prot_000.pdf)
P	Prix Galien (http://register.prix-galien-usa.com/registration/index)
Q	Gary Walsh, *Nature Biotechnology*, volume 36, pages 1136–1145 (2018)
R	Declaration by Daniel Bazarko
S	Alexion's Forms 10-K for FY 2007-2019
T	Hillmen, P., *et al*., Natural history of paroxysmal nocturnal hemoglobinuria. N. Engl. J. Med., 1995. 333(19): p. 1253-8.
U	Hillmen P, *et al. Br J Haematol.* 2013;162(1):62-73.
V	Hillmen P, *et al. Am J Hematol.* 2010;85(8):553-559.
W	Kelly RJ, *et al*. *Blood.* 2011;117(25):6786-6792.

CONCLUSION

Claims 52-59 are pending. *No new matter has been added.*

If a telephone conversation with Applicant's attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call Applicant's attorney at (617) 217-4630. Applicant believes no fee is due with this statement. However, if a fee is due, please charge our Deposit Account No. 50-1196, under Order No. AXJ-114BUSCN5 from which the undersigned is authorized to draw.

Dated: December 11, 2019

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

Samsung Bioepis Exhibit No. 1002
Page 14866

United States Patent and Trademark Office

Office of the Chief Financial Officer

Document Code:WFEE

User :64734

Sale Accounting Date:01/06/2020

Sale Item Reference Number	Effective Date
15642096	12/11/2019

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I202016957594618	1814	STATUTORY OR TERMINAL DISCLAIMER	$160.00	Deposit Account

Application Number * 15/642,096 *	Application/Control No. 15/642,096	Applicant(s)/Patent under Reexamination BELL et al.	
	Examiner ROGERS, JAMES L	Art Unit 1644	

Document Code - DISQ	Internal Document - DO NOT MAIL

TERMINAL DISCLAIMER	☑ APPROVED	☐ DISAPPROVED
Date Filed: 11 December 2019	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:

/JEAN PROCTOR/

Technology Center: OPLC

Telephone: (571)272-1040

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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NOTICE OF ALLOWANCE AND FEE(S) DUE

133485 7590 01/22/2020

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	
ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 01/22/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/642,096	07/05/2017	Leonard BELL	AXJ-114BUSCN5	7606

TITLE OF INVENTION: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	04/22/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PTOL-85 (Rev. 02/11)

Samsung Bioepis Exhibit No. 1002
Page 14869

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
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Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

133485 7590 01/22/2020
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One Post Office Square
30th Floor
Boston, MA 02109-2127

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/642,096	07/05/2017	Leonard BELL	AXJ-114BUSCN5	7606

TITLE OF INVENTION: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	04/22/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
ROGERS, JAMES L	1644	424-133100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1_____
2_____
3_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

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UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/642,096	07/05/2017	Leonard BELL	AXJ-114BUSCN5	7606

133485 7590 01/22/2020

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 01/22/2020

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Samsung Bioepis Exhibit No. 1002
Page 14871

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/642,096	Applicant(s) BELL et al.	
	Examiner JAMES L ROGERS	Art Unit 1644	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 12/11/2019.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 52-59 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JAMES L ROGERS/ Examiner, Art Unit 1644	/DANIEL E KOLKER/ Supervisory Patent Examiner, Art Unit 1644

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

1. Claims 52-59 are pending.

2. Applicant's IDSs, submitted on 12/11/2019 are acknowledged and have been considered.

REASONS FOR ALLOWANCE

3. The following is an Examiner's Statement of Reasons for Allowance:

Applicant's arguments/Response filed 12/11/2019, was considered persuasive and has overcome the obviousness rejections or the prior OA.

The closest prior art was considered to be Evans (US 6,355,245) which was discussed in the prior OA. Evans teaches the antibody 5G1.1, eculizumab. However and as previously noted, Evans does not teach an antibody that binds C5 which have a H and L chain with SEQ ID Nos: 2 and 4, respectively. One of ordinary skill in the art would according need to search for secondary references which provide the missing parts of the currently claimed sequences.

The secondary reference Bowdish (US 2003/232972) which was also discussed in the prior OA teaches an antibody that includes a region where amino acid residues corresponding to at least a portion of two CDRs are replaced with a TPO mimec (see for example claim 1). As was also discussed previously at p. 4 of the prior OA, Bowdish at ¶191 exemplifies this scheme by using an antibody framework, 5G1.1 and refers back to Evans above for this framework. The TPO mimetic peptide was cloned into 5G1.1 so as to replace the native CDR3.

However, and as noted by applicant at p. 12 of applicant's Response, Bowdish is directed to increasing the biological activity of antibody scaffolds rather than to using antibodies against C5 such as the one currently claimed in a method to treating PNH. (see ¶5 of Bowdish).

Even assuming that one of skill in the art would find Bowdish, the fact remains that one of skill in the art would still not end up with the currently claimed antibody. As noted by applicant at pp. 13-14 of the Response, Evan's scaffold 5G1.1 mouse antibody variable regions or the whole 5G1.1 mouse antibody with the sequences for Bowdish's TPO

mimetic compound would still have revealed a mismatch in amino acids beyond those that Bowdish identified as the TPO mimetic peptide insert. Accordingly, it would appear that the scaffold taught by Evans is different from the scaffold used by Bowdish such that even if one of skill in the art were guided to Bowdish by reference to Evans, one of skill in the art would still not end up with the currently claimed antibody.

It is noted that at least some of the secondary considerations are evidence of non-obviousness, particularly that the invention as claimed satisfies a long felt need and that there is objective evidence of copying. The argument as to commercial success as no evidence has been presented that the commercial success is due to the claimed invention as opposed to other efforts undertaken by the seller, such as marketing; see MPEP 716.03, in particular subsection (b).

Accordingly, claims 52-59 are deemed allowable.

4. Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

5. Any inquiry concerning this communication or earlier communications from the examiner should be directed to James Rogers whose telephone number is (571) 270-5374. The examiner can normally be reached Monday through Friday from 8:00 am to 5:30 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Daniel E. Kolker** can be reached on (571) 272-3181. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

December 27, 2019

/JAMES ROGERS/
Examiner, Art Unit 1644

/DANIEL E KOLKER/
Supervisory Patent Examiner, Art Unit 1644

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/642,096	BELL et al.
	Examiner	Art Unit
	JAMES L ROGERS	1644

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
STN: DISSABS MEETINGS BIOSCI CAPLUS MEDICINE	05/01/2019	JR
EAST: PGPUB, USPAT, JPO, EPO, DRWNT, INVENTOR SEARCH	05/01/2019	JR
updated	12/27/2019	JR

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	USPAT, USPAD	12/27/2019	JR

/JAMES L ROGERS/ Examiner, Art Unit 1644	

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/642,096	BELL et al.
	Examiner	**Art Unit**
	JAMES L ROGERS	1644

CPC						
Symbol					**Type**	**Version**
C07K	/	16	/	18	F	2013-01-01
A61K	/	31	/	7105	I	2013-01-01
A61K	/	9	/	0019	I	2013-01-01
A61K	/	38	/	00	A	2013-01-01
A61K	/	2039	/	505	A	2013-01-01
C07K	/	2317	/	24	A	2013-01-01
C07K	/	2317	/	565	A	2013-01-01
C07K	/	2317	/	51	A	2013-01-01
C07K	/	2317	/	515	A	2013-01-01

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/JAMES L ROGERS/ Examiner, Art Unit 1644	27 December 2019	**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	8	
/DANIEL E KOLKER/ Supervisory Patent Examiner, Art Unit 1644	07 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20191227

Samsung Bioepis Exhibit No. 1002
Page 14877

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/642,096	BELL et al.
	Examiner	**Art Unit**
	JAMES L ROGERS	1644

INTERNATIONAL CLASSIFICATION				
CLAIMED				
A61K	/	9	/	00
A61K	/	39	/	00
C07K	/	16	/	18

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US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

/JAMES L ROGERS/ Examiner, Art Unit 1644	27 December 2019	**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	8	
/DANIEL E KOLKER/ Supervisory Patent Examiner, Art Unit 1644	07 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/642,096	BELL et al.
	Examiner	Art Unit
	JAMES L ROGERS	1644

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIMS

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/JAMES L ROGERS/ Examiner, Art Unit 1644	27 December 2019	**Total Claims Allowed:**	
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/DANIEL E KOLKER/ Supervisory Patent Examiner, Art Unit 1644	07 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

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Samsung Bioepis Exhibit No. 1002
Page 14879

Bibliographic Data

Application No: 15/642,096

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /JAMES L ROGERS/ (Examiner's Signature) | (Initials)

Title: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/05/2017	424	1644	AXJ-114BUSCN5
RULE			

APPLICANTS

Alexion Pharmaceuticals, Inc., New Haven, CT,

INVENTORS

Leonard BELL Woodbridge, CT, UNITED STATES

Russell P. ROTHER Oklahoma City, OK, UNITED STATES

Mark J. EVANS Radnor, PA, UNITED STATES

CONTINUING DATA

This application is a CON of 15284015 10/03/2016 PAT 9732149

15284015 is a CON of 15260888 09/09/2016 PAT 9725504

15260888 is a CON of 15148839 05/06/2016 PAT 9718880

15148839 is a CON of 13426973 03/22/2012ABN

13426973 is a CON of 12225040 05/13/2009ABN

12225040 is a 371 of PCT/US2007/006606 03/15/2007

FOREIGN APPLICATIONS

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07/19/2017

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UNITED STATES

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
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Application Number	15642096
Filing Date	2017-07-05
First Named Inventor	Leonard BELL
Art Unit	1644
Examiner Name	J. L. Rogers
Attorney Docket Number	AXJ-114BUSCN5

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	1	6329509		2001-12-11	Jardieu et al.	

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	1	20050027166	A1	2005-02-03	Matsumoto et al.	

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	1	2581113	EP	A1	2013-04-17	Kyowa Hakko Kirin Co., Ltd		
	2	2004/035607	WO	A2	2004-04-29	Genmab As		

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	Attorney Docket Number	AXJ-114BUSCN5

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1	2007 USP Dictionary of USAN and International Drug Names, 85 pages.	
	2	Alexion Form 10-K for FY 2008 (Excerpts) 3 pages.	
	3	Alexion Form 10-K for FY 2009 (Excerpts) 3 pages.	
	4	Alexion Form 10 K for FY 2011 (Excerpts) 3 pages.	
	5	Alexion Form 10-K for FY 2007 (Excerpts) 3 pages.	
	6	Alexion Form 10-K for FY 2010 (Excerpts) 3 pages.	
	7	Alexion Form 10-K for FY 2016 (Excerpts) 3 pages.	
	8	Alexion Form 10-K for FY 2017 (Excerpts) 3 pages.	
	9	Alexion Form 10-K for FY 2018 (Excerpts) 3 pages.	
	10	Alexion Form 10-Q for Q1 of FY 2019 (Excerpts) 3 pages.	

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	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

	11	Alexion Form 10-Q for Q2 of FY 2019 (Excerpts) 3 pages.	
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	13	Alexion Form 10-K for FY 2012 (Excerpts) 3 pages.	
	14	Alexion Form 10-K for FY 2013 (Excerpts) 3 pages.	
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	Attorney Docket Number	AXJ-114BUSCN5

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	23	Amgen Inc., Petitioner v. Alexion Pharmaceuticals, "Amgen's Inc.'s Objections to Evidence," Case IPR2019-00740, filed December 2, 2019, 9 pages.	
	24	Amgen Inc., Petitioner v. Alexion Pharmaceuticals, "Amgen's Inc.'s Objections to Evidence," Case IPR2019-00741, filed December 2, 2019, 9 pages.	
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	Attorney Docket Number	AXJ-114BUSCN5

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	37	DECISION Institution of Inter Partes Review 35 U.S.C. § 314(a), IPR2019-00741, dated August 30, 2019, 61 pages.	
	38	Declaration of Arturo Casadevall and Curriculum Vitae, dated November 22, 2019, 251 pages.	
	39	Declaration of Bernhardt L. Trout, Ph.D. and Curriculum Vitae, dated November 22, 2019, 95 pages.	
	40	Declaration of Daniel Bazarko, dated November 22, 2019, 7 pages.	
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	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

	44	European Application No. 11001632.6 Amgen's Response to the Statement of Grounds of Appeal filed by the Appellant on June 24, 2019, dated November 8, 2019, 47 pages.	
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EXAMINER SIGNATURE

Examiner Signature	/JAMES L ROGERS/	Date Considered	12/27/2019

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	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2019-12-11
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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	Application Number	15642096
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	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

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	1	Rosenberg, A. "Effects of Protein Aggregates: An Immunologic Perspective," AAPS Journal 8, Article 59: E501-E507 (Aug. 4, 2006).	
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	12	Alexion Form 8-K for FY 2019 (Excerpts) 3 pages, September 5, 2019.	

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	Attorney Docket Number	AXJ-114BUSCN5

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2019-12-11
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

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Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	(" (nocturnalnear2hemoglobinuria).clm.").PN.	US-PGPUB; USPAT	OR	OFF	2019/12/27 08:02
L2	410	(nocturnal near2 hemoglobinuria).clm.	US-PGPUB; USPAT	OR	OFF	2019/12/27 08:02
L3	8	L2 and ("300" near2 mg).clm.	US-PGPUB; USPAT	OR	OFF	2019/12/27 08:03
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L5	59	L2 and (SEQ near3 (2 or "4")).clm.	US-PGPUB; USPAT	OR	OFF	2019/12/27 08:04

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	15642096
	Filing Date	2017-07-05
	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

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	1	European Application No. 11001632.6 Hoffmann Eitle's Response to the Statement of Grounds of Appeal filed by the Appellant on June 24, 2019, dated November 8, 2019, 45 pages.	
	2	European Application No. 11001632.6 Ranibizumab, Drug Bank, accessed November 05, 2019, 6 pages, cited in Opposition.	
	3	European Application No. 11001632.6 Salbutamol Drug Guidelines, 5 pages, cited in Opposition.	
	4	European Application No. 11001632.6 Statement of Grounds of Appeal on Behalf of Alexion Pharmaceuticals, Inc., dated June 24, 2019.	
	5	European Application No. 11001632.6 Third Declaration of Dr. Dino Miano, dated June 21, 2019, 6 pages, cited in Opposition.	
	6	European Application No. 16172335.8 Alexion Pharmaceuticals, Inc. Written Submission in Response to Summons to Attend Oral Proceedings, dated July 5, 2019, 23 pages.	
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Examiner Signature	/JAMES L ROGERS/	Date Considered	12/27/2019

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L77             0 FILE ADISINSIGHT
L78             0 FILE ADISNEWS
L79             0 FILE ANABSTR
L80             0 FILE BIOSIS
L81             0 FILE CABA
L82             0 FILE CAPLUS
L83             0 FILE CEABA-VTB
L84             0 FILE CIN
L85             0 FILE ESBIOBASE
L86             0 FILE FSTA
L87             4 FILE IFIALL
L88             0 FILE IMSRESEARCH
L89             0 FILE KOSMET
L90             0 FILE MEDLINE
L91             0 FILE NTIS
L92             0 FILE PCTGEN
L93             0 FILE PS
L94             0 FILE RDISCLOSURE
L95             0 FILE SCISEARCH
L96             0 FILE TOXCENTER
L97             0 FILE USGENE
L98             4 FILE USPATFULL
L99             0 FILE USPATOLD
L100            3 FILE USPAT2
L101            0 FILE IPA
L102            0 FILE NAPRALERT
      TOTAL FOR ALL FILES
L103           11 S L34 AND ECULI
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Samsung Bioepis Exhibit No. 1002
Page 14905

To: ipboston.docketing@nelsonmullins.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 133485

Jan 22, 2020 03:35:53 AM

Dear PAIR Customer:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 133485 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
15642096	NOA	01/22/2020	AXJ-114BUSCN5
	1449	01/22/2020	AXJ-114BUSCN5
	1449	01/22/2020	AXJ-114BUSCN5
	1449	01/22/2020	AXJ-114BUSCN5

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/642,096	07/05/2017	Leonard BELL	AXJ-114BUSCN5	7606

133485 7590 02/03/2020

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
02/03/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com

Applicant-Initiated Interview Summary	**Application No.** 15/642,096	**Applicant(s)** BELL et al.	
	Examiner JAMES L ROGERS	**Art Unit** 1644	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) JAMES L. ROGERS.

(2) DANIEL KOLKER, SPE.

(3) JANE REMILLARD.

(4) JILL SLOPER.

Date of Interview: 29 January 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Upon further consideration and reading of the references cited, it is noted that the second sentence of the paragraph beginning "The closest prior art" from the office action mailed 22 January 2020 is incorrect. That sentence states that Evans teaches the antibody 5G1.1, eculizumab. However the Evans reference does not identify this antibody as eculizumab. The 5G1.1 antibody taught by Evans is a mouse antibody (see at least column 37 line 34 through column 38 line 45 of US Patent 6355245) and the Evans reference does not teach any additional full-length antibodies that have the same CDRs as 5G1.1 (note Evans does mention N19/8 at column 39 but there is no indication this is pertinent to the now-claimed invention). Evans does teach scFVs which comprise the CDRs of 5G1.1 (Example 11) but these lack at least the constant region of the antibodies in the claimed methods..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/JAMES L ROGERS/ Examiner, Art Unit 1644	/DANIEL E KOLKER/ Supervisory Patent Examiner, Art Unit 1644

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

Doc Code: MFEE.C.AD
Document Description: Maintenance Fee Address Change

PTO/AIA/47 (11-18)
Approved for use through 09/30/2021. OMB 0651-0016
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no person is required to respond to a collection of information unless it displays a valid OMB control number.

"FEE ADDRESS" INDICATION FORM

Address to:
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

- OR -

Fax to:
571-273-6500

INSTRUCTIONS: The issue fee must have been paid for the application(s) listed on this form. In addition, only an address represented by a Customer Number can be established as the fee address for maintenance fee purposes (hereafter, fee address). A fee address should be established when correspondence related to maintenance fees should be mailed to a different address than the correspondence address for the application.

When to check the first box below: If you have a Customer Number to represent the fee address.

When to check the second box below: If you have no Customer Number representing the desired fee address, in which case a completed Request for Customer Number (PTO/SB/125) must be attached to this form. For more information on Customer Numbers, see the Manual of Patent Examining Procedure (MPEP) § 403.

For the following listed application(s), please recognize as the "Fee Address" under the provisions of 37 CFR 1.363 the address associated with:

[X] Customer Number: 127532

OR

[ ] The attached Request for Customer Number form (PTO/SB/125)

PATENT NUMBER (if known)	APPLICATION NUMBER
	15/642,096

Completed by (check one):

[ ] Applicant/Inventor

[X] Attorney or Agent of record 60,760 (Reg. No.)

[ ] Assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(c) (Form PTO/AIA/96) is enclosed or was filed on _____ (Date)

/Jill Gorny Sloper, Esq./
Signature

Jill Gorny Sloper, Esq.
Typed or printed name

617-217-4630
Requester's telephone number

February 3, 2020
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. If the assignee is a juristic entity, this form must be signed by a patent practitioner (attorney or agent) of record. Submit multiple forms if more than one signature is required, see below*.

[ ] * Total of 1 forms are submitted.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: February 3, 2020 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Samsung Bioepis Exhibit No. 1002
Page 14910

Electronic Patent Application Fee Transmittal

Application Number:	15642096
Filing Date:	05-Jul-2017
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Filer:	Jill R. Gorny Sloper/Melissa St Germain
Attorney Docket Number:	AXJ-114BUSCN5

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
PRINTED COPY OF PATENT - NO COLOR	8001	1	3	3
		Total in USD ($)		**1003**

Electronic Acknowledgement Receipt	
EFS ID:	38474107
Application Number:	15642096
International Application Number:	
Confirmation Number:	7606
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Melissa St Germain
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN5
Receipt Date:	03-FEB-2020
Filing Date:	05-JUL-2017
Time Stamp:	15:56:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1003
RAM confirmation Number	E202023F57127155
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	2020_02_03_Issue_Fee_Transmittal_--_AXJ-114BUSCN5.pdf	241424 670b9494036390844 3be8a81e6b868c7fdac2964	no	1
Warnings:					
Information:					
2	Change of Address	2020_02_03_Fee_Address_Indication_Form_--_AXJ-114BUSCN5.pdf	172466 1dd9e98e006da3c769094bf711a3991b27a1be53	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32143 ecea64e102cd3d9fba9bd93f1d40f5fb035e99b8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	446033		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PART B–FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

133485 7590 01/22/2020

NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square, 30th Floor
Boston, Massachusetts 02109-2127

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Jill Gorny Sloper, Esq.	(Typed or printed name)
/Jill Gorny Sloper, Esq./	(Signature)
February 3, 2020	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
15/642,096	07/05/2017	Leonard BELL	AXJ-114BUSCN5	7606

TITLE OF INVENTION: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1,000.00	$0	$0	$1,003.00	04/22/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
J. L. Rogers	1644	424-133100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☒ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. Nelson Mullins Riley & Scarborough LLP
2. Jane E. Remillard, Esq.
3. Jill Gorny Sloper, Esq.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Alexion Pharmaceuticals, Inc.

(B) RESIDENCE: (CITY and STATE or COUNTRY): Boston, Massachusetts

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☒ Advance Order - # of Copies 1

4b. Method of Payment (*Please first reapply any previously paid fee shown above*):

☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-1196

5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29. NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status. NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Jill Gorny Sloper, Esq./ Date February 3, 2020

Typed or printed name Jill Gorny Sloper, Esq. Registration No. 60,760

Samsung Bioepis Exhibit No. 1002
Page 14915

To: ipboston.docketing@nelsonmullins.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 133485

Feb 03, 2020 03:36:12 AM

Dear PAIR Customer:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 133485 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15642096	INTV.SUM.APP	02/03/2020	AXJ-114BUSCN5

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/642,096	03/17/2020	10590189	AXJ-114BUSCN5	7606

133485 7590 02/26/2020

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 177 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Leonard BELL, Woodbridge, CT;
Alexion Pharmaceuticals, Inc., New Haven, CT;
Russell P. ROTHER, Oklahoma City, OK;
Mark J. EVANS, Radnor, PA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

To: ipboston.docketing@nelsonmullins.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 133485

Feb 27, 2020 04:08:56 AM

Dear PAIR Customer:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 133485 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15642096	ISSUE.NTF	02/26/2020	AXJ-114BUSCN5

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: April 7, 2020
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN5
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of:
Leonard Bell *et al.*

Patent No.: 10,590,189

Issued: March 17, 2020

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

REQUEST FOR CERTIFICATE OF CORRECTION PURSUANT TO 37 C.F.R. § 1.323

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted typographical errors which should be corrected.

In the List of References Cited by Applicant and Considered by Examiner:

On page 2, column 1, under "Other Publications", line 8, delete "Bood," and insert - - Blood, - -, therefor.

On page 2, column 2, under "Other Publications", line 69, delete "hemoqlobinuria,"" and insert - - hemoglobinuria," - -, therefor.

On page 2, column 2, under “Other Publications”, line 69, delete “Hematot,” and insert - - Hematol., - -, therefor.

On page 3, column 1, under “Other Publications”, line 56, delete “Medelian” and insert - - Mendelian - -, therefor.

On page 4, column 1, under “Other Publications”, line 7, delete “Neisseia” and insert - - Neisseria - -, therefor.

On page 4, column 1, under “Other Publications”, line 48, delete “Hemoglobinuira” and insert - - Hemoglobinuria - -, therefor.

On page 4, column 1, under “Other Publications”, line 52, delete “Phamaceuticals:” and insert - - Pharmaceuticals: - -, therefor.

On page 4, column 1, under “Other Publications”, line 54, delete “/techplat/” and insert - - /techplait/ - -, therefor.

On page 4, column 2, under “Other Publications”, line 42, delete “Pharmaceuitcals” and insert - - Pharmaceuticals - -, therefor.

On page 4, column 2, under “Other Publications”, line 59, delete “545-S50” and insert - - S45-S50 - -, therefor.

On page 5, column 1, under “Other Publications”, line 56, delete “Abbot” and insert - - Abbott - -, therefor.

On page 5, column 2, under “Other Publications”, line 29, delete “Hinter,” and insert - - Winter, - -, therefor.

On page 8, column 2, under “Other Publications”, line 4, delete “huan” and insert - - human - -, therefor.

In the Specification:

In column 1, line 43, delete “Intravasular” and insert - - Intravascular - -, therefor.

In column 1, line 49, delete “activation.5-8” and insert - - activation.5-8 - -, therefor.

In column 8, line 56, delete “anti-CS” and insert - - anti-C5 - -, therefor.

In column 11, line 3, delete “anti-CS” and insert - - anti-C5 - -, therefor.

In column 11, line 48, delete "2d" and insert - - 2nd - -, therefor.

In column 13, line 18, delete "VH, VL" and insert - - VH-VL - -, therefor.

In column 13, line 56, delete "(VH, VL)." and insert - - (VH-VL). - -, therefor.

In column 14, line 45, delete "antibodies"" and insert - - "antibodies" - -, therefor.

In column 19, line 11, after "(mg/mL)]" insert - - . - -.

In column 26, line 7, delete "m" and insert - - in - -, therefor.

In column 28, line 62, after " 73:703-11" insert - - . - -.

In column 29, line 24, delete "infravascular" and insert - - intravascular - -, therefor.

In column 29, line 27, delete "hemoglobiruria." and insert - - hemoglobinuria. - -, therefor.

In column 29, line 62, delete "Hypeension" and insert - - Hypertension - -, therefor.

In column 30, line 4, delete "m" and insert - - in - -, therefor.

In column 30, line 13, delete "Patients." and insert - - Patients - -, therefor.

Some of the errors were found in the application as filed by applicant.

The errors now sought to be corrected are inadvertent typographical errors the correction of which does not involve new matter or require reexamination.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

Please charge our Deposit Account No. 50-1196 in the amount of $150.00 covering the fee set forth in 37 C.F.R. § 1.20(a). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 50-1196, under Order No. AXJ-114BUSCN5.

Dated: April 7, 2020

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square, 30th Floor
Boston, Massachusetts 02109-2127
617-217-4630 (Tel)
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Attorney/Agent For Applicant

PTO/SB/44 (09-07)
Approved for use through 01/31/2020. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.
(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.	:	10,590,189
APPLICATION NO.	:	15/642,096
ISSUE DATE	:	March 17, 2020
INVENTOR(S)	:	Leonard BELL *et al.*

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the List of References Cited by Applicant and Considered by Examiner:

On page 2, column 1, under “Other Publications”, line 8, delete “Bood,” and insert - - Blood, - -, therefor.

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MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
Jill Gorny Sloper, Esq.
NELSON MULLINS RILEY & SCARBOROUGH LLP 1
One Post Office Square, 30th Floor
Boston, Massachusetts 02109-2127

On page 4, column 1, under "Other Publications", line 7, delete "Neisseia" and insert - - Neisseria - -, therefor.

On page 4, column 1, under "Other Publications", line 48, delete "Hemoglobinuira" and insert - - Hemoglobinuria - -, therefor.

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On page 4, column 2, under "Other Publications", line 42, delete "Pharmaceuitcals" and insert - - Pharmaceuticals - -, therefor.

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In the Specification:

In column 1, line 43, delete “Intravasular” and insert - - Intravascular - -, therefor.

In column 1, line 49, delete “activation.5-8” and insert - - activation.5-8 - -, therefor.

In column 8, line 56, delete “anti-CS” and insert - - anti-C5 - -, therefor.

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In column 26, line 7, delete "m" and insert - - in - -, therefor.

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In column 29, line 62, delete "Hypeension" and insert - - Hypertension - -, therefor.

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In column 30, line 13, delete "Patients." and insert - - Patients - -, therefor.

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Dated: April 7, 2020 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

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Jill Gorny Sloper, Esq.
NELSON MULLINS RILEY & SCARBOROUGH LLP 4
One Post Office Square, 30th Floor
Boston, Massachusetts 02109-2127

Electronic Patent Application Fee Transmittal

Application Number:	15642096
Filing Date:	05-Jul-2017
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Filer:	Jill R. Gorny Sloper/Melissa St Germain
Attorney Docket Number:	AXJ-114BUSCN5

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
CERTIFICATE OF CORRECTION	1811	1	150	150

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**150**

Electronic Acknowledgement Receipt	
EFS ID:	39081477
Application Number:	15642096
International Application Number:	
Confirmation Number:	7606
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Melissa St Germain
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN5
Receipt Date:	07-APR-2020
Filing Date:	05-JUL-2017
Time Stamp:	14:00:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$150
RAM confirmation Number	E202047E00342203
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	AXJ-114BUSCN5_Request_for_Certificate.pdf	28321 af855703dd6d5886790f70562fbfb13603a2127c	no	4
Warnings:					
Information:					
2	Request for Certificate of Correction	AXJ-114BUSCN5_Proposed_Certificate.pdf	33210 fc3c18a4543ab9e3781bf0efc80c5fce5c5c1072	no	4
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30358 192a941f3bc983b815486d4fb470eb3a56f7b822	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	91889		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 10,590,189 B2
APPLICATION NO. : 15/642096
DATED : March 17, 2020
INVENTOR(S) : Leonard Bell et al.

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Signed and Sealed this
Twelfth Day of January, 2021

Andrei Iancu
Director of the United States Patent and Trademark Office

On page 4, Column 2, under "Other Publications", Line 59, delete "545-S50" and insert -- S45-S50 --, therefor.

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