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Minireview

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Antibody Engineering for the Development of Therapeutic Antibodies

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Therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry. There are currently 19 monoclonal antibodies in the market that have been approved by the FDA and over 150 in clinical developments. Driven by innovation and technological developments, therapeutic antibodies are the second largest biopharmaceutical product category after vaccines. Antibodies have been engineered by a variety of methods to suit a particular therapeutic use. This review describes the structural and functional characteristics of antibody and the antibody engineering for the generation and optimization of therapeutic antibodies.

Keywords: Affinity Maturation; Antibody Engineering; Antibody Fragments; Effector Functions; Human Monoclonal Antibodies; Humanized Antibodies; Pharmacokinetics; Therapeutic Antibodies.

Introduction

Today, therapeutic monoclonal antibodies (MAbs) represent one of the fastest growing area of the pharmaceutical industry, with exceptional 48.1% growth between 2003 and 2004. There are currently 19 therapeutic antibodies in clinical use that have been approved by the FDA (Fig. 1; Table 1) and over 150 antibodies in clinical trials. The MAbs market is expected to almost triple in value over the next six years from $10.3 billion in 2004 to $30.3 billion in 2010, driven by technological evolution from chimeric and humanized to fully human antibodies. Oncology products will continue to dominate the market. However, sales of arthritis, immune and inflammatory disorders products are forecast to grow strongly and account for 40.1% of the market by 2010. The development focus of the industry is moving away from murine and chimeric antibodies, to humanized and, in particular, fully human antibodies. A wave of fully human antibodies are expected to launch from 2007 onwards.

The features that make antibodies attractive drug candidates are high target specificity and their organization into distinct structural and functional domains. The characteristic domain structure of antibody has facilitated protein engineering for the development of therapeutic antibodies. When an antibody is designed as a drug, all of its different features including immunogenicity, affinity, stability, effector functions, half-life, and tissue penetration and distribution should be taken into consideration and optimized accordingly. From a manufacturing standpoint, ease of production and stability must also be considered. Nowadays, natural antibodies are tailored by a variety of methods to suit a particular therapeutic use. This review describes the structure and function of antibody, and antibody engineering for the generation of humanized antibodies, antibody fragments, and fully human MAbs. In addition, the technologies for enhancing their biological activities such as affinity maturation, improvement of effector functions, and altering pharmacokinetics are reviewed.

Structural and functional features of antibodies

Immunglobulins (Ig) are highly specific and naturally

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Abbreviations: AAR, anti-antibody response; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; Fab, antigen-binding fragment; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin; MAb, monoclonal antibody; scFv, single-chain Fv; V, variable; VH, heavy chain variable domain; VL, light chain variable domain.

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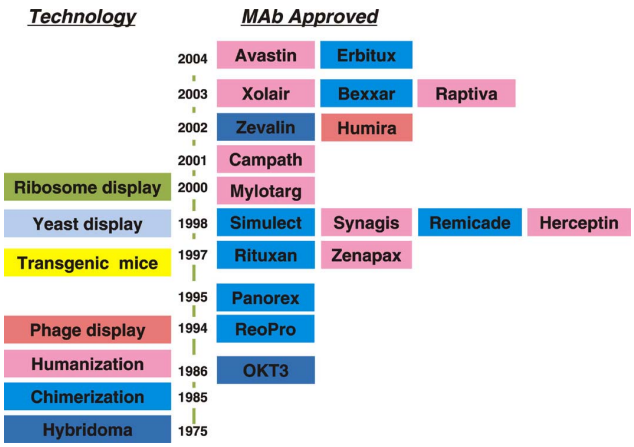

Fig. 1. Antibody technologies and approved therapeutic antibodies.

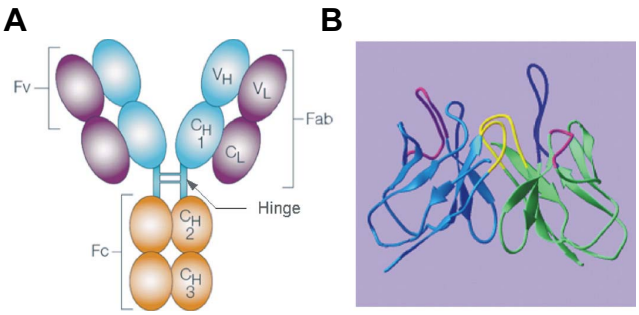

Fig. 2. The modular structure of antibody molecule (A) and the CDR1, 2, and 3 within the heavy and light chain variable domains (B).

evolved molecules that recognize and eliminate foreign antigens. There are five classes of immunoglobulins: IgM, IgG, IgE, IgA, and IgD. From a biotechnology perspective, IgG is the most important class of antibodies. IgG is organized into distinct structural and functional domains (Fig. 2). IgG consists of two identical heavy chains (50 kDa) and two identical light chains (25 kDa). Light chains consist of one V domain (VL) and a single constant domain (CL), whereas heavy chains comprise one V domain (VH) and three constant domains (CH1, CH2, and CH3).

Functionally, antibody is divided into two antigen-binding fragments (Fabs) and a constant (Fc) region, which are linked via a flexible hinge region. The heavy (VH) and light (VL) chain variable domains in the amino-terminal part of Fabs bind antigens. Each domain features the characteristic immunoglobulin fold consisting of two antiparallel β-sheets with an intramolecular disulfide bond. Within each V domain are three regions that are hypervariable in sequence and that form loops at the ends of rigid β-sheets. The hypervariable loops are primarily responsible for antigen recognition and are referred to as complementarity determining regions (CDRs). The remaining V region amino acids act as a scaffold to support the loops and are referred to as framework residues (FR).

The Fc portion of antibody mediates effector functions, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, antibodies bind to Fc receptors (FcγRs) on the surface of effector cells such as natural killer (NK) cells and macrophages, and trigger phagocytosis or lysis of the targeted cells. In CDC, antibodies kill the targeted cells by triggering the complement cascade at the cell surface. The human IgG1 is most efficient in both CDC and ADCC, and therefore the most suitable for therapeutic use against pathogens or tumor cells. On the other hand, human IgG4 is not active in CDC or ADCC, and therefore may be suitable for diagnostic imaging or blocking molecular interactions. The interaction of IgG with the FcγRs or the first component (C1q) of complement depends on the hinge region and the CH2 domain.

Fc region is associated with serum half-life of antibody. The half-life of human IgG1 is generally 3 weeks, whereas that of antibody fragment is just a few hours. This is because FcRn receptor on the endothelial cells binds the interface between the CH2 and CH3 domains and protects IgG1 from degradation. The most notable feature of this binding is that it is pH-dependent with good binding at slightly acidic pH such as 6.0 which exists in endosomes where the FcRn protects the pinocytosed and endocytosed IgG by binding from degradation. On recycling, the IgG is released into the extracellular regions because there is almost no binding at pH 7.4 of the extracellular space. Therefore, to reduce the half-life of an antibody drug, for example, for radio-imaging, antigen binding fragments instead of whole IgGs can be used.

Humanization of murine antibodies

Since the hybridoma technology for the production of murine MAb was established in 1975, an anti-CD3 murine MAb (OKT3) was approved as the first therapeutic antibody in 1986. Despite the high expectations of MAb therapy, OKT3 failed as a good treatment for transplantation rejection primarily because OKT3 induced severe human anti-murine antibody (HAMA) response in patients.

To reduce the immunogenicity of murine antibodies in humans, chimeric antibodies with mouse variable regions and human constant regions were constructed by genetic engineering (Morrison *et al.*, 1984) (Fig. 3). In 1994, ReoPro, a chimeric Fab, was introduced as the second therapeutic antibody. Since then, five more chimeric antibodies have been marketed and clinically used (Table 1). However, although chimeric antibodies were less immunogenic than murine MAbs, human anti-chimeric antibody (HACAs) responses have been observed (Bell and Kamm, 2000).

Table 1. Approved therapeutic antibodies.

Year (FDA)	Trade name (Generic)	Type of antibody (Target antigen)	Disease indication	Company
1986	OKT 3 (muromanab-CD3)	Murine (CD3)	Allograft rejection	Ortho Biotech
1994	ReoPro (abciximab)	Chimeric Fab (GPIIb/IIIa)	Adjunct to PTCA	Centocor
1995	Panorex (edrecolomab)	Chimeric (CA17-1A)	Colorectal cancer	GSK/Centocor
1997	Rituxan (rituximab)	Chimeric (CD20)	Non-Hodgkins lymphoma	IDEC
1997	Zenapax (daclizumab)	Humanized (IL2R)	Prevention of kidney transplant rejection	PDL
1998	Herceptin (trastuzumab)	Humanized (Her2/neu)	Metastatic breast cancer	Genentech
1998	Synagis (palivizumab)	Humanized (RSV F)	RSV propylaxis	MedImmune
1998	Simulect (basiliximab)	Chimeric (IL2R)	Prevention of kidney transplant rejection	Norvatis
1998	Remicade (infliximab)	Chimeric (TNF-α)	Rheumatoid arthritis, Crohn's disease	Centocor
2000	Mylotarg (gemtuzumab ozogamicin)	Humanized-calicheamicin(CD33)	CD33-acute myeloid leukemia	Celltech
2001	Campath (alemtuzumab)	Humanized (CD52)	B-cell Chronic Lymphocytic Leukemia	Millennium
2002	Zevalin (ibritumomab tiuxetan)	Murine-Y-90 (CD20)	Non-Hodgkins lymphoma	IDEC
2002	Humira (adalimumab)	Human (TNF-α)	Crohn's disease, RA	CAT/BASF
2003	Xolair(omalizumab)	Humanized (IgE)	Asthma	Tanox/Genentech/ Novartis
2003	Raptiva (efalizumab)	Humanized (CD11a)	Psorisis	Xoma/Genentech
2003	Bexxar (tositumomab)	Murine-I-131 (CD20)	Non-Hodgkins lymphoma	Corixa/GSK
2004	Erbitux (cetuximab)	Chimeric (EGFR)	Colorectal cancer	Imclone
2004	Avastin (bevacizumab)	Humanized (VEGF)	CRC, breast, renal, NSCL cancer	Genentech

To further minimize the mouse component of antibodies, humanized antibodies were constructed by protein engineering (Jones *et al.*, 1986) (Fig. 3). In such 'humanized' antibodies, only the CDR loops that are responsible for antigen binding are grafted onto human variable-domain framework, which is referred to as CDR-grafting. However, simple CDR-grafting often results in significant loss of antigen-binding affinity. Therefore, to restore the affinity of parental murine MAb, key framework residues that support the conformations of CDR loops in the murine antibody are also grafted onto the human template, which is the most important part in the development of humanized antibody by CDR-grafting. Since the first humanized antibody, Zenapax was marketed in 1997, seven more humanized antibodies have been approved as therapeutics and are in clinical use (Table 1).

CDR-grafting has been designed with the aid of computer-guided modeling of antibody variable domain (Tsurushita *et al*., 2005). Recently, several other approaches to humanization are executed. Hwang *et al.* (2005) select human templates on the basis of structural similarity between human and murine CDRs and identify human templates that can support the grafted murine CDRs in binding to their cognate antigen. Dall'Acqua *et al*. (2005) use framework shuffling in which the CDRs of a murine antibody are fused in frame to pools of synthetic human germline frameworks, and screen the resulting combinatorial library for the combinations most able to support binding. Kashmiri *et al*. (2005) identify the CDR residues most crucial to the antigen-antibody interaction using structural and experimental information and graft those residues, not whole CDRs, onto a human template.

Generation of fully human monoclonal antibodies

While the production of murine MAbs is routine, the production of human MAbs by conventional hybridoma technology has been difficult because human hybridomas and immortalized cell lines do not stably produce high levels of antibody and *in vivo* immunization of humans is

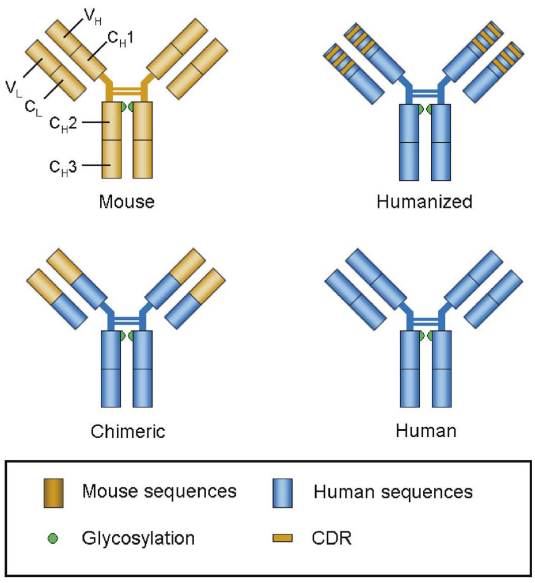

Fig. 3. Antibody engineering for humanization.

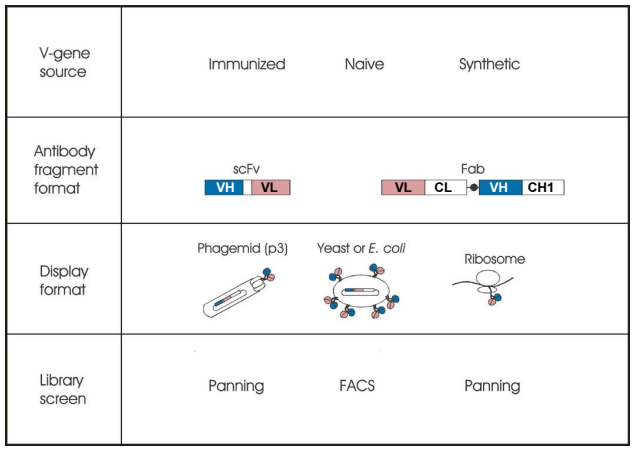

Fig. 4. The display and screening system of antibody libraries.

not feasible for many antigens. However, the development of methods for the expression of antibody fragments such as Fab or ScFv in bacteria and the display of antibody fragments on filamentous bacteriophage as well as powerful techniques for the screening of antibody libraries made it possible to generate human MAbs. This phage-display technology is the most used and well-established technology for the development of new human antibodies (Fig. 4). As an alternative strategy for producing human MAbs, transgenic mice containing human immunoglobulin germ line locus are used. Immunization of such transgenic mice results in a human antibody response, from which hybridomas that produce human antibodies can be generated, as in traditional hybridoma technology.

In 2002, the first human MAb, Humira, developed by phage display, was approved by the FDA and marketed (Table 1). The human MAbs derived from transgenic mice are in clinical trials. Recently, human MAbs are more developed than chimeric or humanized antibodies.

Phage display technology

In the mammalian immune system, antibodies with high affinities are created by combinatorial recombination of germline antibody gene segments and somatic hypermutation in antibody genes, preferentially within the CDRs (Neuberger *et al*., 1998; Wabl *et al*., 1999). B-lymphocytes expressing affinity-matured antibodies are then selected and expanded (Kuby, 1997). Phage antibody technology can successfully mimic the immune system by cloning large libraries of antibody genes and selecting for binding to a desired target.

Immune library Humans exposed to a desired antigen through vaccination or disease have high levels of circulating antibodies to the antigen. Therefore, even small libraries (~10^5) from immunized donors give rise to specific antibodies. In addition, because the antibody genes have experienced affinity maturation *in vivo*, antibodies that do not require further affinity maturation can potentially be isolated. However, isolation of human MAbs from immune library has certain limitations because human antibodies in general cannot be generated by immunization for ethical reasons. Also, immunological tolerance mechanisms make it very difficult and often impossible to isolate antibodies against self antigens, many of which are potentially important therapeutic targets, particularly in cancer.

Naïve library Naïve libraries represent the V-gene repertoire created by cloning the antibody genes found in non-immunized individuals. mRNA is isolated from peripheral lymphocytes, bone marrow, tonsils, and cadaver spleens. Subsequently, IgM and/or IgG variable regions are amplified from mRNA by PCR using degenerate oligonucleotide primer sets and cloned into vectors suitable for screening. IgM repertoires are preferred to IgG because they have not been subjected to tolerance or antigen selection and are thus more diverse. In contrast, IgG chains can be biased by host immune responses and will not react to self antigens (Griffiths and Duncan, 1998). The affinity and specificity of antibodies that can be isolated from naïve libraries is intrinsically linked to library size. For example, a library consisting of 10^{10} different clones yielded antibodies with affinities in the low nanomolar range (Vaughan *et al*., 1996), whereas a similar library of 3×10^7 clones only resulted in antibodies with micromolar affinities (Marks *et al*., 1991). Once a library has been made, it can be propagated and used repeatedly to isolate antibodies against numerous antigens. However, poor expression and toxicity to the host bacteria are often issues with antibodies isolated from naïve libraries. These problems may be circumvented by using

synthetic antibody repertoire libraries.

Synthetic library In principle, synthetic libraries have potentials of encoding antibodies to self-antigens. To create fully synthetic repertoires, germline antibody gene segments, V_H, D_H, and J_H or $V_{\kappa/\lambda}$ and $J_{\kappa/\lambda}$ are cloned and arranged combinatorially *in vitro* so as to reconstitute genes encoding complete V_H and V_L chains (Winter, 1998). Such synthetic libraries of 10^7–10^{10} clones gave rise to antibodies with specificity to self-antigens (de Kruif *et al*., 1995; Griffiths *et al*., 1994; Hoogenboom and Winter, 1992; Nissim *et al*., 1994), but their affinities are generally not high (Griffiths and Duncan, 1998).

Semi-synthetic libraries have also been generated by selecting one or more antibody frameworks as a scaffold and randomizing sequences within the CDR loops. Because CDR3 contributes most of the antigen-binding contacts, it has been predominantly used as a target for randomization (Barbas *et al*., 1992). Early semi-synthetic libraries gave rise to antibodies with rather low K_D values for antigen binding, typically in the 100 nM range (de Wildt *et al*., 1996). Randomization of key residues in the light chain CDRs, in addition to the heavy chain CDR3, has been employed to improve diversity, resulting in the recovery of higher affinity antibodies (Huls *et al*., 1999).

Recently, Knappik *et al*. (2000) developed fully synthetic Human Combinatorial Antibody Library (HuCAL). They constructed 49 combinations of seven VH and seven VL consensus frameworks that represented each of the human V gene families and were optimized for expression in *E. coli*, then randomized the heavy and light chain CDR3 residues of the 49 master genes to build the synthetic library. This highly diverse library has been used to isolate numerous antibodies with high affinities and good expression characteristics in *E. coli*.

Antibody library screening The screening of combinatorial antibody libraries is one of the most important tools in antibody engineering. Efficient high throughput screening of large libraries has enabled the isolation of specific antibody clones and engineering of antibodies with high affinity, increased stability and improved effector functions. Currently, the most widely used technique for library screening is based on the display of antibodies on the surface of filamentous bacteriophages (Hoogenboom *et al*., 1998). Antibody library in the Fab or ScFv format is fused to a surface protein of phages, most commonly pIII encoded by the gene III. Phages displaying an antibody specific for an antigen can be readily enriched by selective adsorption onto immobilized antigen, a process known as panning. Then the bound phage is eluted from the surface and amplified through infection of *E. coli* cells. Usually, 5–8 rounds of panning, elution, and amplification are sufficient to select for phages displaying specific antibodies, even from very large libraries (up to 10^{11} clones). A variety of techniques for the efficient enrichment of specific and high-affinity clones have been developed (Duenas and Borrebaeck, 1994; Griffiths and Duncan, 1998; Hawkins *et al*., 1992; Hoogenboom *et al*., 1998; Spada *et al*., 1997).

Cell display Antibodies can also be displayed on the surface of microbial cells such as *E. coli* and *Saccharomyces cerevisiae* (Boder and Wittrup, 1997; Francisco *et al*., 1993; Fuchs *et al*., 1991; Georgiou *et al*., 1997; Gunneriusson *et al*., 1996). For screening purposes, a library of cells, each displaying multiple copies of a different antibody variant, is incubated with a fluorescently tagged ligand in buffer. Cells displaying antibodies that bind the ligand become fluorescently labeled and are isolated by fluorescence-activated cell sorting (FACS). With flow cytometry, the binding of each clone in the library to a particular ligand is determined quantitatively. Parameters such as ligand concentration or time for the dissociation of antibody:ligand complexes can be easily optimized to ensure the isolation of only the highest affinity antibodies. At present, cell surface display and FACS cannot be used for the de novo isolation of antibodies from natural or synthetic repertoire libraries because the maximum library diversity that can be realistically screened by FACS is about 5×10^8. These features are particularly significant for antibody affinity maturation. However, repertoire libraries can be screened by 1–2 rounds of panning to reduce the library diversity, followed by cell display technologies that guarantee the isolation of high-affinity, well expressed antibodies.

Ribosome display Ribosome display relies on the formation of a ternary complex between ribosomes, mRNA, and the polypeptide (Hanes and Pluckthun, 1999; Hanes *et al*., 1998; He and Taussig, 1997; Mattheakis *et al*., 1994). Complexes containing folded antibodies with a desired specificity are enriched by panning against immobilized ligand. The mRNA of the ribosome-mRNA-protein complexes is reverse transcribed to produce the DNA encoding the antibodies responsible for the binding of the complexes to the immobilized ligand. The DNA is then transcribed by RNA polymerase to begin another cycle of ternary complex formation and selection. Alternatively, a covalent link may be established directly between mRNA and the protein it encodes (Roberts and Szostak, 1997). Covalent mRNA-protein complexes are formed via the reactive amino acid analogue puromycin. The advantages of this technology are that covalent mRNA-protein is more stable and can be subjected to harsh screening conditions to enrich antibodies or other proteins with increased stability and the entire process is performed entirely *in vitro* without transformation. Using this technology, Hanes *et al*. (2000) obtained picomolar affinity antibodies from the synthetic HuCAL library.

Human antibodies from transgenic mice

A radically different approach to antibody humanization is to develop murine hybridomas that produce fully human antibodies. For this purpose, transgenic mice in which the native immunoglobulin repertoire has been replaced with human V-genes in the murine chromosome have been generated (Bruggemann and Taussig, 1997; Fishwild *et al*., 1996; Mendez *et al*., 1997). These mice appear to carry out VDJ recombination and somatic hypermutation of the human germline antibody genes in a normal fashion, thus producing high-affinity antibodies with completely human sequences and differing only in glycosylation patterns. The transgenic mice can be injected with a desired antigen (including human proteins), and the resulting antibody genes are recovered by cloning and screening an immune library, or by conventional hybridoma technology. Antibodies with mid-picomolar affinities have been isolated and these appear promising for therapeutic applications (Fishwild *et al*., 1996; Yang *et al*., 1999a; 1999b). Importantly, their half-life in *Cynomologus* monkeys was found to be similar to antibodies recovered from humans [9.5–11 days for transgenic mouse-derived human antibodies, versus 9.6 days for human IgG, 1.9 days for murine IgG, and 7.1 days for chimeric mouse-human IgG (Yang *et al*., 1999a; 1999b)]. These mice present a major advance for hybridoma technology.

Immunogenicity of chimeric, humanized, and fully human antibodies in humans

Recently, Hwang and Foote reviewed reported immunogenicity of murine, chimeric, and humanized antibodies in humans (Hwang and Foote, 2005). They grouped the anti-antibody response (AAR) incidence into three operational categories: *negligible*, *tolerable*, and *marked*. *Negligible* AAR was classified when AAR was reported in less than 2% of patients, which represents an ideal in terms of safety. *Tolerable* AAR was classified when it was detectable in 2–15% of patients. In this case, the MAbs were essentially flawed, though use was arguably warranted for catastrophic or life-limiting disease. *Marked* AAR was classified when AAR was detected in more than 15% of patients. Products with *marked* AAR were usually clinical failures and regulatory concerns were likely to preclude clinical use except for one time use as radioimmunoconjugates. As shown in Fig. 5, among the 44 murine MAbs analyzed, 84%, 7%, or 9% had marked, tolerable, or negligible HAMA responses, respectively. Among the 15 chimeric antibodies with clearly reported AAR, 40%, 27%, or 33% had marked, tolerable, or negligible HACA responses, respectively. On analyzing 22 humanized antibodies with AAR, 9%, 36%, or 55% had marked, tolerable, or negligible HAHA responses, respectively. The

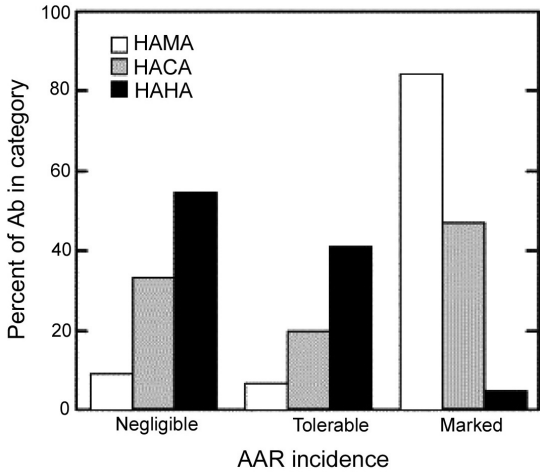

Fig. 5. Anti-antibody response incidence compared to molecular format.

data clearly show that chimerization largely decreases the immunogenicity of therapeutic antibodies and humanization decreases it further. However, both humanized and chimeric antibodies have about equal proportions of tolerable and negligible examples, which suggests that it may be necessary to further reduce the immunogenicity of humanized antibodies. Likewise, whether fully human MAbs are less immunogenic than humanized antibodies or not cannot be answered at present because full immunogenicity data are available for just one MAb Humira, developed from phage-displayed human libraries. Repetitively dosed Humira showed AAR in 12% of patients - at the higher end of HAHA responses. More data on the immunogenicity of human antibodies will be needed to answer the question.

Antibody fragments

Whole antibody with a molecular weight of about 150 kDa diffuses poorly from the vascular bed into a solid tumor mass and clears slowly from the body. Antibody fragments such as Fab, scFv, diabodies, and minibodies can be generated by removing the entire constant region or part or whole of the Fc portion (Fig. 6). These antibody fragments are known to have better clearance from whole body and also better tissue/tumor penetration characteristics. Thus antibody fragments are better suited for imaging and/or radio-therapy (Chang *et al*., 2002; Colcher *et al*., 1998; Wu, 2004; Wu and Yazaki, 2000). Antibody fragments are thought to be easy to produce in bacteria in large amounts and are therefore considered to bypass the hurdles associated with mammalian cell based production of whole antibodies.

In general, the smallest fragment of an antibody that retains the antigen binding specificity of whole antibody is the Fv in which the VH and VL domains are noncova-

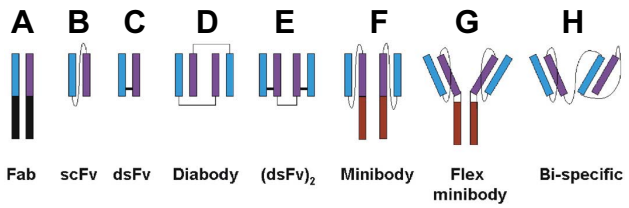

Fig. 6. Schematic representation of antibody fragments.

lently associated, although even single V domain can bind to antigens (Ward *et al*., 1989). Because of its instability at low concentrations, the VH and VL domains of Fv are linked by a flexible peptide linker to make a single-chain Fv (scFv) (Fig. 5B) (Bird *et al*., 1988). The most common peptide linker is a flexible $(Gly_4Ser)_3$. Also, Fv is engineered to form a disulfide bond by introducing two cysteine residues in the framework regions of VH and VL to yield a disulfide stabilized Fv (dsFv) (Fig. 5C) (Brinkmann *et al*., 1993). Converting a whole IgG to a scFv or dsFv is usually associated with a decrease in the antigen binding activity because of the loss of avidity. However, this loss in binding activity can be compensated by engineering the Fvs to increase affinity.

Diabodies are homodimers of scFvs that are covalently linked by a short peptide linker of four amino acids which forces the V domains to make inter-molecular complexes with their cognate domains (Fig. 5D) (Holliger *et al*., 1993). In a slightly different format called $(dsFv)_2$, the VH and VL are engineered to form a dsFv but the VH is expressed twice in tandem separated by a flexible linker. When the VH and VL are co-expressed, a divalent molecule is formed (Fig. 5E) (Bera *et al*., 1998). Minibodies are homodimers of scFv-CH3 fusion proteins (Fig. 5F). In a different variant called Flex minibody (Fig. 5G), the scFv is fused to the hinge region of IgG1 which in turn is fused to via an additional 10 residues to the CH3 domain (Hu *et al*., 1996). The dimeric forms of antibody fragment usually do not suffer any loss of apparent antigen binding affinity compared to the parental IgG and show excellent tumor targeting and short half-life (Santimaria *et al*., 2003).

Improvement of antigen binding affinity

Engineering antibodies for improving their antigen binding affinity has been a very active and probably one of the most extensively studied areas of antibody engineering research. This may be due to the belief that increasing the affinity of an antibody would allow lower doses to elicit a more profound biological activity which in turn would increase the therapeutic window and lower dose related toxicity. In addition, cost can also be reduced. Comparison and analysis of several independent studies now reveal that the relation between antibody affinity and bioactivity is linear only up to a threshold level (Adams *et al*., 2001). It is dictated to a large extent by the nature of the antigen and the target tissue, the density of the antigen on the target tissue, and the mode of action of the therapeutic antibody. Affinity maturation of antibodies has been aided by the phage display and yeast display due to its simplicity and high throughput effect in screening high affinity variants.

The approaches to improve antibody affinity can be basically divided into two broad categories. One approach is to create very large libraries of randomly mutated CDRs or the entire variable domains and then select for higher affinity variants from this large collection of mutants (Adams *et al*., 1998; Barbas *et al*., 1994; Chames *et al*., 2002; Maynard *et al*., 2002; McCall *et al*., 2001; Schier *et al*., 1996a; 1996b; Yang *et al*., 1995; Zhang *et al*., 2004). The large libraries are created by random CDR mutagenesis, chain shuffling, or error prone PCR and DNA shuffling. The other approach is to make small libraries by focused mutagenesis or hot spot mutagenesis mimicking *in vivo* affinity maturation. In this focused approach, every single position in each of the six CDRs or certain discrete spots of the variable domains called hot spots are randomized and high affinity variants are selected. It is a common practice to combine different mutations that lead to small increases in affinity. Often these combinations of different mutations have an additive or synergistic effect and lead to a greater improvement in affinity (Chowdhury, 2003; Chowdhury and Pastan, 1999; Glaser *et al*., 1992; Hassan *et al*., 2002; Ho *et al*., 2005; Kuan *et al*., 2000; Salvatore *et al*., 2002; Wu *et al*., 1998; 1999).

Improvement of effector functions

Therapeutic antibodies work by one of two basic mechanisms. One is by blocking ligand-receptor interaction or by triggering an intracellular signal, such as apoptosis. The action of these antibodies is largely dependent on their antigen binding function and not on their effector functions. The other way therapeutic antibodies work is by recruiting immune system components following antigen binding. The therapeutic efficacy of these antibodies is therefore dependent on their antigen binding ability as well as their ability to trigger effector activity. These effector functions include ADCC and CDC. It is therefore conceivable that engineering antibodies to improve their binding to FcγRs or the complement factors may lead to improvement in their therapeutic efficacy. However, under certain circumstances, abrogating ADCC activity may be desirable, for example, in the treatment of diseases where lysing of target cells can cause release of pro-inflammatory cytokines. In these situations, one may be able to use those IgG variants whose binding to FcγRs (especially IIIA) or to complement factors are greatly reduced.

Improvement of ADCC

In humans, there are several types of FcγRs present on the effector cells. These include FcγR1 (CD64), FcγRII (CD32), and FcγRIII (CD16). The FcγRII and III have subtypes A and B. The FcγRIIA has two naturally occurring allotypes for position 131, namely Arg^{131} and His^{131}. The FcγRIIIA has three naturally occurring allotypes at position 48 (Leu, His, and Arg) and two at position 158 (Val and Phe). The different allotypes vary in their ability to bind IgGs and in triggering effector activities. The FcγRI, IIA and IIIA and IIIB are called stimulatory receptors and facilitate ADCC or other cellular effector functions while FcγRIIB is an inhibitory receptor and down regulates ADCC (Daeron, 1997). The three receptors also differ in their affinity for IgG. The FcγRI binds monomeric IgG with high affinity (K_a = 10^{-8}–10^{-9} M^{-1}), while FcγRII and FcγRIII have lower affinity for monomeric IgG ($K_a \leq 10^{-7}$) and interact effectively only with multimeric immune complexes. Regarding the expression of the different receptors on the effector cells, NK cells which are believed to be the main effector cell in ADCC express only the stimulatory Fcγ RIIIA, while macrophages express the stimulatory FcγRI, IIA, and IIIA but also the inhibitory FcγRIIB, and neutrophils express the stimulatory FcγRI, IIA, and IIIB but also the inhibitory FcγRIIB. Therefore, to improve ADCC activity, one can improve the affinity of IgG Fc for the stimulatory receptors. The interaction of the IgG with the FcγRs depend on the oligosaccharide in the Fc region and on several residues located in the hinge and CH2 regions. Consequently, engineering for improved ADCC activity has involved glycoengineering of IgG Fc and mutating the residues that contribute to FcγR binding.

Glycoengineering An IgG molecule contains two N-linked glycan chains attached to Asn^{297} in each of its heavy chains in the Fc region. It is well known that IgG is produced as a heterogeneous population of gylcoforms in mammalian cells (Fig. 7). Fc glycosylation is important for the interaction with FcγR. This interaction is known to be sensitive to changes in the oligosaccharide structures of the Fc region (Lund *et al*., 1996; Wright and Morrison, 1998). The oligosaccharide core normally found attached to the human IgG Fc is of the bi-antennary type and consists of Asn^{297}-linked GlcNAc(Fuc)-GlcNAc-Man-(Man-GlcNAc)$_2$. Individual IgG molecules vary with respect to terminal galactose or galactose-sialic acids at one or both of the terminal GlcNAc and/or attachment of a third GlcNAc (bisecting GlcNAc). They also differ with respect to the presence or absence of a fucose residue attached to the GlcNAc that is linked to Asn^{297}.

Recently, it was reported that elimination of the fucose moiety from the core of the Fc N-linked glycan profoundly improved binding to FcγRs and the ADCC activity (Shields *et al*., 2002; Shinkawa *et al*., 2003). The hypo-fucosylated IgG showed increased binding to FcγRIIIA. In this case, there was at least 42-fold increased binding to the FcγRIIIA(Phe^{158}) allotype and about 19-fold increased binding to the FcγRIIIA(Val^{158}) allotype by the hypo-fucosylated IgG dimers compared to the hyper-fucosylated IgG dimers. In terms of ADCC activity, the hypo-fucosylated IgG showed enhanced activity compared to the parental hyper-fucosylated IgG when PBMCs from individuals with Fcγ RIIIA(Val^{158}/Phe^{158}) and FcγRIIIA(Phe^{158}/Phe^{158}) were used. Therefore, the data suggest that improved binding to FcγRIIIA by the hypo-fucosylated IgG translated into improved ADCC activity.

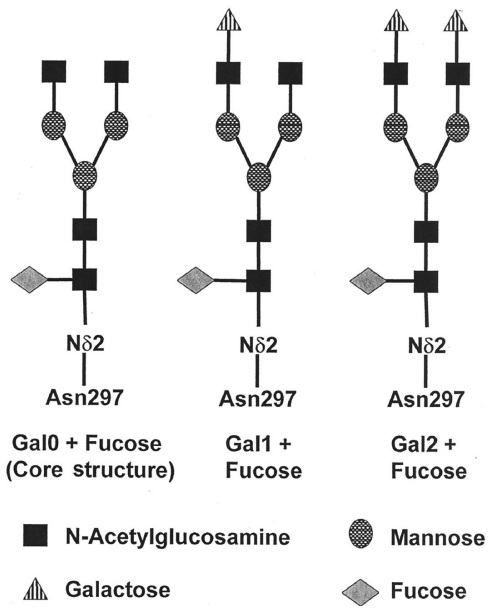

Fig. 7. Schematic representation of immunoglobulin glycoforms.

Protein engineering A number of studies have been done to map the residues in human IgG1 that involve binding to FcγRs. Shields *et al*. (2001) using alanine scanning mutagenesis of all the solvent exposed residues in IgG1 Fc, have extensively mapped the binding site on human IgG1 for FcγRI, II, and III. Based on this study they dissected out the contribution of different residues in the IgG CH2–CH3 domains for binding to FcγRs in general and also in the differential binding to the various subtypes of the FcγRs such as FcγRII and IIIA. From this and other studies (Lazar *et al*., 2004; Stavenhagen and Vijh, 2004) that focused on screening for IgG1 variants with improved binding to the ADCC stimulatory FcγRIIIA, and decreased binding to the inhibitory receptor, FcγRIIB, several mutants have been reported which had improved ADCC activity. Thus, by grading the contribution of the different residues, Shields *et al.* identified three mutants which when combined together (S298A/E333A/K334A) (EU numbering system; Kabat *et al*., 1991) showed im-

proved binding to FcγRIIIA with corresponding improvement in ADCC activity. Additionally, Lazar *et al.* (2004) in a study involving substitution of wild type residues with all different amino acids, identified a mutant S239D/A330L/I332E with greatly improved ADCC activity over the wild type IgG1.

It is interesting to see that the Fc variant, S298A/E333A/K334A, identified by Shields *et al.* (2002) can be combined with de-fucosylation for synergistic ADCC improvement. It should also be kept in mind that the improvement in ADCC activity reported varies to some extent based on the antibody used and also on the types of the cells being employed for assays (Lazar *et al.*, 2004; Shields *et al.*, 2001; Stavenhagen and Vijh, 2004). In addition, the variants discussed in this section have not yet been tested *in vivo* for ADCC improvement. Using transgenic mice expressing human FcγRs will aid the understanding of the *in vivo* activities of these mutations.

Improvement of CDC

CDC is dependent upon binding of two molecules of C1qs to the hinge and CH2 domain of antibodies. Klein *et al.* (1981) had suggested that the hinge region, by modulating the rotational flexibility between the Fab and Fc region, confers on the Fc the ability (or inability) to bind complement and support CDC. A series of studies led by Morrison and co-workers (Sensel *et al.*, 1977; Tao *et al.*, 1991; 1993) employing domain switching and site directed mutagenesis of IgG constant domains, indicated that the CH2 domain had an important contribution to CDC. Based on these and other studies, Idusogie *et al.* (2000) have applied alanine scanning mutagenesis to study the CDC function of Rituxan. This has led to the mapping of the C1q binding epicenter in the CH2 domain of human IgG1. This epicenter involves Asp^{270}, Lys^{322}, Pro^{329}, and Pro^{331} that are spatially close to each other. Idusogie *et al.* (2001) have further identified two residues at the edges of the C1q binding epicenter that could be engineered to increase the C1q binding which consequently improved CDC activity in a human IgG1 format. These residues are Lys^{326} and Glu^{333}, which do not interact with the oligosaccharide chains in CH2. Several different types of residues that do not conform to any particular size, hydrophilicity, and charge were found to be tolerated at each of these two positions. The residues that increased CDC most were Ala, Met, and Trp at position 326, and Ala and Ser at position 333. Combination of some but not all of the single mutations at these positions demonstrated an additive effect on C1q binding and CDC activity.

Introduction of new effector functions

As mentioned above, the two effector functions of therapeutic antibodies are ADCC and CDC. While these effector pathways are important for therapeutic antibody such as Rituxan and probably also for others like Herceptin, there are indications of resistance to CDC and ADCC among cancer cells (Gelderman *et al.*, 2004; Golay *et al.*, 2000; Treon *et al.*, 2001). It is also important to note that ADCC and CDC require the presence of certain minimum level of antigen expression on the target cells and the response is affected by the polymorphic state of FcγRs in the case of ADCC. Therefore, in some therapeutic settings, additional engineering of novel effector functions would be beneficial.

Novel effector activities are provided by conjugating MAbs or their antigen binding domains with diverse classes of molecules, such as cytotoxic drug (for example, calicheamycin conjugated to Mylotarg, approved by FDA), toxin (ricin, Smallshaw *et al.*, 2003), cytokine (IL-2, Neal *et al.*, 2004), enzyme (bacterial carboxyl peptidase, Medzihradszky *et al.*, 2004) in antibody-directed enzyme prodrug therapy, radio-isotopes (^{131}I, ^{90}Y), or even another antibody (anti-activating Fc receptors, Valone *et al.*, 1995a; 1995b) to attract effector cells to the target as in bi-specific antibodies. In these cases, the antibody simply acts as a homing device to carry its payload to the target where the latter precipitates a biologic activity with minimum side effects to non-target tissues.

Altering pharmacokinetics

Increased FcRn binding The plasma half-life of IgG1 is dictated by its binding to FcRn receptor. The site on IgG that is responsible for binding to FcRn has been mapped and well characterized. Hence, the trend in the field of antibody engineering is to mutate the FcRn binding site such that binding to FcRn is increased at pH 6.0 but not at pH 7.4. Dall'Acqua *et al.* (2002) found that major improvement in FcRn binding occurred when mutations were introduced at positions 252, 254, 256, 433, 434, and 436 which are at the interface of the Fc-FcRn binding region. More recently, Hinton *et al.* (2003) discovered two mutations, T250Q and M428L, that caused respectively a 3- and 7-fold increase in FcRn binding and when combined together caused a 28-fold increase in FcRn binding. When tested in rhesus monkeys, the M428L or T250Q/M428L mutant showed about 2-fold increase of plasma half-life compared to the parental molecule. Because these positions are conserved among different human IgG isotypes they proposed that the beneficial effect of these mutations should hold good for other isotypes as well.

PEGylation The huge potential advantage of antibody drugs are somewhat offset by factors such as their high manufacturing cost in mammalian cell lines and limita-

tions in current world-wide manufacturing capacity. An alternative option is to use *Escherichia coli* for the manufacture of antibody fragments. As discussed earlier, these fragments have exceedingly shorter plasma half-lives compared to whole antibodies. Therefore, development of the methods to increase in the half-lives of antibody fragments is an important area of research. One method is called PEGylation, the process of conjugating polyethylene glycol chains to the antibody fragments. PEGylation of proteins and liposomes has been a time tested and successful technique that offered the advantage of reducing immunogenicity, increasing the plasma half-life, increasing solubility, and reducing protease sensitivity (Chapman, 2002). Therefore, the science of antibody PEGylation has two primary aims which are (a) to preserve the antigen binding activity completely and (b) to link the PEG molecule to the antibody in a stable manner. These are achieved by doing site specific PEGylation using maleimide chemistry. Site specific PEGylation was done by introducing a free cysteine to the end of the hinge region in a Fab (Chapman *et al*., 1999) or by incorporating the hinge region on the C-terminus of a Fab and scFv (King *et al*., 1994). In the case of a scFv-immunotoxin, the free cysteine was introduced in the linker between the scFv and the toxin for PEGylation (Tsutsumi *et al*., 2000). The increase in half-life observed with PEGylated antibody fragments is usually due to a prolongation of the α phase, a phase that represents the redistribution of a molecule in the extravascular environment. It therefore appears that PEGylation slows the redistribution of the molecules from the plasma to the interstitial compartment.

Conclusions

This review briefly updates the different aspects and avenues of antibody engineering that have been attempted to generate therapeutic antibodies and increase their therapeutic efficiencies. While many of these have been tested for their effectiveness *in vivo* and in patients, immunogenicity is a potential risk factor and only clinical trials in humans will evaluate how well humans can tolerate engineered antibodies. Other areas that have not been covered in this review but that have high impact on the success of antibodies as drugs include the improvement of production levels to control the cost of manufacturing and shelf-life of antibody drugs. Antibody drugs for various indications are pursued vigorously and will be clinically used for the treatment of cancer, immune disorders, and infectious diseases.

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