CCE IX: Review

Glycosylation of Recombinant Antibody Therapeutics

Royston Jefferis*

Division of Immunity & Infection, The School of Medicine, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, U.K.

The adaptive immune system has the capacity to produce antibodies with a virtually infinite repertoire of specificities. Recombinant antibodies specific for human targets are established in the clinic as therapeutics and represent a major new class of drug. Therapeutic efficacy depends on the formation of complexes with target molecules and subsequent activation of downstream biologic effector mechanisms that result in elimination of the target. The activation of effector mechanisms is dependent on structural characteristics of the antibody molecule that result from posttranslational modifications, in particular, glycosylation. The production of therapeutic antibody with a consistent human glycoform profile has been and remains a considerable challenge to the biopharmaceutical industry. Recent research has shown that individual glycoforms of antibody may provide optimal efficacy for selected outcomes. Thus a further challenge will be the production of a second generation of antibody therapeutics customized for their clinical indication.

Contents

Structure of Human IgG Antibodies	**12**
The Oligosaccharide	**12**
Cell Engineering To Influence Glycoform Profiles	**13**
IgG Glycoforms and Fc Effector Functions	**14**
Glycosylation Engineering	**15**

Recombinant antibody therapeutics (rMAbs) are likely to form the largest family of disease-modifying drugs available to clinicians. Their efficacy results from specificity for the target antigen and biological activities (effector functions) activated by the immune complexes formed. Currently, there are 14 rMAbs licensed and hundreds in clinical trials or under development. The biopharmaceutical industry has met the challenge to produce rMAbs; however, productivity, cost, and potency remain to be optimized. All antibody therapeutics that are currently licensed have been produced by mammalian cell culture, utilizing Chinese hamster ovary (CHO) cells or mouse NSO or Sp2/0 plasma cell lines; other systems under development and evaluation include transgenic animals, yeasts, fungi, plants, etc. The efficacy of an antibody therapeutic is critically dependent on appropriate posttranslational modifications (PTM) and each production vehicle offers a different challenge since PTMs show species, tissue, and site specificity. Essential PTMs are relevant not only to product potency but also to stability, potential for immunogenicity, etc. All rMAbs have evidenced a potential for immunogenicity, whether presented as mouse, chimeric, humanized, or fully human sequences; the promise that fully human antibodies may not be immunogenic has not been realized for Humira (Adalimumab) since a 12% incidence of patients producing anti-Humira antibodies is reported. It should be appreciated that antibodies generated from a phage display library or translocus mice that target human antigens are, essentially, anti-self and represent forbidden clones; it is not surprising, therefore, that unique structures that ensure unique specificities carry with them the potential to be immunogenic.

The further development of the potency of rMAbs depends on optimizing the "downstream" biological effector functions activated in vivo by the immune complexes formed. This offers a considerable challenge as a result of the difficulty of monitoring events in vivo; a number of genetically modified animal models are under development that might mimic human biology, e.g., transgenic and knock-out mice that produce human antibodies and express human cellular IgG-Fc receptors. At the present time we presume to extrapolate from effector functions activated in vitro to activity in vivo, e.g., complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), the induction of apoptosis, etc. In recent years in vitro studies have unequivocally established that essential effector functions are dependent on appropriate glycosylation of the antibody molecule. Thus, glycosylation has been a focus of attention for the biopharmaceutical industry for the past several years, and the FDA requires that a consistent human-type glycosylation be maintained for rMAbs, irrespective of the system in which they are produced. Glycosylation of rMAbs is the focus of this review.

* Email: r.jefferis@bham.ac.uk.

10.1021/bp040016j CCC: $30.25 © 2005 American Chemical Society and American Institute of Chemical Engineers
Published on Web 12/22/2004

FORMYCON-1088

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp040016j by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

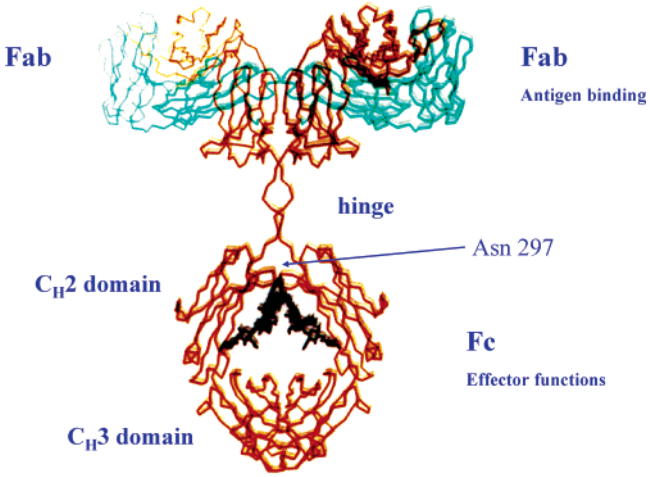

Figure 1. Alpha backbone structure of human IgG showing functional regions (see text): light chain, green; heavy chains, orange.

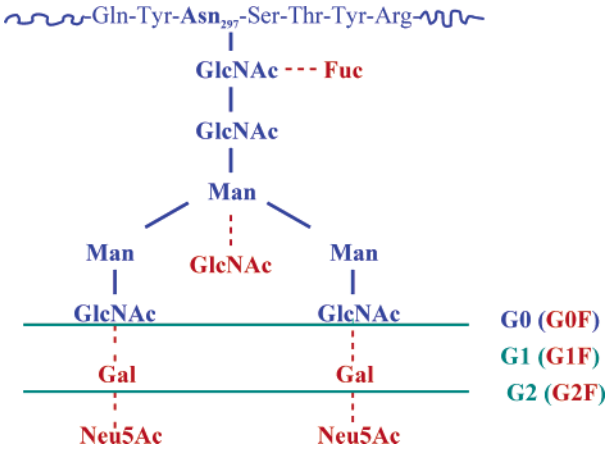

Figure 2. Structures of the possible diantennary oligosaccharide structure attached to IgG-Fc at asparagine 297 (Asn_{297}). The "core" heptasaccharide present in normal human IgG is shown in blue; this generates the G0 structure. Additional sugar residues that may be attached to the "core" are shown in red; thus G0F represents a fucosylated GO (G0F) oligosaccharide.

Structure of Human IgG Antibodies. To date all licensed therapeutic rMAbs have been of the IgG class. The basic structure of an IgG molecule is of two light and two heavy chains in covalent and noncovalent association to form three independent protein moieties connected through a flexible linker (the hinge region), Figure 1. Two of these moieties, referred to as Fab regions, are of identical structure and each expresses a specific antigen-binding site; the third, the Fc, expresses interaction sites for ligands that activate clearance mechanisms. These effector ligands include three structurally homologous cellular Fc receptor types (FcγRI, FcγRII, FcγRIII), the C1q component of complement, the neonatal Fc receptor (FcRn), etc. (*1*−*3*). There are four subclasses of human IgG, which although highly homologous (> 95% sequence homology) express unique profiles of effector functions.

The IgG-Fc region is a homodimer comprising covalent disulfide-bonded hinge regions and noncovalently paired C_H3 domains; the C_H2 domains are glycosylated through covalent attachment of oligosaccharide at asparagine 297 (Asn-297). X-ray crystallographic analysis reveals a discreet structure for the oligosaccharide that is integral to the IgG-Fc structure and forms multiple noncovalent interactions with the protein surface of the C_H2 domain; thus each exerts a reciprocal influence on protein−oligosaccharide conformation (*4*). There is cumulative evidence that interaction sites on IgG-Fc for effector ligands comprise the protein moiety only; however, generation of the essential IgG-Fc protein conformation is dependent on the presence of the oligosaccharide. Thus, effector mechanisms mediated through FcγRI, FcγRII, FcγRIII, and C1q are severely compromised or ablated for aglycosylated or deglycosylated forms of IgG (*2*, *5*−*7*).

The Oligosaccharide. ***IgG-Fc Glycosylation.*** The oligosaccharide of normal polyclonal human IgG-Fc is of the diantennary complex type and shows considerable heterogeneity; a "core" heptasaccharide can be defined with variable addition of outer arm sugar residues, Figure 2. This allows for a possible total of 32 different oligosaccharides and, potentially, more than 400 glycoforms, given random pairing of heavy chain glycoforms (*3*). Analysis of the oligosaccharide released from normal polyclonal IgG-Fc shows a paucity of sialylation (<10%) and 12 of the possible 16 neutral oligosaccharides to predominate (Figure 3) (*8*), providing the potential to generate a total of 72 glycoforms. Because glycosylation is essential for the expression of effector functions it may be anticipated that efficacy may vary between glycoforms. The glycoform profile of monoclonal mouse antibodies, produced by hybridomas, and human monoclonal IgG, produced by plasma cell tumors (multiple myeloma) in vivo, are mostly heterogeneous and present a clone-specific glycoform profile. The IgG-Fc glycoform profile of chimeric and humanized rMAbs produced by CHO, NSO, or Sp2/0 cells can vary widely from clone to clone and are dependent on the mode of production and culture conditions.

Glycosylation is a co-translational PTM that results in the attachment of a glucosylated high mannose oligosaccharide ($GlcNAc_2Man_9Glu_3$), which is trimmed to a $GlcNAc_2Man_9Glu$ structure that is bound by chaperones that aid and monitor folding fidelity (*9*, *10*). The glycoprotein, being targeted for secretion, then transits the Golgi apparatus where the oligosaccharide is initially trimmed back by glycosidases to a $GlcNAc_2Man_5$ structure before being processed by the successive action of glycosyltransferases to generate the complex diantennary structure (*9*, *10*). Early studies established that CHO cells are able to produce IgG antibodies with major glycoforms identical to glycoforms present in polyclonal human IgG (predominantly G0F and G1F glycoforms); however, under nonoptimal conditions CHO, NS0, and Sp2/0 cells can produce a number of abnormally glycosylated products that lack potency or are potentially immunogenic and unacceptable as therapeutics to be delivered to human patients in vivo. In addition to incorrectly processed oligosaccharides CHO and murine cells can also add sugars that are not found on normal human IgG and are known to be immunogenic, e.g., galactose $\alpha(1-3)$ galactose and *N*-glycolylneuraminic acid structures (*11*−*14*). A major achievement of the biopharmaceutical industry has been the establishment of cell lines that can be expanded in serum- and protein-free media with scale-up to manufacturing levels while maintaining glycosylation fidelity and minimizing the content of abnormal glycoforms. Regulatory authorities require that the glycoform profile be maintained within strict limits.

IgG-Fab Glycosylation. It has been demonstrated that 15−20% of polyclonal human IgG molecules bear N-linked oligosaccharides within the IgG-Fab region, in addition to the conserved glycosylation site at Asn 297 in the IgG-Fc (*15*; Holland et al., manuscript in preparation). There are no consensus sequences for N-linked

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp040016j by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

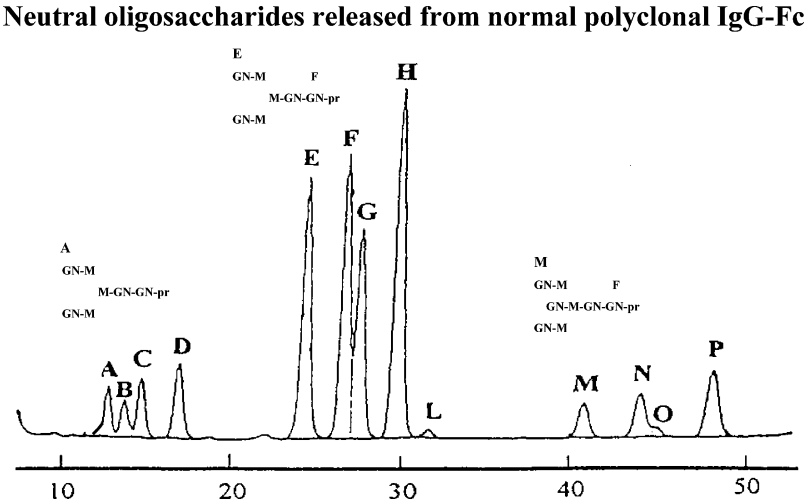

Figure 3. HPLC profile of the neutral oligosaccharides released from normal human IgG-Fc (*8*).

oligosaccharide within the constant domains of either the kappa or lambda light chains or the C_H1 domain of heavy chains. Therefore, when present they are attached in the variable regions of the kappa ($V\kappa$), lambda ($V\lambda$), or heavy (V_H) chains and sometimes both. In the immunoglobulin sequence database ~20% of IgG V regions have N-linked glycosylation consensus sequences (Asn-X-Thr/Ser; where X can be any amino acid except proline). Interestingly, these consensus sequences are mostly not germline encoded but result from somatic mutation, suggesting positive selection for improved antigen binding (*16*−*18*). The functional significance for IgG-Fab glycosylation of polyclonal IgG has not been fully evaluated, but data emerging for monoclonal antibodies suggest a positive, neutral, or negative influence on antigen binding.

The rMAb Cetuximab (Erbitux), which has specificity for the epidermal growth factor receptor and is licensed for the treatment of colon, head, and neck cancer, bears an N-linked oligosaccharide at Asn 88 of the V_H region. Three rMAbs with specificity for α(1−6) dextran and differing only in potential N-glycosylation sites in CDR2 at Asn 54, Asn 58, or Asn 60 in the V_H region were evaluated for antigen-binding affinity. The Asn 54 and Asn 58 molecules were equivalent in antigen binding and each bore a complex diantennary oligosaccharide rich in sialic acid, in contrast to the IgG-Fc oligosaccharide; the glycosylated forms had a 10- to 50-fold higher affinity for antigen compared with that of the aglycosylated forms. In contrast the Asn 60 molecule bore a high mannose oligosaccharide and had a lower affinity for antigen (*19*, *20*). A significant proportion of IgG-Fab oligosaccharides also bore Gal α(1−3) Gal structures (*21*). By contrast humanization of a mouse anti-CD33 antibody with concomitant removal of a potential glycosylation site at Asn 73 of the V_H resulted in higher affinity for antigen; similarly, deglycosylation of the original mouse antibody resulted in increased affinity (*22*). A mouse antibody specific for ovomucoid was shown to be N-glycosylated in CDR2 of the light chain, and increased affinity was reported for the deglycosylated antibody (*23*). A multispecific human monoclonal antibody, produced in mouse−human heterohybridoma cells, has been reported to bear both diantennary and tetra-antennary oligosaccharides attached at Asn 75 of the V_H region and to include antigenic *N*-glycolylneuraminic acid sugar residues (*24*).

The consistent observation of higher levels of galactosylation and sialylation for IgG-Fab N-linked oligosaccharides, in comparison to IgG-Fc, is thought to reflect increased exposure and/or accessibility. It might be

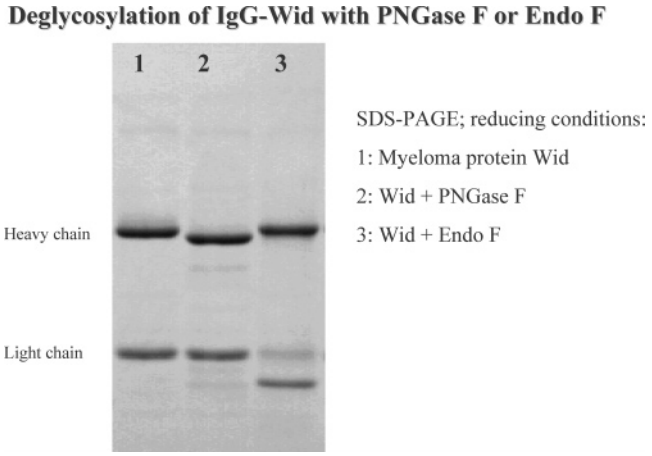

Figure 4. SDS−PAGE analysis of a human monoclonal protein (Wid) isolated from a patient with multiple myeloma. Tracks: (1) the native protein; (2) after exposure to peptide endoglycosidase F (PNGase F); (3) after exposure to endoglycosidase F (Endo F).

expected, therefore, that the enzyme PNGase F would more readily cleave IgG-Fab oligosaccharides; however, we have contrary experiences. In one study a human IgG1 myeloma protein was shown to be glycosylated in the V_L region, in addition to IgG-Fc. Exposure to PNGase F resulted in the release of oligosaccharide from the IgG-Fc but not the light chain (Figure 4); in contrast the enzyme endoglycosidase F released the oligosaccharide from the light chain but not the heavy chain. We have observed, similarly, that PNGase F releases oligosaccharide from the IgG-Fc of Cetuximab but not that attached at Asn 88 of the V_H region.

Cell Engineering To Influence Glycoform Profiles. Therapeutic antibody production cell lines yield a product with a restricted glycoform profile relative to that observed for normal polyclonal human IgG, and the G0F and G1F glycoforms predominate. Concern has been expressed for the relatively low levels of galactosylation and its possible impact on activation of the classical complement pathway. It has been reported that galactosylation impacts positively on the ability of Rituximab to lyse CD20 expressing cells (*25*); however, to date, this appears to be a solitary experience.

Following an earlier paper suggesting that antibodies with oligosaccharides bearing a bisecting *N*-acetylglucosamine (peaks M, N, O, P; Figure 3) are more efficient at recruiting ADCC, the company Glycart generated a

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp040016j by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

CHO cell line transfected with the GNTIII enzyme and produced an anti-neuroblastoma antibody product bearing bisecting GlcNAc residues that exhibited a 15- to 20-fold improvement in ADCC (*26*). A similar improvement was reported by IDEC for the rMAb Rituximab that is licensed for the treatment of non-Hodgkin's lymphoma and is being developed for other indications (*27*). It is thought that the efficacy of Rituximab is determined by antibody sensitization of CD20 expressing malignant B cells and inducing killing by ADCC and/or CDC. The density of the CD20 antigen on the B cell surface is probably an important parameter and a paucity of expression may be linked with lack of clinical response. If the increased efficacy for in vitro killing of B cells observed for the Rituximab glycoform bearing bisecting *N*-acetylglucosamine translates to in vivo, substantial gains may be anticipated and tumor cells with a relatively low level of CD20 expression may be killed, resulting in higher clinical response rates. Similarly, clinical responses for tumors with a high level of CD20 expression may be achieved with reduced doses of the therapeutic.

Further exploration of the influence of rMAb glycoform on effector functions was reported from Genentech. A mutant CHO cell line (LEC 13) was employed that does not add fucose to the primary *N*-acetylglucosamine residue to produce nonfucosylated glycoforms of Herceptin. They report a 40- to 50-fold increase in the efficacy of FcγRIII-mediated ADCC and some improvement in binding to certain polymorphic forms of FcγRII but no effect on binding to FcγRI or C1q (*28*); the LEC 13 cell line was reported not to be suitable for development as a production vehicle. These findings provide an obvious incentive to generate a new production cell line by knockout of the appropriate fucosyltransferase. A similar improvement in ADCC was reported for the nonfucosylated fraction of a recombinant anti-human IL-5 receptor (rhIL-5-R) antibody (*29*) produced in the rat-derived YB2/0 cell line. This cell line had previously been reported to express the GNTIII enzyme and to produce antibody expressing bisecting *N*-acetylglucosamine residues, which appeared to correlate with improved ADCC. Physical separation of the nonfucosylated and bisecting *N*-acetylglucosamine glycoforms suggested that it was the absence of fucose rather than the presence of bisecting *N*-acetylglucosamine that resulted in enhanced ADCC (*29*). The separation protocol has been applied to the isolation of nonfucosylated Rituximab produced in the YB2/0 cell line, and improved ADCC has been correlated with increased affinity of the antibody for the FcγRIII receptor (*30*). It is claimed that the protocol can be scaled up, and fractionation of customer products is offered through the company Biowa.

IgG Glycoforms and Fc Effector Functions. It is established that glycosylation of the IgG-Fc is essential for optimal expression of biological activities mediated through FcγRI, FcγRII, FcγRIII, and the C1q component of complement. Present evidence suggests that it does not influence interactions with FcRn and consequently, presumably, the catabolic half-life or transport across the placenta. Bacterial IgG-Fc binding proteins, e.g., SpA, SpG are also unaffected (*1*−*3*). The association constant of aglycosylated IgG1 or IgG3 binding for FcγRI is reduced by 2 orders of magnitude, relative to that observed for the normally glycosylated form. However, we have shown that aglycosylated IgG3 antibody can mediate ADCC if a high level of target cell sensitization is achieved; cellular activation through FcγRII and FcγRIII appears to be completely ablated (*1*, *2*). The association constant for C1q binding to aglycosylated IgG is also reduced by 1 order of magnitude and results in a complete loss of CDC (*31*). Protein engineering, employing alanine scanning, has been used to "map" amino acid residues deemed to be critical for FcγR and C1q binding. These studies "map" the binding site for all four of these ligands to the hinge proximal or lower hinge region of the C_H2 domain (*31*−*33*). Formal proof for FcγRIII has been obtained recently through X-ray crystallographic analysis of IgG-Fc in complex with soluble recombinant forms of FcγRIII (*34*, *35*). Interestingly, one structure reveals a possible contribution of the primary *N*-acetylglucosamine residue to binding while the other structure holds that there is no direct contact. Clearly, any contribution is minimal, and thus the oligosaccharide contributes indirectly to the binding of these ligands.

The X-ray crystallographic analysis of the IgG-Fc fragment, residues 216−446, reveals electron density for residues 238−443 only (*4*); thus the lower hinge region is assumed to be mobile and without defined structure. This might appear to be incompatible with the suggestion that the lower hinge region is directly involved in the generation of structurally distinct interaction sites for FcγR and C1q ligands. However, we have proposed that this region of the molecule is not without structure but is composed of multiple conformers in equilibrium, resulting from reciprocal interactions between the oligosaccharide and the protein moiety with individual conformers being compatible with specific ligand recognition (*1*−*3*, *41*). In the absence of the oligosaccharide a different set of conformers will be generated that are not compatible with ligand binding. The complex of IgG-Fc with FcγRIII supports this thesis since the interaction site on the IgG-Fc is seen to include asymmetric binding to discrete conformations of the lower hinge residues of each heavy chain. Other residues of the hinge proximal region of the C_H2 domain also form contacts with the FcγRIII. Another critical requirement is explained by this structure, i.e., that the IgG-Fc is univalent for the FcγR. This is essential because if monomeric IgG were divalent, it could crosslink cellular receptors and hence constantly activate inflammatory reactions. X-ray crystal structures of IgG-Fc in complex with SpA, FcRn, SpG, and the autoantibody rheumatoid factor show that each of these ligands interacts with sites embracing residues of both the C_H2 and C_H3 domain at their junction (*4*, *36*−*38*) and the IgG-Fc is divalent for these ligands. It is evident, therefore, that the distribution of effector ligand binding and activation sites on IgG-Fc results from evolutionary selection for valency, among other properties.

Currently there are only two functional activities for which direct binding to the oligosaccharide is established, the mannan binding ligand (MBL) and the cellular mannose receptor (MR) (*39*, *40*). Each of these lectins recognize arrays of sugar residues that may be presented on the surface of microorganisms, and they form a link between the innate and adaptive immune response. The sugar residues recognized include *N*-acetylglucosamine, and there is evidence that immune complexes of G0F IgG can present arrays that bind and activate these lectins. Such activation may be presumed to be beneficial; however, there is evidence that immune complexes composed predominantly of G0 IgG can promote the adaptive immune response by uptake through the mannose receptor expressed on dendritic cells (*40*). There is a possible downside to this activity for G0/G0F glycoforms of chimeric rMAb since they express mouse V region sequences and are potentially immunogenic. The development of human antibody responses to human−mouse

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp0400164 by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

chimeric antibodies (HAMA) and humanized or human (HAHA) has been reported for a variable proportion of patients receiving rMAb therapeutics.

Glycosylation Engineering. It is evident that there could be advantages to being able to produce selected homogeneous glycoforms of recombinant antibody molecules. Manipulation of culture conditions appears to have limited potential, contributing only to minor changes in the proportions of the glycoforms produced. Cell engineering is being undertaken in order to knock-out and/or knock-in genes encoding for selected glycosyltransferases, as illustrated above. Subtle structural parameters also influence the glycosylation profile. Thus, in our alanine scanning studies we showed that single amino acid replacements could result in gross changes in the glycoform profile of product, resulting in increased galactosylation and sialylation (*31*). Similarly, radical differences in glycoform profile were observed for a series of truncated IgG molecules (*41*). It is also possible to modify the glycoform profile in vitro using glycosidases or glycosyltransferases and activated sugar precursors. We used this approach to determine the minimal oligosaccharide structure that could provide both structural stability and biological function for IgG-Fc (*5*−*7*). The study showed that the initial GlcNAc-GlcNAc-Man trisaccharide conferred significant stability and activity in comparison with the aglycosylated form. There is promise that similar in vitro modifications to the glycoform profile could be economic on a commercial scale.

In summary, the presence of "core" oligosaccharide is essential for the expression of IgG-Fc effector functions, and the addition of outer arm sugar residues has a variable influence of the efficacy of specific functions. Thus, glycosylation offers a challenge and opportunity to the biopharmaceutical industry in producing improved and consistent product.

References and Notes

(1) Jefferis, R.; Lund, J.; Pound, J. IgG-Fc mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol. Rev.* **1998**, *163*, 50−76.

(2) Jefferis, R.; Lund, J. Interaction sites on human IgG-Fc for Fc$_\gamma$R: current models. *Immunol. Lett.* **2002**, *82*, 57−65.

(3) Jefferis, R. Glycosylation of human IgG antibodies: relevance to therapeutic applications. *BioPharm* **2002**, *14*, 19−26.

(4) Deisenhofer, J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* **1981**, *20*, 0**,** 2361−2370.

(5) Mimura, Y.; Church, S.; Ghirlando, R.; Dong, S.; Goodall, M.; Lund, J.; Jefferis, R. The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol. Immunol.* **2000**, *37*, 697−706.

(6) Mimura, Y.; Sondermann, P.; Ghirlando, R.; Lund, J.; Young, S. P.; Goodall, M.; Jefferis, R. The role of oligosaccharide residues of IgG1-Fc in FcγIIb binding. *J. Biol. Chem.* **2001**, *276*, 45539−45547.

(7) Krapp, S.; Mimura, Y.; Jefferis, R.; Huber, R.; Sondermann, P. Structural analysis of human IgG glycoforms reveals a correlation between oligosaccharide content, structural integrity and Fcγ-receptor affinity. *J. Mol. Biol.* **2003**, *325*, 979−989.

(8) Takahashi, N.; Nakagawa, H.; Fujikawa, K.; Kawamura, Y.; Tomiya N. Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. *Ann. Biochem.* **1995**, *226*, 139−146.

(9) High, S.; Lecomte, F. J.; Russell, S. J.; Abell, B. M.; Oliver, J. D. Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett.* **2000**, *476*, 38−41.

(10) Jakob, C. A.; Burda, P.; Roth, J.; Aebi, M. Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. *Cell Biol.* **1998**, *142*, 1223−1233.

(11) Galili, U. The alpha-gal epitope (Gal alpha 1-3 Gal beta 1-4GlcNAc-R) in xenotransplantation. *Biochimie* **2001**, *83*, 557−563.

(12) Dor, FJ.; Alt, A.; Cooper, D. K. Gal alpha 1,3 Gal expression on porcine pancreatic islets, testis, spleen, and thymus. *Xenotransplantation* **2004**, *11*, 101−106.

(13) Cooper, D. K. Xenoantigens and xenoantibodies. *Xenotransplantation* **1998**, *5*, 6−17.

(14) Magnusson, S.; Mansson, J. E.; Strokan, V.; Jussila, R.; Kobayashi, T.; Rydberg, L.; Romano, E.; Breimer, M. E. Release of pig leukocytes during pig kidney perfusion and characterization of pig lymphocyte carbohydrate xenoantigens. *Xenotransplantation* **2003**, *10*, 432−445.

(15) Youings, A.; Chang, S. C.; Dwek, R. A.; Scragg, I. G. Site-specific glycosylation of human immunoglobulin G is altered in four rheumatoid arthritis patients. *Biochem. J.* **1996**, *314*, 621−30.

(16) Jefferis, R. Glycosylation of antibody molecules: functional significance. *Glycoconjugate J.* **1993**, *10*, 357−361.

(17) Dunn-Walters D.; Boursier L.; Spencer J. Effect of somatic hypermutation on potential N-glycosylation sites in human immunoglobulin heavy chain variable regions. *Mol. Immunol.* **2000**, *37*, 107−13.

(18) Zhu, D.; Ottensmeier, C. H.; Du, M. Q.; McArthy, H.; Stevenson, F. K. Incidence of potential glycosylation sites in immunoglobulin variable regions distinguishes between subsets of Burkitt's lymphoma and mucosa-associated lymphoid tissue lymphoma. *Br. J. Haematol.* **2003**, *120*, 217−22.

(19) Coloma, M. J.; Trinh, R. K.; Martinez, A. R.; Morrison, S. L. Position effects of variable region carbohydrate on the affinity and in vivo behavior of an anti-(1→6) dextran antibody. *J. Immunol.* **1999**, *162*, 2162−70.

(20) Gala, F. A.; Morrison, S. L. V region carbohydrate and antibody expression. *J. Immunol.* **2004**, *172*, 5489−94.

(21) Wright, A.; Morrison, S. L.; Kobata, A. Glycosylation of the variable region of immunoglobulin G−site specific maturation of the sugar chains. *Mol. Immunol.* **1995**, *32*, 931−40.

(22) Co, M. S.; Scheinberg, D. A.; Avdalovic, N. M.; McGraw, K.; Vasquez, M.; Caron, P. C.; Queen, C. Genetically engineered deglycosylation of the variable domain increases the affinity of an anti-CD33 monoclonal antibody. *Mol. Immunol.* **1993**, *30*, 1361−7.

(23) Fujimura, Y.; Tachibana, H.; Eto, N.; Yamada, K. Antigen binding of an ovomucoid-specific antibody is affected by a carbohydrate chain located on the light chain variable region. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2298−305.

(24) Leibiger, H.; Wustner, D.; Stigler, R. D.; Marx, U. Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. *Biochem. J.* **1999**, *338*, 529−538.

(25) http://www.fda.gov/cder/biologics/review/ritugen112697-r2.pdf

(26) Umana, P.; Jean-Mairet, J.; Moudry, R.; Amstutz, H.; Bailey, J. E. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **1999**, *17*, 176−180.

(27) Davies, J.; Jiang, L.; LaBarre, M. J.; Anderson, D.; Reff, M. Expression of GTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies of altered glycoforms leads to an increase in ADCC thro' higher affinity for FcRIII. *Biotechnol. Bioeng.* **2001**, *74*, 288−294.

(28) Shields, R. L.; Lai, J.; Keck, R.; O'Connell, L. Y.; Hong, K.; Meng, Y. G.; Weikert, S. H.; Presta, L. G. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J. Biol. Chem.* **2002**, *277*, 26733−40.

(29) Shinkawa, T.; Nakamura, K.; Yamane, N.; Shoji-Hosaka, E.; Kanda, Y.; Sakurada, M.; Uchida, K.; Anazawa, H.; Satoh, M.; Yamasaki, M.; Hanai, N.; Shitara, K. The absence of fucose but not the presence of galactose or bisecting *N*-acetylglucosamine of human IgG1 complex-type oligosaccha-

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp040016j by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

rides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem.* **2003**, *278*, 3466−3473.
(30) Okazaki, A.; Shoji-Hosaka, E.; Nakamura, K.; Wakitani, M.; Uchida, K.; Kakita, S.; Tsumoto, K.; Kumagai, I.; Shitara, K. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. *J. Mol. Biol.* **2004**, *336*, 1239−1249.
(31) Lund, J.; Takahashi, N.; Pound, J.; Goodall, M.; Jefferis, R. Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human FcγRI and influence the synthesis of its oligosaccharide chains. *J. Immunol.* **1996**, *157*, 4963−4969.
(32) Idusogie, E. E.; Presta, L. G.; Gazzano-Santoro, H.; Totpal, K.; Wong, P. Y.; Ultsch, M.; Meng, Y. G.; Mulkerrin, M. G. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J. Immunol.* **2000**, *164*, 4178−4184.
(33) Shields, R. L.; Lai, J.; Keck, R.; O'Connell, L. Y.; Hong, K.; Meng, Y. G.; Weikert, S. H.; Presta, L. G. High-resolution mapping of the binding site on human IgG1 for FcRI, FcRII, FcRIII, and FcRn and design of IgG1 variants with improved binding to the FcR. *J. Biol. Chem.* **2001**, *276*, 6591−6604.
(34) Sondermann, P.; Huber, R.; Oosthuizen, V.; Jacob, U. The 3.2-A crystal structure of the human IgG1 Fc fragment-FcγRIII complex. *Nature* **2000**, *406*, 267−273.
(35) Radaev, S.; Motyka, S.; Fridman, W. H.; Sautes-Fridman, C.; Sun, P. D. The structure of human type III Fcγ receptor in complex with Fc. *J. Biol. Chem.* **2001**, *276*, 16469−16477.
(36) Burmeister, W. P.; Huber, A. H.; Bjorkman, P. J. Crystal structure of the complex of rat neonatal Fc receptor with Fc. *Nature* **1994**, *372*, 379−383.
(37) Sauer-Eriksson, A. E.; Kleywegt, G. J.; Uhl, M.; Jones, T. A. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. *Structure* **1995**, *3*, 265−278.
(38) Corper, A. L.; Sohi, M. K.; Bonagura, V. R.; Steinitz, M.; Jefferis, R.; Feinstein, A.; Beale, D.; Taussig, M. J.; Sutton, B. J. Structure of human IgM rheumatoid factor Fab bound to its autoantigen IgG Fc reveals a novel topology of antibody−antigen interaction. *Nat. Struct. Biol.* **1997**, *4*, 374−381.
(39) Malhotra. R.; Wormald, M. R.; Rudd, P. M.; Fischer, P. B.; Dwek, R. A.; Sim R. B. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat. Med.* **1995**, *1*, 237−243.
(40) Dong, X.; Storkus, W. J.; Salter, R. D. Binding and uptake of agalactosyl IgG by mannose receptor on macrophages and dendritic cells. *J. Immunol.* **1999**, *163*, 5427−5434.
(41) Lund, J.; Takahashi, N.; Popplewell, A.; Goodall, M.; Pound, J.; Tyler, R.; King, D.; Jefferis, R. Expression and characterisation of truncated glycoforms of humanised L243 IgG1: architectural features can influence synthesis of its oligosaccharide chains and affect superoxide production triggered through human FcγRI. *Eur. J. Biochem.* **2000**, *267*, 7246−7257.

Accepted for publication November 8, 2004.

BP040016J

15206033, 2005, 1, Downloaded from https://aiche.onlinelibrary.wiley.com/doi/10.1021/bp040016j by NHMRC National Cochrane Australia, Wiley Online Library on [16/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License