Criteria for Selection of IgG Isotype and Glycoform of Antibody Therapeutics

Royston Jefferis

The efficacy of recombinant antibodies (rMAbs) results from their specificity for target antigens and the biological activities (effector functions) activated by the immune complexes formed. Numerous parameters influence the effector function profile (including antibody class, IgG subclass, and glycoform), and their definition offers opportunities for optimizing a recombinant MAb for a given disease indication. Five classes of human antibodies (immunoglobulins) are defined: IgM, IgG, IgA, IgD, and IgE; four subclasses of IgG and two of IgA are designated IgG1, IgG2, IgG3, or IgG4 and IgA1 or IgA2 according to their relative concentrations in serum.

Each antibody class and subclass expresses a unique profile of effector functions (**1**). All currently licensed rMAbs are of the IgG class, predominantly of the IgG1 subclass, and produced in Chinese hamster ovary (CHO), mouse NS0, or Sp2/0 plasma cell lines.

Product Focus: Protein therapeutics

Process Focus: Mammalian cell engineering

Who Should Read: Process development and manufacturing

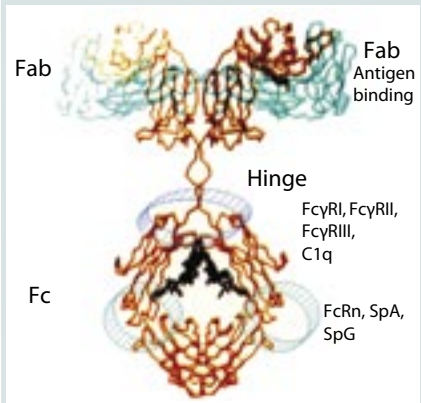
Keywords: Glycosylation, immunoglobulins, antibodies, MAbs, posttranslational modifications

Level: Intermediate

The choice of IgG subclass is increasingly appreciated as a critical decision when developing a recombinant MAb therapeutic (**2**). In oncology it would seem beneficial to maximize the potential to induce antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) to eliminate targeted cancer cells. However, in chronic diseases, neutralization of a soluble target (a cytokine, for example) may be the central objective, and excessive effector activity could be detrimental. One example is when a target is also expressed as a membrane protein on certain cells, such as targeting of TNFα by infliximab (Remicade, www.centocor.com) in patients with rheumatoid arthritis. Consequently, IgG1 may not be the automatic choice, and the other IgG subclasses are now being evaluated, both in native and engineered forms.

Some companies have selected IgG4 as the preferred alternative subclass, and two IgG4 rMAbs are already licensed. However, recent studies suggest that IgG4 may not be the best alternative because it has been shown to activate inflammatory reactions through cellular IgG-Fc receptors (**3–5**). It should be noted that the "superagonistic" anti-CD28 antibody TGN 1412, which appears to have induced a "cytokine storm" in healthy volunteers, is a humanized IgG4 recombinant antibody (**6, 7**). In addition IgG4 is secreted as a mixture of covalent and noncovalently assembled molecules; the latter dissociate to "half molecules" in nonreducing SDS gel electrophoresis (**5, 8**).

Figure 1: The alpha backbone structure of human IgG indicating the approximate location of interaction site for antigen and effector ligands; light chain = green, heavy chains = orange



The proportions of each molecular species vary between cell lines and clones, posing a problem for product consistency. A preferred alternative may be the IgG2 subclass because it is very stable, exhibits a minimum of IgG-Fc effector activities, and has a long serum half-life. Aglycosylated IgG may also be explored because it also exhibits minimal effector activity and retains the long catabolic half-life of glycosylated forms.

The Structure of Human IgG Antibodies

The basic structure of an IgG molecule is of two light and two heavy chains (H2L2) in covalent and noncovalent association to form three independent protein moieties connected through a flexible linker (the hinge region, Figure

1). In an individual antibody molecule, two of these moieties, Fab regions, are identical in 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, and FcγRIII), the C1q complement component, and the neonatal Fc receptor (FcRn) (**1, 8, 9**). The Fc receptors are expressed constitutively but differentially on leukocytes, and their engagement results in cellular activation and mediation of ADCC, phagocytosis, enzyme release, and so on (**10**).

Engagement and activation of the C1q molecule of the complement C1 component results in the classical complement cascade, culminating in CDC and generation of inflammatory and immunoregulatory molecules. The FcRn receptor controls the catabolic half-life of the IgG molecule and has a role in placental transport from mother to fetus (**9, 11**). The four subclasses of human IgG are structurally homologous (>95% primary sequence homology), but each has a unique effector function profile, Table 1.

Table 1: The ligand binding and activation properties of the human IgG

Isotype	IgG1	IgG2	IgG3	IgG4
C1[1]	++	–	+++	–
FcγRI	+++	–	+++	++
FcγRII	+	±[2]	+	?
FcγRIIIa/b	+	–	+	±[2, 3]
FcRn	+	+	+	+

[1] Immune complexes may also activate the alternative pathway

[2] Dependent on FcgR polymorphisms

[3] Dependent on glycoform

IgG-Fc Glycosylation

The IgG-Fc region is a homodimer comprising covalent inter–heavy-chain disulphide-bonded hinge regions and noncovalently paired CH3 domains. The CH2 domains are not paired, and the exposed hydrophobic surface is "overlaid" by oligosaccharide moieties covalently attached at asparagine 297 (Asn-297). X-ray crystallographic analysis reveals discreet structure for the oligosaccharide that is integral to the IgG-Fc structure because of multiple noncovalent interactions of the oligosaccharide with the protein surface. Thus the protein and oligosaccharide exert reciprocal influences on each other's conformation (**12**). Effector mechanisms mediated through FcγRI, FcγRII, FcγRIII, and C1q are severely compromised or ablated for aglycosylated or deglycosylated forms of IgG (**8**).

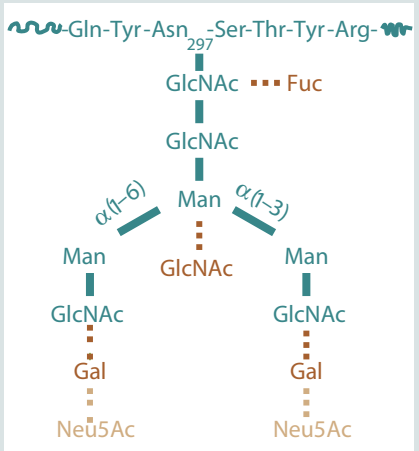
The oligosaccharide of normal polyclonal human IgG-Fc is of the diantennary complex type and shows considerable heterogeneity. A "core" heptasaccharide can be defined with variable addition of outer arm sugar residues (Figure 2). Analysis shows a paucity of sialylation (<10%): Only twelve of the possible sixteen neutral oligosaccharides are present (Figure 3), providing the potential to generate a total of 72 glycoforms. Because glycosylation is essential for expression of effector functions, efficacy may vary between glycoforms.

The IgG-Fc glycoform profile of currently licensed therapeutic rMAbs (produced in CHO, NS0, or Sp2/0 cells) predominantly comprises core fucosylated structures with low levels of galactosylation. These cell lines do not express the GnTIII transferase that adds the bisecting *N*-acetylglucosamine sugar residue. Although these antibodies are functionally active, it has been shown that effector activities can be enhanced by manipulation of antibody glycoform through cell engineering (**8, 13–17**).

IgG Glycoforms and Fc Effector Functions

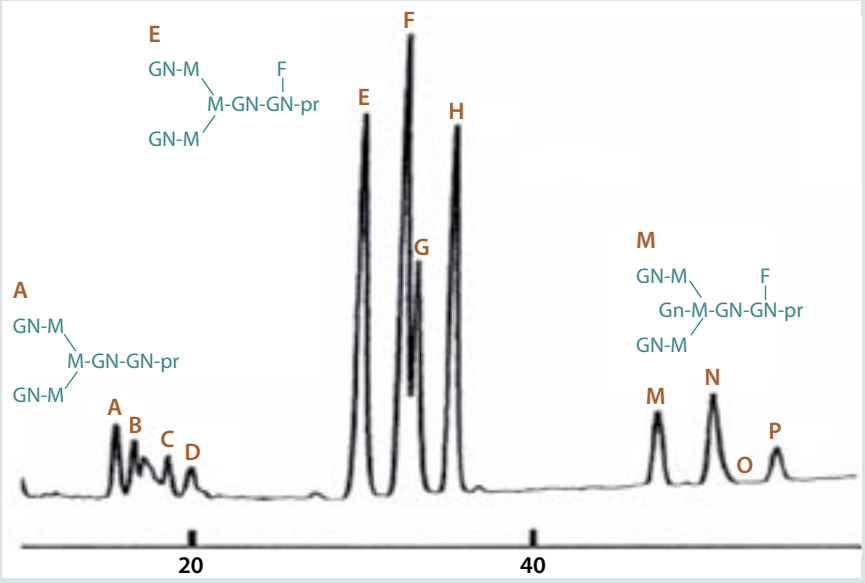
IgG-Fc glycosylation is therefore essential for expression of biological activities mediated through FcγRI, FcγRII, FcγRIII, and the C1q complement component. But present evidence suggests that it does not influence interactions with FcRn and, consequently, the catabolic half-life or interactions with bacterial IgG-Fc binding proteins such as SpA and SpG (**1, 8, 9**). The influence on placental transport has not been reported, but evidence suggests that galactose-bearing IgG-Fc oligosaccharides enhance placental transport (**11**). Interaction sites for FcγRI, FcγRII, FcγRIII, and C1q have been "mapped" to the hinge proximal and lower hinge region of the CH2 domain (Figure 1) (**8**). Cumulative evidence suggests that interaction sites on IgG-Fc for FcγR and C1q effector ligands contain the protein moiety only; however, the required protein conformation depends on the presence of the oligosaccharide (**8, 18, 19**).

Figure 2: The structures of the possible diantennary oligosaccharide structure attached to IgG-Fc at asparagine 297 (Asn297). The "core" heptasaccharide present in normal human IgG is shown in blue. Additional sugar residues that may be attached to the "core" are shown in red; GlcNAc = *N*-acetylglucosamine; Man = mannose; Fuc = fucose; Gal = galactose; Neu5Ac = *N*-acetylneuraminic acid.



Some evidence points to NK (natural killer) cell–mediated ADCC as a major mechanism recruited to the killing of cancer cells by rMAb (**2**). Because NK cells express only FcγRIII, it would seem that maximizing this activity might enhance ADCC and hence therapeutic efficacy. NK-cell activity can be enhanced by orders of magnitude for IgG1 rMAb bearing nonfucosylated oligosaccharides. This has been achieved by transfecting CHO cells with the GnTIII transferase that adds bisecting N-acetylglucosamine to the oligosaccharide, consequently inhibiting addition of fucose; or by the alternative strategy of "knocking-out" the α1-6 fucosyl transferase from CHO cells (**13–17**). Importantly, it has been shown that FcγRIII- mediated ADCC is enhanced for nonfucosylated forms of IgG3 and IgG4 also; at high levels of target cell sensitization, some activity for IgG2 was observed (**3**). We wait to see whether the

Figure 3: An HPLC profile of the neutral oligosaccharides released from normal human IgG-Fc; peak A represents the basic core oligosaccharide structure; peak B has galactose on the 1–6 arm, peak C has galactose on the 1–3 arm, peak D has galactose on both harms; the same pattern is repeated with fucose in peaks F–H, after peak E showing the core with galactose; peak M is the core with fucose and bisecting GlcNAc, then peaks N–P follow the same pattern as B–D and F–H

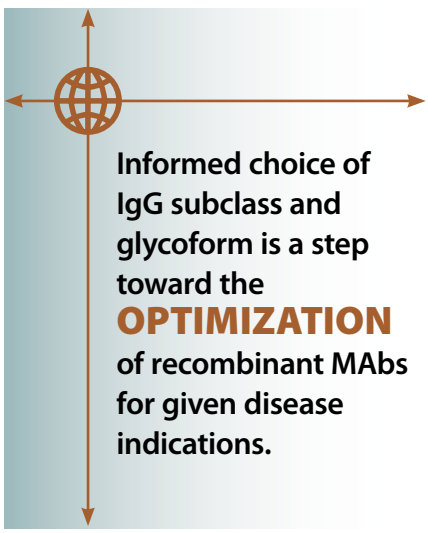


dramatic impact on ADCC revealed by in vitro assays translates into in vivo therapeutic benefit.

Influence of Production Vehicle on Product Fidelity

Production vehicles and processes affect glycosylation and other posttranslational modifications (PTMs) of protein therapeutics (**20**). The ideal is for a recombinant product to have structural fidelity with the "natural" protein. One particular concern is that proteins lacking such structural fidelity may provoke an immune response that affects therapeutic efficacy and may precipitate adverse reactions.

It is important to recognize, however, that the structure assigned to a "natural" protein is determined for molecules that have been subject to residence in body fluid(s) before isolation and purification using multiple protocols. Similarly, recombinant proteins are synthesized in an "alien" tissue (such as CHO or NS0 cells), exposed to a culture medium and products of a producer cell line, and subjected to rigorous downstream processes. One example is production of an endogenous carboxypeptidase by CHO, NS0, and Sp2/0 cell lines. This results in cleavage of C-terminal lysine residues from antibody heavy chains generating structural and charge heterogeneity. Cell culture conditions may be manipulated to influence productivity and PTMs — conditions such as temperature, growth rate, and media composition.



Considerable success has been reported for increased productivity of antibodies in CHO cell lines by repeated rounds of clonal selection, achieving levels of 5 g/L and setting 10 g/L as a goal (**21**). However, high production levels may compromise the PTM machinery to yield poor product quality. It is therefore essential to apply qualitative as well as quantitative criteria at an early stage in clone selection. Downstream processing also can compromise product fidelity if it allows selective purification or enrichment of PTM products.

Engineering of mammalian cells to maximize production of selected antibody glycoforms has been referred to above. Similarly, yeast (*Pichia pastoris*) has been engineered to produce selected antibody glycoforms (**22**), and an aquatic plant (*Lemna*) has been engineered to produce selected, homogeneous glycoforms of antibody products while simultaneously suppressing expression of glycosyl transferases that add the immunogenic α1-3 fucose and β1-3 xylose sugars (**23**). Production in prokaryotic systems such as *Escherichia coli* result in production of an aglycosylated protein; however, an N-linked glycosylation machinery is expressed in some bacteria species (e.g., *Campylobacter jejuni*), and this machinery has been successfully transferred and expressed in *E. coli* (**24**).

In summary, the presence of "core" oligosaccharide is essential for expression of IgG-Fc effector functions, and addition of outer arm sugar residues has a variable influence on efficacy of specific functions. Thus, informed choice of IgG subclass and glycoform is a step toward optimization of rMAb for given disease indications. It is important to note that the generalizations given here are for guidance only. You need to consider each antibody molecule, its target, and the disease indication case-by-case. Control of glycosylation offers a challenge and opportunity to the biopharmaceutical industry for production of improved and consistent products.

References

1 Burton DR, Woof JM. Human Antibody Effector Function. *Advances in Immunology* (Vol. 51), Dixon F, Ed. Elsevier (Academic Press): Burlington, MA, 1992; 1–84.

2 Reff ME, Hariharan K, Braslawsky G. Future of Monoclonal Antibodies in the Treatment of Hematologic Malignancies. *Cancer Control* 9(2) 2002: 152–166.

3 Niwa R, et al. IgG Subclass-Independent Improvement of Antibody-Dependent Cellular Cytotoxicity By Fucose Removal from Asn297-Linked Oligosaccharides. *J. Immunol. Methods.* 306(1–2) 2005: 151–160.

4 Holland M, et al. Anti-Neutrophil Cytoplasm Antibody IgG Subclasses in Wegener's Granulomatosis: A Possible Pathogenic Role for the IgG4 Subclass. *Clin. Exp. Immunol.* 138(1) 2004: 183–192.

5 Aalberse RC, Schuurman J. IgG4 Breaking the Rules. *Immunology* 105(1) 2002: 9–19.

6 TeGenero AG (Würzburg, Germany). I*nvestigator's Brochure: TGN1412 Humanized Agonistic Anti-CD28 Monoclonal Antibody* (19 December 2005); www.circare.org/foia5/tgn1412investigatorbrochure.pdf.

7 Urgent Changes Needed for Authorisation of Phase 1 Trials (Editorial). *The Lancet* 367(9518) 2006: 1214.

8 Jefferis R. Glycosylation of Recombinant Antibody Therapeutics. *Biotechnol. Prog.* 21(1) 2005: 11–16.

9 Nezlin R, Ghetie V. Interactions of Immunoglobulins Outside the Antigen-Combining Site. *Advances in Immunology* (Volume 82), Alt F, Ed. Elsevier (Academic Press): Burlington, MA, 2004: 155–215.

10 Nimmerjahn F, Ravetch JV. Fc Gamma Receptors: Old Friends and New Family Members. *Immunity* 24(1) 2006: 19–28.

11 Kibe T, et al. Glycosylation and Placental Transport of Immunoglobulin G. *J. Clin. Biochem. Nutr.* 21(1) 1996: 57–63.

12 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* 20(9) 1981: 2361–2370.

13 Umana P, et al. Engineered Glycoforms of an Antineuroblastoma IgG1 with Optimized Antibody-Dependent Cellular Cytotoxic Activity. *Nat. Biotechnol.* 17(2) 1999: 176–180.

14 Davies J, et al. Expression of GnTIII in a Recombinant Anti-CD20 CHO Production Cell Line: Expression of Antibodies of Altered Glycoforms Leads to an Increase in ADCC Through Higher Affinity for Fc Gamma RIII. *Biotech. Bioeng.* 74(4) 2001: 288–294.

15 Shields RL, et al. Lack of Fucose on Human IgG1 N-Linked Oligosaccharide Improves Binding to Human Fcgamma RIII and Antibody-Dependent Cellular Toxicity. *J. Biol. Chem.* 277(30) 2002: 26733–26740.

16 Shinkawa T, et al. The Absence of Fucose but Not the Presence of Galactose or Bisecting *N*-Acetylglucosamine of Human IgG1 Complex-Type Oligosaccharides Shows the Critical Role of Enhancing Antibody-Dependent Cellular Cytotoxicity. *J. Biol. Chem.* 278(5) 2003: 3466–3473.

17 Ferrara C, et al. Modulation of Therapeutic Antibody Effector Functions By Glycosylation Engineering: Influence of Golgi Enzyme Localization Domain and Co-Expression of Heterologous Beta1, 4-*N*-Acetyl glucosaminyltransferase III and Golgi Alpha-Mannosidase II. *Biotechnol. Bioeng.* 93(5) 2006: 851–861.

18 Sondermann P, et al. The 3.2-A Crystal Structure of the Human IgG1 Fc Fragment-FcγRIII Complex. *Nature* 406(6973) 2000: 267–273.

19 Radaev S, et al. The Structure of Human Type III Fcγ Receptor in Complex with Fc. *J. Biol. Chem.* 276(1) 2001: 6469–16477.

20 Walsh G, Jefferis R. Post-Translational Modifications in the Context of Therapeutic Proteins. *Nat. Biotech.* (2006). In press.

21 Birch J, Racher AJ. Antibody Production. Adv. Drug Delivery Rev. 58(5–6) 2006: 671–685.

22 Hamilton SR, et al. Humanization of Yeast to Produce Complex Terminally Sialylated Glycoproteins. *Science* 313(5792) 2006: 1441–1443.

23 Stomp AM. The Duckweeds: A Valuable Plant for Biomanufacturing. *Biotechnology Annual Review* (Volume 11), El-Gewely MR, Ed. Elsevier BV: Burlington, MA, 2005; 69–99.

24 Wacker M, et al. Substrate Specificity of Bacterial Oligosaccharyltransferase Suggests a Common Transfer Mechanism for the Bacterial and Eukaryotic Systems. *Proc. Natl. Acad. Sci. USA* 103(18) 2006: 7088–7093.

Royston Jefferis *is professor of molecular immunology with the Division of Immunity and Infection at the School of Medicine, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT UK; 44-121-414-4063, fax 44-121-414-6815, r.jefferis@bham.ac.uk.* *This article has been adapted with permission from an earlier version that appeared in a Touch Briefings report,* Bioprocessing and Biopartnering 2006*, www.touchbriefings.com/cdps/cditem.cfm?nid=1920&cid=5.*