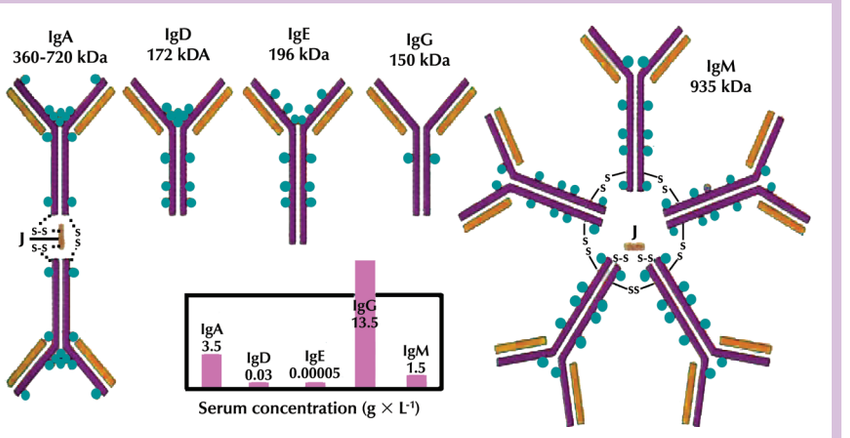
Glycosylation Variations with Expression Systems

and Their Impact on Biological Activity of Therapeutic Immunoglobulins

T. Shantha Raju

Immunoglobulins are highly soluble serum glycoproteins involved in the defense mechanism of the immune system. IgGs are the major components of the five different classes of immunoglobulins (namely IgA, IgD, IgE, IgG, and IgM; Figure 1 provides a schematic representation) (1). The serum concentration of IgG is up to 13.5 mg/mL (Figure 1). The four isotypes of IgGs are classified as IgG_1, IgG_2, IgG_3 and IgG_4 (Figure 2). These IgG isotypes have different numbers and arrangements of interchain disulfide bonds (2). In IgG_1 the disulfide bond linking the light and heavy chains goes to the hinge region (Figure 2), whereas in the IgG_2, IgG_3, and IgG_4 subclasses the disulfide bond linking the light and heavy chains goes to the junction between the variable and constant regions.

Figure 1: Classes of immunoglobulins and their concentrations in serum



PRODUCT FOCUS: ANTIBODIES

PROCESS FOCUS: CELL CULTURE, RECOVERY, ANALYSIS, RESEARCH

WHO SHOULD READ: SCIENTISTS, DIRECTORS, VPS, REGULATORS

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LEVEL: BASIC TO ADVANCED

Among the four isotypes, IgG_1 molecules exhibit predominant effector functions (Table 1) (3). The IgG_2, IgG_3, and IgG_4 isotypes contain very minimal effector functions. The N-linked oligosaccharides present in the constant CH_2 domain of the Fc have greater influence on the effector functions of IgG_1 molecules (Figure 3 shows domain structure). These N-linked oligosaccharides of IgGs are highly heterogeneous (also referred to as microheterogeneity) and vary considerably with the expression system (4).

Variations in conditions of production systems greatly influence the heterogeneity of IgG oligosaccharides (5). Thus, glycosylation of rIgGs varies from lot to lot and among different cell-culture conditions. These variations often affect the biological activities of therapeutic antibodies. This might lead to potency changes in drug substances and drug products. Because of this, regulatory agencies around the world are very vigilant about glycosylation variations of therapeutic antibodies. This article examines the issues related to glycosylation variations with particular emphasis on expression systems and their impact on the effector functions of therapeutic antibodies.

HUMAN IgG GLYCOSYLATION

N-Linked glycans of human IgG have been extensively characterized (6). These glycans are mainly complex biantennary structures with

Figure 2: Schematic representation of human IgG isotypes

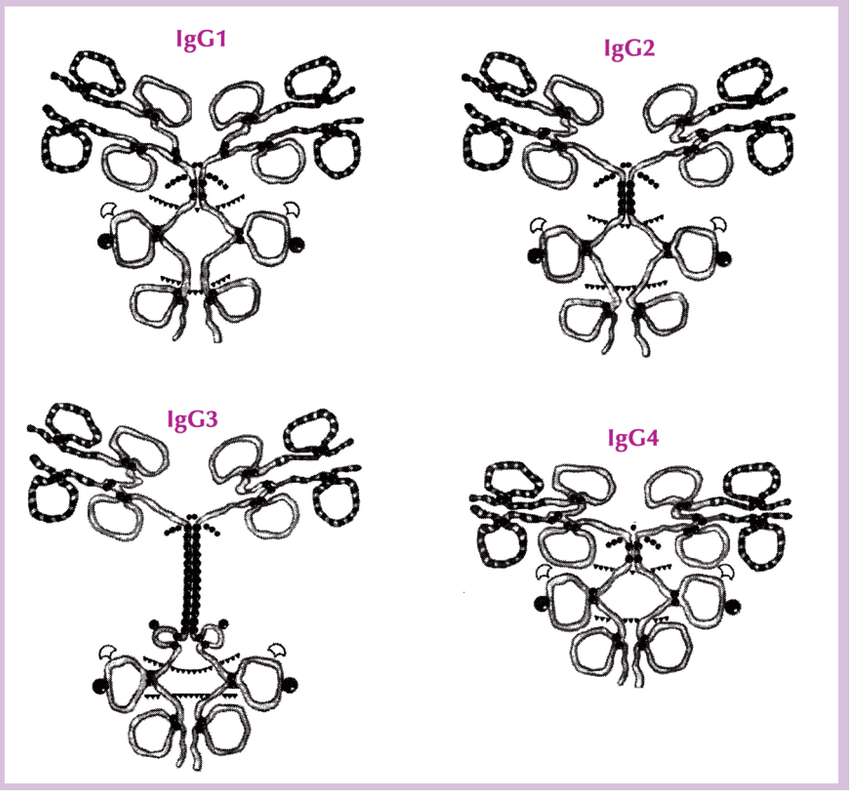


Table 1: Effector functions of human IgG isotypes

	IgG_1	IgG_2	IgG_4
Complement	++	+	–
FcγRI	+++	–	+
FcγRII	+++	+	+
FcγRIII	++	–	–

Note: Receptors for FcγRI, FcγRII, and FcγRIII located on human monocytes, macrophages, and polymorphonuclear cells

core fucose and often terminated with sialic acid residues. Figure 4 shows the largest N-linked oligosaccharide structure found in human IgG. This structure is present as a very minor component. The majority of oligosaccharides found in human IgG are truncated versions of the structure shown in Figure 4 due to the microheterogeneity of glycans. The microheterogeneity of human IgG glycans is mainly due to the presence and/or the absence of either core fucose, bisecting GlcNAc, terminal sialic acid, exposed Gal, and often the exposed GlcNAc residues (in either the α1,6- or α1,3-branch; Figures 4 and 5 show the structures of human IgG glycans). Unlike other serum glycoproteins in which sialic acid residues are mainly α2,3-linked to penultimate Gal residues of N-glycans, in human IgG the sialic acid residues are reported to be mainly α2,6-linked to the penultimate Gal residues of N-glycans (**7**).

The microheterogeneity of human IgG glycans varies with age and gender and is often indicative of disease status (**8**). For example, IgGs from rheumatoid arthritis patients contain more nongalactosylated oligosaccharides (G0) than do those from nonarthritic humans (**9**). The microheterogeneity of human IgG glycans reportedly affects biological functions such as complement-dependent cytotoxicity (CDC), antibody-dependent cytotoxicity (ADCC), binding to various Fc receptors, and binding to C1q protein (**10**). Further, nongalactosylated antibodies such as G0 glycoforms have been reported to bind to mannose binding protein present in serum and activate the alternative complement cascade (**11**). Also, terminal Gal, bisecting GlcNAc, and core fucose residues affect the effector functions of therapeutic antibodies (**12, 13**). Hence, producing properly glycosylated antibodies for therapeutic purposes seems to be very important.

Recombinant IgG Glycosylation

About 11 rIgGs produced by recombinant DNA technology are currently being marketed as human therapeutics to treat life-threatening diseases. Table 2 lists different IgGs and their indications. Additionally, more than 200 rIgGs are in various phases of human clinical trials for development as human therapeutics. Biotechnology companies are using different methods of production to make rIgG molecules. Most of the currently marketed rIgGs are being produced using in vitro cell culture methods.

Because the demand for recombinant antibodies far exceeds the current in vitro cell culture production capacity, alternative methods to producing antibodies are being explored. These alternative methods include producing the antibodies using transgenic mammals, plants, or avian eggs and also amplifying the cell lines to improve their yields. Several antibodies have been successfully produced in transgenic goats, sheep, cows, and also in plants such as corn, alfalfa, and tobacco. Additionally, rIgGs have been successfully produced in avian eggs. For in vitro cell culture production, some cell lines have been amplified and/or cell culture conditions manipulated to produce up to 4–6g of antibody per liter of culture medium.

Although the integrity of polypeptide chains seems to be largely unchanged in the various expression systems and different culture conditions, significant changes in glycosylation have been noticed. Glycosylation varies with cell line and animal species. Glycosylation of antibodies also varies with culture conditions (**14**). Hence, glycosylation of antibodies expressed under different cell

Figure 3: Domain structure of IgG molecule

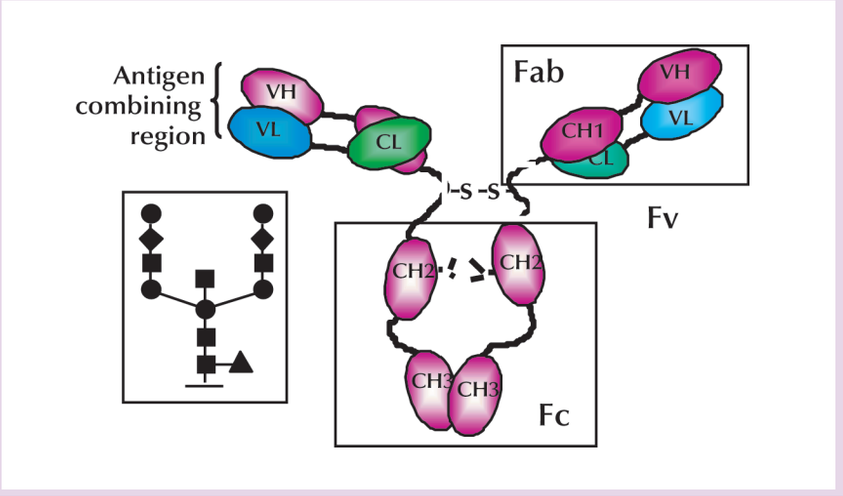


Table 2: FDA-approved recombinant monoclonal antibodies in the market to treat human diseases

Type	Product	Marketer	Approved
Murine	**Orthoclone OKT3** allograft rejection	Johnson & Johnson	June 1986
Chimeric	**ReoPro** PTCA adjunct	Lilly	December 1994
Chimeric	**Rituxan** non-Hodgkin's lymphoma	Genentech	November 1997
Chimeric	**Simulect** organ rejection prophylaxis	Novartis	May 1998
Chimeric	**Remicade** rheumatoid arthritis, Crohn's disease	Johnson & Johnson	August 1998
CDR-grafted	**Zenapax** organ rejection prophylaxis	Roche	December 1997
CDR-grafted	**Synagis** respiratory syncytial virus (RSV)	Medimmune	June 1998
CDR-grafted	**Herceptin** metastatic breast cancer	Genentech	September 1998
CDR-grafted	**Mylotarg** acute myeloid leukemia	American Home Products	May 2000
CDR-grafted	**Campath** chronic lymphocytic leukemia	Millennium	July 2001
Human	**Humira** disease modifying antirheumatic drugs (DMARDs)	Abbott Laboratories	December 2002

culture conditions will vary from batch to batch. Because it varies from species to species, the glycosylation of antibodies produced in transgenic animals or plants or in avian eggs will be significantly different from the glycosylation of antibodies produced using in vitro cell culture processes (**4–6, 15**). Depending on the mechanism of action for each rIgG, these variations will affect product quality as well as product potency.

Regulatory agencies are paying close attention to glycosylation variations and their impact on product quality. It is therefore advisable to address these issues at the early phases of development. It is also recommended that the impact of glycosylation on a particular antibody product needs to be addressed at the proof-of-concept stage. Specific differences in glycosylation for different animal species and under varied cell culture conditions are detailed below.

Changes Due to Variations in Cell Culture Conditions

Many marketed recombinant antibodies are being produced either using Chinese hamster ovary (CHO) cells or mouse-derived cell lines such as NS0s or hybridomas as host cells. The glycosylation machinery of CHO cells is almost similar to the human glycosylation machinery (**16**) – with several minor differences. Notable among these differences is that normal CHO cells do not contain N-acetylglucosaminyltransferase-III (GnTIII), an enzyme that mediates the transfer of bisecting GlcNAc (Figure 4) to complex N-glycans (**17**). However, the amount of bisecting GlcNAc-containing oligosaccharides in human IgG is <10% (**4**). Hence the absence of bisecting GlcNAc-containing oligosaccharides in antibodies produced using CHO cells as hosts may not significantly affect product quality and bioactivity.

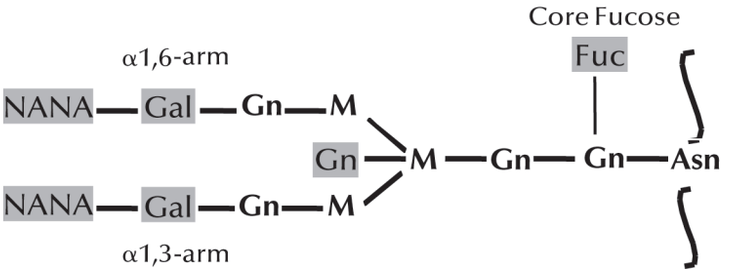
Another difference between CHO cell derived IgG and human IgG is in the nature of sialic acid linkages. It has been reported that human IgG contain α2,6-linked sialic acid residues whereas the CHO cell derived antibodies contain α2,3-linked sialic acid residues (**18**). However, the amount of sialylated oligosaccharides in both human and CHO cell derived antibodies is very small (<5% of glycans contain sialic acid residues) (**4, 19**). So this difference in sialic acid linkages may not affect product quality. Moreover, the impact of sialic acid residues on the antibody function is not yet fully understood. Further, many of the human serum glycoproteins such as Fetuin and α1-acid glycoprotein contain α2,3-linked sialic acid residues. Because of this, regulatory agencies may not be very concerned about the linkage difference in sialic acid residues between human and CHO cell derived antibodies. In addition to these variations, there are several

differences in O-glycosylation of human and CHO cell derived glycoproteins. Most of the antibodies on the market and in clinical trials do not seem to contain O-linked oligosaccharides. Hence, differences in O-glycosylation between human and CHO cells are beyond the scope of this article.

Antibodies produced in CHO cells mainly contain core fucosylated biantennary complex oligosaccharides terminated with 0, 1, or 2 Gal residues (Figure 5) (**15**). These are commonly designated as G0, G1, and G2 structures. The relative proportions of G0, G1, and G2 oligosaccharides vary from batch to batch and are dependent on cell culture conditions (**20, 21**). Depending on the mechanism of action for a given therapeutic antibody, variations in G0, G1, and G2 glycans affect product quality and bioactivity (**22**). For example, G2 glycan significantly improves the CDC activity of Rituxan but does not affect the antigen binding (**23**). Hence, these variations need to be monitored and often controlled using tightly regulated culture conditions. This is especially necessary for antibodies that require effector functions for their biological activity. However, for antibodies that bind only to the antigen, such variations in glycosylation may not affect product quality.

Glycosylation of cell lines derived from mouse and other mammals is also very similar to human glycosylation (**4, 19**). However, several significant differences might affect product quality as well as bioactivity. Most mouse-derived cell lines contain an additional glycosylation enzyme. The enzyme is referred as α1,3-galactosyltransferase; it mediates the transfer of Gal residues from UDP-Gal in α-configuration to the internal and/or exposed Gal residues (Figure 6). Humans have antibodies against the α-Gal epitopes. Although no evidence in the literature suggests that the presence of α-Gal epitopes on rIgG is immunogenic to humans, regulatory agencies might express concerns about α-Gal residue–containing therapeutic glycoproteins. Such concerns might be on a case-by-case basis.

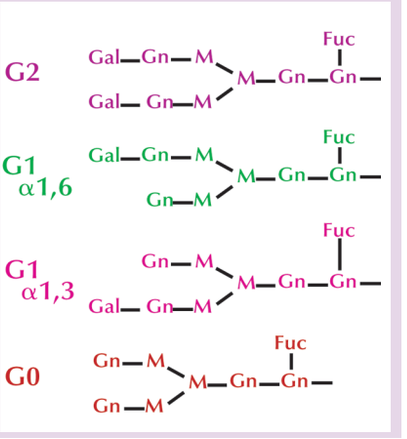
Figure 4: Largest oligosaccharide structure found in human IgG. Asn = asparagine; Gn = N-acetylglucosamine; Fuc = fucose; M = mannose; Gal = galactose; NANA = N-acetyl neuraminic acid



Another difference between glycoprotein glycans from humans and mouse cell lines is that the mouse cell lines produce N-glycolylneuraminic acid (NGNA) containing oligosaccharides in addition and/or instead of those N-acetylneuraminic acid (NANA). The difference between NANA and NGNA is the presence of only an additional oxygen atom in NGNA (Figure 7). However, glycoproteins containing NGNA residues have been attributed to be immunogenic to humans. Although this is yet to be proved, regulatory agencies are concerned about NGNA-containing glycoprotein therapeutics. Although no published data is available to date on the immunogenicity of NGNA containing glycoproteins, it is speculated that some of the marketed glycoprotein therapeutics that have caused adverse reactions in patients contain NGNA residues. Such adverse reactions may not be due to the presence of NGNA, but even a single adverse reaction is enough to cause significant concerns by regulatory agencies.

In addition to the differences due to α-Gal and NGNA residues, mouse cell line derived antibodies often contain higher amounts of nonfucosylated oligosaccharides. More than 95% of the human and CHO cell derived antibody oligosaccharides contain core Fuc residues. However, about 10–40% of the mouse cell derived antibody glycans do not seem to contain core Fuc residues. Their absence in antibodies has been shown to significantly improve ADCC activity (**12**). Further, antibodies that do not contain core Fuc residues have been shown to bind better to various Fc receptors (**12**). This in turn might affect the quality and the bioactivity of therapeutic antibodies. Hence these issues need to be addressed at the earliest possible stages of antibody drug development. Despite all these variations in the glycosylation of mouse cell line derived antibodies, it should be noted that regulatory agencies have approved several therapeutic antibodies that are produced in mouse-derived cell lines for human therapy.

Figure 5: Structure of major N-linked oligosaccharides found in human IgG and rIgGs expressed in CHO cells



Antibodies Produced in Transgenic Animals

Intact antibodies have been produced using transgenic animals such as goats, sheep, pigs, and cows (**24, 25**). Although the glycosylation machinery of those animals is very similar to human glycosylation

Figure 6: Structure of G2 oligosaccharide containing α-Gal residues at the nonreducing termini

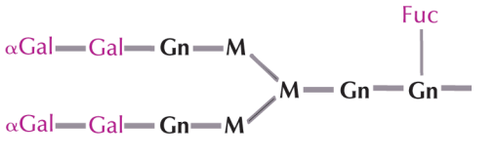


Figure 7: Structure of N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA)

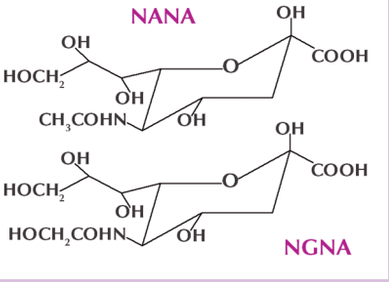


Figure 8: MALDI-TOF-MS analysis of neutral glycans of human and chicken IgG molecules

machinery, several significant differences do exist (**4, 19, 26**). These are very similar to the differences described above for the antibodies derived from mouse cell lines. Currently, several glycoproteins including antibodies that were produced in transgenic animals are undergoing human clinical trials. Genzyme Transgenics reported a successful completion of pivotal phase III trials of anti-thrombin-III that was produced in transgenic goat milk (**27**). However, the company did not follow it through by filing a biological license application to market the product. Despite a certain amount of skepticism in the industry, still, about the success of transgenic technology, there are several significant benefits of pursuing it to produce human therapeutics. Notable among these benefits are potential savings in cost of goods and services (COGS) and production of unlimited amounts of protein therapeutics using easily available resources. Additionally, it should be noted that no significant changes have been observed in the polypeptide chains of protein therapeutics produced using transgenic animals other than the differences in glycosylation (**28**).

In addition, efforts are under way to produce animals lacking the α-galactosyltransferase gene. Already Vanhoue et al. reported that the intracellular expression in pig cells of anti-α1,3galactosyltransferase single-chain FV antibodies reduces Gal α1,3Gal expression and inhibits cytotoxicity mediated by anti-Gal xenoantibodies (**29**). This type of technology development should advance the field significantly. Such advanced transgenic technology is not only useful to produce human therapeutics; it is also useful for human tissue grafting and organ transplantation. Hence it is very important to continue the research in this area to advance the biotechnology field.

Antibodies Produced in Avian Eggs

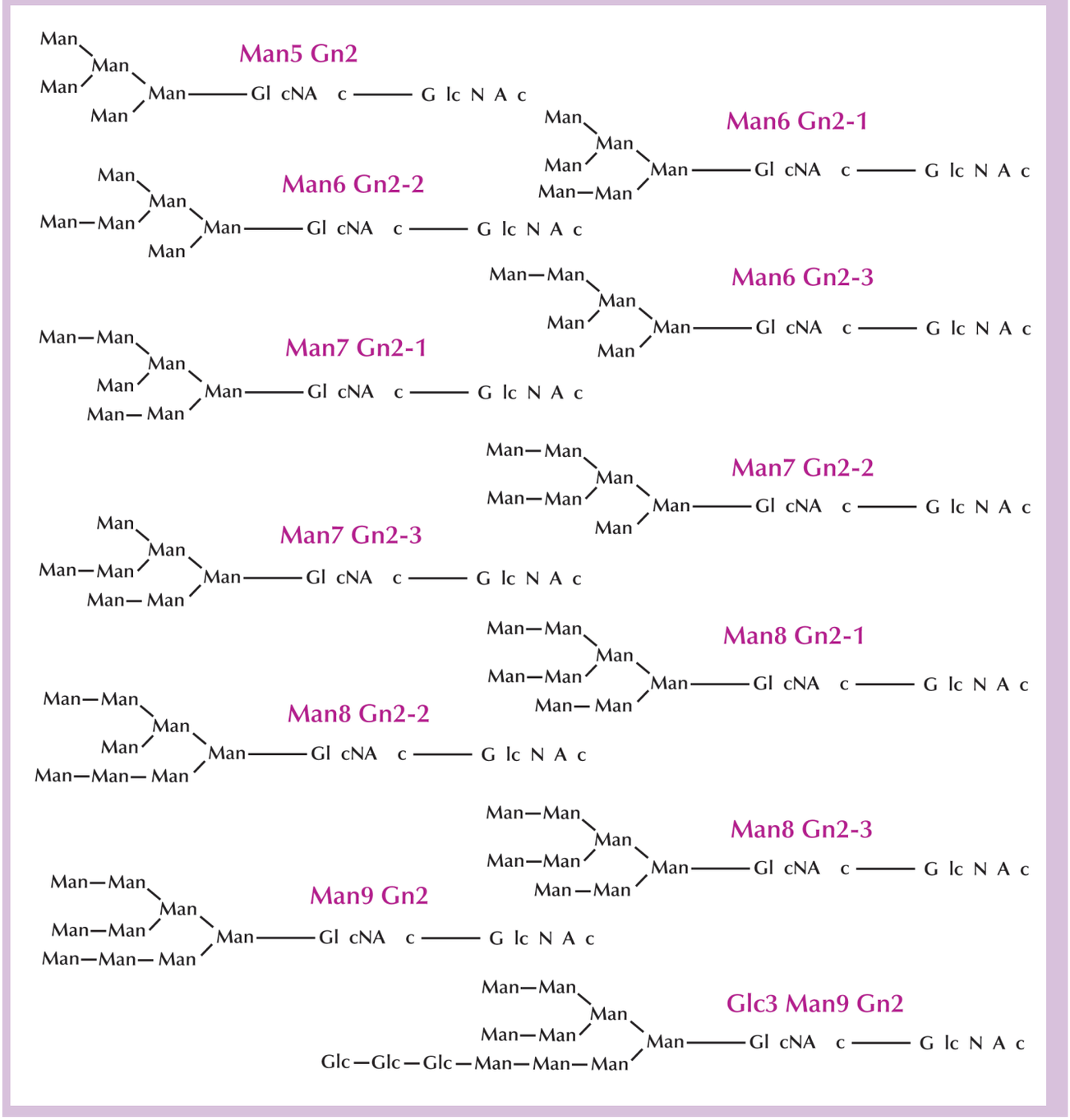
Therapeutic antibodies to treat human diseases have been successfully produced in avian eggs (**30**). However, to date no published glycosylation analysis data are available in the literature about the recombinant antibodies produced in avian eggs. Some information is available in the literature about the glycosylation of IgG purified from chicken serum (**4, 19, 30, 31**). We recently reported that chicken IgG contains a highly heterogeneous mixture of complex biantennary oligosaccharides as well as high mannose structures (**4**). Figure 8 shows a comparative analysis of chicken IgG neutral glycans with human IgG neutral glycans. Major glycan structures of chicken IgG were found to be Glc3Man9GlcNAc2 and a complex biantennary structure containing core Fuc, bisecting GlcNAc and terminated with Gal residues (see Figure 9 for structures of high mannose glycans and Figure 4 for the complex biantennary structure). This observation suggests that the chicken IgG contains both fully processed glycans (complex glycans) as well as unprocessed glycans (high mannose structures) in almost equal amounts. The glycosylation of rIgG produced in avian eggs might be very similar to that observed in chicken IgG.

It should be noted here that no potential immunogenic epitopes such as α-Gal and NGNA residues were observed in chicken IgG. All the sialylated oligosaccharides of chicken IgG contained only NANA; no NGNA-containing oligosaccharides were seen. It has been reported that the IgGs containing high mannose type oligosaccharides were cleared faster than those containing complex type oligosaccharides (**32–34**). Because chicken IgG contains significant amounts of high mannose type oligosaccharides (~50% glycans), the pharmacokinetic properties of avian egg derived rIgGs might be different from those of the rIgGs containing complex type oligosaccharides. This will be the case if the avian-egg–derived rIgGs contain glycans similar to the chicken IgG glycans. This might pose some hurdles during clinical trials as well as during product approval to market a drug. Hence, research studies should be continued to understand the physicochemical and biological properties of rIgGs produced in avian eggs.

Antibodies Produced in Transgenic Plants

Both glycosylated and nonglycosylated antibodies have been successfully produced in transgenic plants such as corn, alfalfa, and tobacco for therapeutic purposes (**35–41**). Plant glycosylation is somewhat different from human glycosylation, but the initial steps in

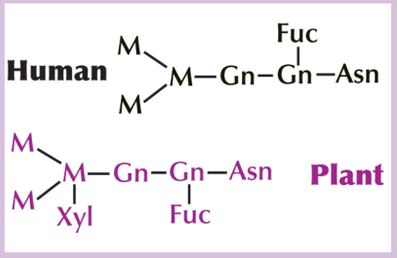
Figure 9: Structure of high-mannose type oligosaccharides found in chicken IgG



the biosynthetic pathway are very similar (**38**). The core N-glycan structure that constitutes the two GlcNAc residues and the three Man residues is similar in both plants and animals including humans (Figure 10). Even the two GlcNAc residues that are β1,2-linked to both α1,3-Man and α1,6-Man are the same in plants and animals (Figure 4). Although plants add Gal residues to the GlcNAc residues in the antennae, the Gal residues are β1,3-linked in plants whereas they are β1,4-linked in animals (**42**). In addition many plants add a Xyl residues to the β1,4-Man residues at O-2 position in β-configuration. Further, plants add α1,3-Fuc to the GlcNAc residue, which in turn links to the Asn residue of the polypeptide chain. In animal glycans, the core Fuc residue is α1,6-linked to the very same GlcNAc (Figure 10).

Both β1,2-Xyl and α1,3-Fuc residues are reported to produce IgE responses in humans. However, at this point, consequences of the presence of β1,3-Gal residues is largely unknown. Because of the presence of these potential immunogenic epitopes in transgenic plant derived antibodies (also called *plantibodies*), nonglycosylated antibodies have been produced in transgenic plants (**35**). The nonglycosylated plantibodies are generated using site-specific mutations. Such plantibodies do not

Figure 10: Comparison of the trimannose core region structure in human and plant glycans



contain the conserved glycosylation site in the Fc. Several glycosylated and nonglycosylated plantibodies have entered human clinical trials, and their results should be available soon.

Efforts are under way to obtain plantibodies with human type glycosylation. A research group in Holland has produced plants that generate plantibodies without β-Xyl and α1,3-Fuc residues (**36, 41–43**). This was achieved using gene knockout technology. If this research effort continues to show improvements at the production scale, it will be advantageous to the biotechnology industry to produce human therapeutics using transgenic plants. They could produce large amounts of economically viable human protein therapeutics.

Long-Term Planning

Literature is replete with the role of N-linked glycans on antibody function. Considering the fact that so many therapeutic antibodies are in development to treat human diseases, regulatory agencies and researchers are making additional efforts to understand the impact of glycans on antibody functions. Hence it is necessary for biotechnology companies to allocate necessary resources for studying the role of glycans on their drug candidate/s.

Although many antibodies in development and on the market are structurally similar, their biophysical and biochemical properties are quite different, as is the impact of glycans on their functions. As long as the antibody drug is a full-length molecule containing glycosylation in the Fc, it is necessary to understand the impact of glycans as much as possible and at the earliest possible stages. From the literature it is very clear that antibody glycosylation is species-specific and varies from cell line to cell line. Within a particular cell line, antibody glycosylation varies from batch to batch and also with scale-up. So it is necessary to develop proper analytical tools to detect those minor variations— because antibody-based drugs are here to stay.

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T. Shantha Raju *is a senior scientist and leader of the CMC (Chemistry, Manufacturing, and Controls) subteam for an antibody development project at FibroGen, where he oversees development and CMC activities for IND and BLA submissions. FibroGen Inc., 225 Gateway Blvd, South San Francisco, CA 94080; 650-866-6456; fax 650-866-7255; traju@fibrogen.com.*