Galactosylation variations in marketed therapeutic antibodies

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Abbreviations: IgG, immunoglobulin G; rIgG, recombinant IgG; ADCC, antibody dependent cellular cytotoxicity; ADCP, antibody dependent cellular phagocytosis; CDC, complement dependent cytotoxicity; TNF, tumor necrosis factor; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; MALDI-TOF-MS, matrix assisted laser desorption time-of-flight mass spectrometry; CHO, Chinese hamster ovary; SP2/0 or NS0, mouse myeloma cells; COGS, cost of goods and services

There are currently~25 recombinant full-length IgGs (rIgGs) in the market that have been approved by regulatory agencies as biotherapeutics to treat various human diseases. Most of these are based on IgG1 framework and are either chimeric, humanized or human antibodies manufactured using either Chinese hamster ovary (CHO) cells or mouse myeloma cells as the expression system. Because CHO and mouse myeloma cells are mammalian cells, rIgGs produced in these cell lines are typically N-glycosylated at the conserved asparagine (Asn) residues in the CH2 domain of the Fc, which is also the case with serum IgGs. The Fc glycans present in these rIgGs are for the most part complex biantennary oligosaccharides with heterogeneity associated with the presence or the absence of several different terminal sugars. The major Fc glycans of rIgGs contain 0 or 1 or 2 (G0, G1 and G2, respectively) terminal galactose residues as non-reducing termini and their relative proportions may vary depending on the cell culture conditions in which they were expressed. Since galcosylation is strongly associated with antibody effector functions and terminal galactosylation may affect some of those functions, a panel of commercially-available therapeutic rIgGs expressed in CHO cells and mouse myeloma cells were examined for their galactosylation patterns. The results suggest that the rIgGs expressed in CHO cells are generally less galactosylated compared with the rIgGs expressed in mouse myeloma cells. Accordingly, rIgGs produced in CHO cells tend to contain higher levels of G0 glycans compared with rIgGs produced in mouse myeloma cell lines. Despite the apparent wide variability in galactose content, adverse events or safety issues have not been associated with specific galactosylation patterns of therapeutic antibodies. Nevertheless, galactosylation may have an effect on the mechanisms of action of some therapeutic antibodies (e.g., effector pathways) and hence further studies to assess effects on product efficacy may be warranted for such antibodies. For antibodies that do not require effector functions for biological activity, however, setting a narrow specification range for galactose content may be unnecessary.

Introduction

Recombinant IgGs (rIgGs) have become important therapeutic agents for the treatment of human diseases, including life-threatening pathologies such as cancer, and more than two dozen rIgGs are currently marketed as human therapeutics. Many of these rIgGs are produced using mammalian cell culture processes. Although different methods of production of rIgGs continue to be investigated to improve yields and to reduce cost of goods and services (COGS), the majority of the currently approved full length human rIgGs are produced using either Chinese hamster ovary (CHO) cells or mouse myeloma-derived cells (either SP2/0 or NS0 cells).

Mammalian serum-derived IgGs are glycosylated in the CH2 domain of the Fc, and the Fc glycans are important determinants for effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). These result from the affect of Fc glycans on antibody interactions with Fc receptors on immune effector cells and C1q in the complement system. Recombinant IgGs also possess Fc region glycan structures that affect antibody effector functions. Since glycosylation patterns vary among species, the glycosylation pattern of rIgGs produced in CHO cells is slightly different than those of rIgGs produced in mouse myeloma-derived cells. For example, rIgGs produced in standard CHO cells do not contain bisecting GlcNAc residues whereas rIgGs produced in mouse myeloma cells contain a fraction of glycans with bisecting GlcNAc residues. This difference likely derives from the fact that standard CHO cells do not express an active GnT-III enzyme, a glycosyltransferase that mediates the transfer of bisecting GlcNAc residues from UDP-GlcNAc whereas mouse myeloma cells express the active GnT-III enzyme. However,
both CHO and mouse myeloma cells express active β1,4GalTs, a group of glycosyltransferases that mediate the transfer of β1,4-gal residues to recombinant glycoproteins, including rlgGs, produced in these cell lines.2 Hence, it could be anticipated that the galactosylation pattern might be similar in rlgGs produced in CHO cells and mouse myeloma cells. However, variations in cell culture conditions have also been shown to affect the glycosylation of therapeutic proteins, including rlgGs,3 and the terminal galactosylation of rlgGs may be affected by such variations.9–11 Although, terminal galactosylation of rlgGs does not appear to affect the antibody binding to antigen, it has been reported that changes in galactosylation may result in noticeable changes in CDC activity of some rlgGs.12

Rituximab (Rituxan®), first approved in 1997, is a chimeric monoclonal antibody produced in CHO cells that is indicated for treatment of non-Hodgkin lymphoma and other B-cell related diseases.13 Rituximab is glycosylated in the Fc, and the Fc glycans are highly heterogeneous.14,15 The heterogeneity of the glycosylation of rituximab is mainly due to the variable presence of terminal galactose residues. The effect of the terminal galactose residues of rituximab on CDC activity stems from the involvement of such residues in the binding of rituximab to complement C1q.12

Although very few other marketed rlgGs have shown similar galactose-dependent CDC activity, the precedent has spurred various regulatory agencies to require manufacturers of rlgGs to carefully monitor the terminal galactosylation patterns of their drug substances and drug products.2–4 With this background, the present investigation was performed to measure the terminal galactose content of a panel of rlgGs (Table 1) that are currently approved as human therapeutics.2–4,16 The results suggest that wide variations in terminal galactosylation exist across rlgGs produced in CHO cells and mouse myeloma cells. However, these variations of Fc galactosylation have not been specifically associated with adverse safety or efficacy outcomes despite the fact that many of these products have been in widespread use for over a decade.

### Results

N-glycans present in the Fc regions of rlgGs were released by treatment with peptide-N-glycosidase F (PNGase F) and the enzymatic deglycosylation reactions were monitored by MALDI-TOF-MS. MALDI-TOF-MS of intact Mab3 before (A) and after (B) the enzymatic deglycosylation (Fig. 1) showed that the removal of the N-glycan side chains appeared to be complete, as indicated by the corresponding decrease in molecular weight (MW) of +1 molecular ion (m/z) of enzymatically deglycosylated Mab3 by ~3 KDa (Fig. 1B). In the mass spectra, the corresponding decrease in the mass of doubly charged (+2) and triply charged (+3) molecular ions of the Mab3 were also observed (Fig. 1). Similar decreases in MW were observed for other Mabs upon treatment with PNGase F to release N-glycans from their respective Fc regions (data not shown).

The N-glycans of rlgGs released by PNGase F were purified by cation-exchange chromatography and the purified glycans were analyzed by MALDI-TOF-MS in the positive mode using sDHB as a matrix. A representative mass spectral profile for Mab3 is shown in Figure 2A. The data in Figure 2A suggests that the major neutral glycans present in Mab3 were complex biantennary structures containing core fucose and terminating with 0, 1 or 2 galactose residues (G0, G1 and G2, respectively). In addition to G0, G1 and G2 structures, minor amounts of mono-α-galactosylated and di-α-galactosylated G2 glycans were also observed for Mab3 at m/z ~ 1,969 and ~2,131, respectively. Additionally, the mass spectra also contained molecular ions of minor glycans that are derivatives due to the absence of core fucose and also glycans containing bisecting GlcNAc residues.17 The minor glycans including afucosylated, bisecting GlcNAc-containing and high mannose type represented less than 1% of total glycans and their variations were harder to measure highly accurately. Hence, only the major glycans of rlgGs were considered for relative quantification and all the major neutral glycans observed in Mabs1–6 were mainly due to galactose heterogeneity.

In addition to the MALDI-TOF-MS analysis, PNGase F-released glycans of rlgGs were also analyzed by normal-phase HPLC (NP-HPLC) after labeling with anthranilic acid by the reductive amination method.18 This is because positive mode MALDI-TOF-MS analysis afforded only neutral glycans and we wanted to see the acidic glycans due to sialylation. Hence, NP-HPLC analysis was performed to measure both neutral as well as acidic glycans in a more quantitative manner. A representative NP-HPLC profile of anthranilic acid labeled N-glycans released from Mab3 is shown in Figure 2B. The major glycan peaks were identified by comparing the retention time with authentic standard glycans and also by using MALDI-TOF-MS analysis of the corresponding de-N-glycosidase digestions. The data in Figure 2B indicated that the Mab3 antibody contained ~35% sialylated glycans, i.e., G2S1 and G2S2. The remaining 65% of N-glycans of Mab3 antibody were neutral glycans of which a majority exhibited galactose heterogeneity.17,18 Schematic diagram of the N-glycans that were identified either by MW using MALDI-TOF-MS analysis or comparing the retention time of the relevant glycan standards using NP-HPLC analysis are shown in Figure 3.

Both the MALDI-TOF-MS and NP-HPLC analyses indicated that the major neutral glycans in Mab3 were G0, G1 and G2. In addition, NP-HPLC data showed that Mab3 also contained appreciable amounts of sialylated G2S1 and G2S2 N-glycans. However, other rlgGs contained significantly lower amounts of

### Table 1. Therapeutic IgGs used in the study

| Name    | Cell lines used to manufacture | IgG Isotype | Category | Antigen        |
|---------|-------------------------------|-------------|----------|----------------|
| MAb1    | SP2/0                         | IgG1        | Chimeric | TNF            |
| MAb2    | SP2/0                         | IgG1        | Human    | TNF            |
| MAb3    | SP2/0                         | IgG1        | Human    | IL-12/23       |
| MAb4    | CHO                           | IgG1        | Humanized| IgE            |
| MAb5    | CHO                           | IgG1        | Humanized| VEGF           |
| MAb6    | CHO                           | IgG1        | Human    | TNF            |

Table 1. Therapeutic IgGs used in the study
produced using mouse myeloma cells whereas MAb4, MAb5 and MAb6 were produced in CHO cells (see Table 1). The G0 content in MAb1, MAb2 and MAb3 varies from 35% to 45%. However, the G0 content of MAb4, MAb5 and MAb6 varied from ~62% to ~83%. Based on these data, it appears that CHO cell-derived rIgGs are less galactosylated and contain higher amounts of G0 glycans compared with the rIgGs produced in mouse myeloma cells. However, it has been observed that cell culture conditions, if not carefully regulated, can affect galactosylation of antibodies, leading to potential lot-to-lot variations. \[1\] Such variations in galactosylation may affect the CDC activity of rIgGs and hence regulatory agencies require manufacturers of rIgGs to carefully monitor the galactosylation levels of marketed antibody-based products. These considerations led to the present examination of the galactosylation patterns of different lots of rIgGs produced in CHO and mouse myeloma cells.

To further investigate the variations in galactosylation of marketed rIgGs, we selected three different lots of anti-TNF MAb1 and MAb6, which are chimeric and phage-derived human antibodies, respectively. Both antibodies contain the same human Fc region, but they are manufactured in two different cell types: MAb1 in SP2/0 mouse myeloma cells and MAb6 in CHO acidic glycans that varied from 0.5 to 15% (data not shown). Based on the MALDI-TOF-MS data described above, MAb3 also contained very minor amounts (<1%) of non-fucosylated glycans along with some glycans with bisecting GlcNAc residues. Also, the MALDI-TOF-MS profile showed the presence of very small amounts of high mannose structure with five mannose residues (Man5 glycans) along with G0-GlcNAc glycans (Fig. 3). The levels of these glycans were minor and appeared not to be present in all the rIgGs analyzed. Hence, we focused on the relative proportions of major glycans containing 0, 1 or 2 galactose residues (G0, G1 and G2 respectively). The relative proportions of G0, G1 and G2 glycans of rIgGs obtained by NP-HPLC analyses are shown in Table 2 for six different commercially-available therapeutic mAbs.

The data in Table 2 indicates that the relative proportions of G0 glycan of rIgGs varied from ~35% to ~83%. MAb3, produced using SP2/0 cells, contained the lowest levels of G0 glycan whereas MAb5, produced using CHO cells, contained the highest amount of G0 glycan with corresponding changes in G1 and G2 glycans (Table 1).

The comparison of galactosylation profiles of rlgG mAbs 1–6 is shown in Table 2. These rIgGs were produced in either CHO cells or mouse myeloma cells. MAb1, MAb2 and MAb3 were produced using mouse myeloma cells whereas MAb4, MAb5 and MAb6 were produced in CHO cells (see Table 1). The G0 content in MAb1, MAb2 and MAb3 varies from 35% to 45%. However, the G0 content of MAb4, MAb5 and MAb6 varied from ~62% to ~83%. Based on these data, it appears that CHO cell-derived rIgGs are less galactosylated and contain higher amounts of G0 glycans compared with the rIgGs produced in mouse myeloma cells. However, it has been observed that cell culture conditions, if not carefully regulated, can affect galactosylation of antibodies, leading to potential lot-to-lot variations in galactosylation patterns. \[1\] Such variations in galactosylation may affect the CDC activity of rIgGs and hence regulatory agencies require manufacturers of rIgGs to carefully monitor the galactosylation levels of marketed antibody-based products. These considerations led to the present examination of the galactosylation patterns of different lots of rIgGs produced in CHO and mouse myeloma cells.

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![Figure 1. MALDI-TOF-MS of glycosylated and deglycosylated MAb3. An aliquot of MAb3 was treated with PNGase F and analyzed by MALDI-TOF-MS using Sinapic acid as matrix. (A) Glycosylated MAb3 and (B) Deglycosylated MAb3. In the figure +1 is a singly charged molecular ion, +2 is a doubly charged molecular ion, +3 is a triply charged molecular ion and LC is free light chain (mass values are not corrected).](image-url)
The Fc glycans are highly heterogenous and may contain several different terminal sugars that may affect antibody functions. For example, IgGs and rIgGs lacking core fucose exhibit higher levels (>100-fold increase) of ADCC activity compared to their fucosylated counterparts. Terminal galactosylation has also been shown to affect the C1q binding of rituximab. However, the difference in CDC activity between the G0 and G2 glyco-forms is only about 2-fold. Because of the small increase in CDC activity between antibodies with 0% galactosylation and 100% galactosylation, minor differences in galactosylation are unlikely to result in substantial differences in the CDC activity of such rIgGs.

The galactosylation pattern of a panel of six marketed rIgGs (Tables 1 and 2) clearly shows product-specific variability known to exist in such rIgGs. At least to some extent, the variability appears to result from use of different cell lines for their expression. Terminal galactosylation may also vary by lot, suggesting that even minor variations in cell culture conditions may have an effect, although these are generally smaller than the variations observed in products manufactured in different cell types. They are marketed for the same indications by two different manufacturers. Comparison of G0, G1 and G2 glycans of different lots of MAAb1 and MAAb6 is shown in Figure 4. It is evident from Figure 4 that the G0 glycan content of MAAb6 is approximately 2-fold higher than the level of G0 glycan present in MAAb1. The proportions of G1 and G2 glycans in these two rlgGs are also markedly different, but the proportions of galactosylation of different lots of either MAAb1 or MAAb6 vary only slightly. Hence, our data show that the variations in galactosylation of rlgGs produced in different cell types is greater than the lot-to-lot variations observed within a given cell type. This may be due to cell type-specific galactosylation attributable to branch specificity of different galactosyltransferases, a group of enzymes that mediates the transfer of β-galactose residues from UDP-Gal.

Discussion

N-glycosylation in the Fc region modulates antibody effector functions of IgGs, rlgGs and other Fc containing molecules. The Fc glycans are highly heterogenous and may contain several different terminal sugars that may affect antibody functions. For example, IgGs and rlgGs lacking core fucose exhibit higher levels (>100-fold increase) of ADCC activity compared to their fucosylated counterparts. Terminal galactosylation has also been shown to affect the C1q binding of rituximab. However, the difference in CDC activity between the G0 and G2 glyco-forms is only about 2-fold. Because of the small increase in CDC activity between antibodies with 0% galactosylation and 100% galactosylation, minor differences in galactosylation are unlikely to result in substantial differences in the CDC activity of such rlgGs. The galactosylation pattern of a panel of six marketed rlgGs (Tables 1 and 2) clearly shows product-specific variability known to exist in such rlgGs. At least to some extent, the variability appears to result from use of different cell lines for their expression. Terminal galactosylation may also vary by lot, suggesting that even minor variations in cell culture conditions may have an effect, although these are generally smaller than the variations observed in products manufactured in different cell types. They are marketed for the same indications by two different manufacturers. Comparison of G0, G1 and G2 glycans of different lots of MAAb1 and MAAb6 is shown in Figure 4. It is evident from Figure 4 that the G0 glycan content of MAAb6 is approximately 2-fold higher than the level of G0 glycan present in MAAb1. The proportions of G1 and G2 glycans in these two rlgGs are also markedly different, but the proportions of galactosylation of different lots of either MAAb1 or MAAb6 vary only slightly. Hence, our data show that the variations in galactosylation of rlgGs produced in different cell types is greater than the lot-to-lot variations observed within a given cell type. This may be due to cell type-specific galactosylation attributable to branch specificity of different galactosyltransferases, a group of enzymes that mediates the transfer of β-galactose residues from UDP-Gal.
types as evidenced for Mab1 and Mab6 (Fig. 4). It is important to note that the variations in terminal galactosylation are unlikely to affect the antibody binding to antigen and appear to have minimum influence on the ADCC activity of rlgGs.\textsuperscript{1,12} Terminal galactosylation appears not to be a determinant of the pharmacokinetic properties of rlgGs because both G0 and G2 glycoforms have been shown to have similar pharmacokinetic properties.\textsuperscript{24-26} From the data now available, it appears that galactose residues have an effect on rlgGs functions due to their impact on CDC activity.\textsuperscript{1,12} Hence, if the mechanism of action of rlgG is based on CDC activity, as is the case with rituximab, then it may be necessary to monitor and control galactosylation of those molecules. This suggests that a very narrow specific range for galactosylation of rlgGs may be warranted for biosimilars or biobetters of rituximab, or similar molecules.

The two anti-TNF antibodies used in this study (MAB1 and MAB6) exhibited a large difference in terminal galactosylation. Both MAB1 and MAB6 have found widespread applications in the effective treatment of patients with a variety of immunological disorders. To date, we are unaware of any reports of adverse events of rlgGs related to Fc glycosylation in general or terminal galactosylation in particular, which, especially given the wide disparity of the G0 glycoform level of ~39% in mAb1 and ~79% in Mab6, suggests the lack of a direct safety-related role for antibody galactosylation. Hence, it may be unnecessary to set a narrow specification range for galactosylated glycans of rlgGs that do not use CDC activity as one of their mechanisms of action. This may be applicable to many different rlgGs in development, including biosimilars and biobetters.

**Materials and Methods**

**Materials.** rlgGs produced in CHO and mouse myeloma cells were obtained from commercial sources.\textsuperscript{15,27,28} PNGase F was obtained from New England Biolabs (Boston, MA). All other reagents are analytical grade and obtained from commercial reagent suppliers or manufacturers.

**MALDI-TOF-MS analysis of IgG samples.** rlgG samples were exchanged into 10 mM TRIS-HCl buffer, pH 7.0 and their concentrations were adjusted to ~1 mg/mL buffer. About 1 μl of IgG solution was mixed with 1 μl of matrix solution (the matrix solution was prepared by dissolving 10 mg sinapic acid in 1.0 ml of 50% acetonitrile in water containing 0.1% trifluoroacetic acid). Approximately 2 μl of this sample-matrix solution was loaded onto the gold surface target plate and allowed to air dry. MALDI-TOF-MS was acquired using a Voyager DE instrument from Applied BioSystems (Foster City, CA).

**MALDI-TOF-MS analysis of oligosaccharides.** IgG samples (~50 μg) were digested with PNGase F in 10 mM TRIS-HCl buffer (50 μl) pH 7.0 for 4 h at 37°C. The digestion was stopped by acidifying the reaction mixture with 50% acetic acid (~5 μl)
and then passed through a cation-exchange resin column as described previously in reference 19. These samples containing a mixture of acidic and neutral oligosaccharides were analyzed by MALDI-TOF-MS in the positive and negative ion modes, as described in reference 19 and 20, using a Voyager DE instrument from Applied BioSystems (Foster City, CA).

**NP-HPLC analysis of oligosaccharides.** IgG samples (~50 μg) in 10 mM TRIS-HCl buffer (~50 μl) pH 7.0 were digested with PNGase F at 37°C for 4–8 h. Derivatization of the released oligosaccharides with anthranilic acid (2-aminobenzoic acid) was performed as described previously in reference 18. A solution of 4% sodium acetate-3H2O (w/v) and 2% boric acid (w/v) in methanol was prepared first. The derivatization reagent was freshly prepared by dissolving ~30 mg of anthranilic acid (Aldrich) and ~20 mg of sodium cyanoborohydride (Aldrich) in 1.0 ml of methanol-sodium acetate-borate solution. IgG-derived oligosaccharides (<3 nmol in 20–50 μl of water) were mixed with 0.1 ml of the anthranilic acid (AA) reagent solution in 1.6 ml polypropylene screw cap freeze vials with “O” rings (Sigma) and capped tightly. The vials were heated at 80°C in an oven or heating block (Reacti-Therm, Pierce) for 1–2 h. After cooling the vials to room temperature, the samples were diluted with water to bring the volume to ~0.5 ml. Derivatized oligosaccharides were purified by using NAP-5 columns as described previously in reference 19 and 20. Purified AA labeled oligosaccharides were separated on an amine (-NH2) bonded polymeric column (Polymer-NH2, 5 μm, 0.46 x 25 cm, Astec, Whippany, NJ). Solvent system A consisted of 2% acetic acid and 1% tetrahydrofuran (inhibited) in acetonitrile and solvent system B consisted of 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran (inhibited) in water. The following gradient for mapping was used for optimum resolution of the oligosaccharides present in IgG samples: 30% B isocratic for 2 min followed by a linear increase to 95% B over 80 min and was held at 95% B for additional 15 min. The column was equilibrated with initial conditions for 15 min prior to the next injection. HPLC systems and their impact on biological Activity of recombinant glycoprotein therapeutics. Glycobiology 2011; 108:1591-602; PMID:21328321; http://dx.doi.org/10.1002/bip.23475.

Figure 4. Comparison of relative proportions of G0, G1 and G2 glycans present in different lots of MAb1 (SP2/0) and MAb6 (CHO). Three different lots of each of MAb1 and MAb6 were separately treated with PNGase F to release N-glycans. The released glycans were labeled with anthranilic acid and analyzed by NP-HPLC. The relative proportions of G0, G1 and G2 glycans from each lots were compared.

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Disclosure of Potential Conflicts of Interest Statement

No potential conflicts of interest were disclosed.
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