High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans

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Glycan structures attached to the C1r2 domain of the Fc region of immunoglobulin G (IgG) are essential for specific effector functions but their role in modulating clearance is less clear. Clearance is of obvious importance for therapeutic monoclonal antibodies (Mabs) as it directly impacts efficacy. Here, we study the impact of Fc glycan structure on the clearance of four therapeutic human IgGs (one IgG1 and three IgG2s) in humans. The therapeutic IgGs were affinity purified from serum samples from human pharmacokinetic studies, and changes to the glycan profile over time were determined by peptide mapping employing high-resolution mass spectrometry. Relative levels of high-mannose 5 (M5) glycan decreased as a function of circulation time, whereas other glycans remained constant. These results demonstrate that therapeutic IgGs containing Fc high-mannose glycans are cleared more rapidly in humans than other glycan forms. The quantitative effect of this on pharmacokinetic area under the curve was calculated and shown to be relatively minor for three of the four molecules studied, but, depending on the dosing regimen and the relative level of the high-mannose glycan, this can also have significant impact. High-mannose content of therapeutic Mabs should be considered an important product quality attribute which may affect pharmacokinetic properties of therapeutic antibodies.

Keywords: Fc glycans / high mannose / monoclonal / pharmacokinetics

Introduction

It is well established that the serum half-life of IgG antibodies is regulated by the neonatal Fc receptor, FcRn (Raghavan and Bjorkman 1996; Roopenian and Akilesh 2007). This receptor binds to the C1r2–C1r3 interface of IgG and is unaffected by the presence or nature of the Fc glycan (Jeffersis 2005). Other receptors in mammalian tissues, such as the asialoglycoprotein and mannose receptors, have the ability to bind and clear proteins containing specific glycans. The asialoglycoprotein receptors preferentially bind terminal Gal residues (Ashwell and Morell 1974; Ashwell and Harford 1982; Stockert 1995), whereas the mannose receptors clear glycoproteins, including endogenous glycopolypeptides, with terminal Man or GlcNAc sugars (Stahl 1992; Lee et al. 2002; Allavena et al. 2004). For many glycopolypeptides, highly sialylated structures with minimally exposed terminal Gal residues extend circulating half-life by reducing asialoglycoprotein receptor-mediated clearance (Ashwell and Morell 1974). The clearance mechanism of endogenous human IgG, on the other hand, is likely to be less influenced by sialylation, as only ∼11% of these molecules are terminally sialylated (Flynn et al. 2010). Recombinant antibodies expressed in Chinese hamster ovary (CHO) tissue culture contain predominantly biantennary fucose-syalted neutral glycans with varying levels of terminal Gal (G0F, G1F and G2F, where G# refers to the number of Gal on the two arms and F is fucosylation of the initial GlcNAc residue) and some high-mannose (M5–M9) structures at the Fc site (Bailey et al. 2005; Kamoda et al. 2006; Chen and Flynn 2007). Levels of high-mannose glycans in recombinant antibodies can range from 1% to greater than 20%, whereas endogenous human IgG contains only trace levels (<0.1%) of high-mannose glycans (Flynn et al. 2010). As mannose receptor functions to bind both high-mannose-containing pathogens as well as endogenous proteins (Allavena et al. 2004), the potential exists for differential clearance of certain therapeutic antibody glycoforms, especially those containing high-mannose structures, due to binding to mannose receptor. If so, this would have obvious implications for antibody drug efficacy.

There are inconsistencies in the published literature regarding whether Fc glycans do, in fact, impact antibody clearance in mammals. In two separate studies in mice, an IgG1 with M5 glycan cleared faster than one with complex glycans (Wright and Morrison 1994; Kanda et al. 2007). An Fc fusion protein studied in both humans and cynomolgus monkeys was found to clear faster when non-Fc glycans, but not Fc
glycans, contained terminal GlcNAc (Jones et al. 2007). This was attributed to uptake by the mannose receptor. Unfortunately, high-mannose Fc glycans were not studied. In another study, Fc glycans had no effect on antibody clearance, although, again, high-mannose structures were not examined (Huang et al. 2006). With another biotherapeutic molecule, rapid clearance of mannosylated antibody–enzyme fusion protein in humans (Kogelberg et al. 2007) was observed, although in this case the enzyme, and not the antibody fragment, contained the high-mannose chains. Clearance of omalizumab, a recombinant IgG1, in mice was found not to be affected by Fc glycan structure, including high mannose (Harris 2005). In a highly relevant experiment, with the same antibody [monoclonal antibody 1 (Mab1)] used in the present study, it was concluded that antibody clearance in humans was not significantly affected by the Fc glycan structure (Chen et al. 2009).

In this study, we have followed the distribution of Fc glycans of one IgG1 and three IgG2 therapeutic Mabs administered at different doses as a function of circulation time in human subjects. Ligand-specific affinity chromatography was used to isolate Mab from serum samples, and a highly specific analytical methodology, which renders the analysis insensitive to the majority of potentially interfering impurity glycoproteins, was employed. The enhanced specificity of the method allowed data to be collected from later time points from samples containing lower Mab concentrations, thereby increasing confidence in any observed trends. Changes to the glycan pattern with circulation time were used to draw conclusions about differences in the clearance rates of individual glycoforms.

Results

Glycopeptide quantification by peptide mapping

A high-sensitivity peptide map-based method (Zhang 2009; Zhang and Shah 2010) for identifying and quantifying IgG Fc glycans was optimized for use with serum samples containing low therapeutic Mab concentrations obtained from pharmacokinetic (PK) studies. In contrast to methods that quantify enzymatically released glycans such as the previously described reversed-phase high-performance liquid chromatography (HPLC) glycan map (Chen and Flynn 2007), this method offers the advantage of being specific for the consensus Fc glycosylation site of either human IgG1 or human IgG2, depending on the therapeutic Mab being studied. This technique has been used to identify glycans on recombinant IgG1 and IgG2 glycoforms (Zhang 2009) as well as endogenous antibodies (Flynn et al. 2010).

With IgG1s, our peptide map method utilizes a high-resolution LTQ Orbitrap mass spectrometer to specifically identify and quantify individual glycoforms of the TKPREEQNYSTRVVSVLTSLHQDWLNGK (N297) Lys-C generated peptide found only in human IgG1 Fc and covering the IgG1 glycosylation site. In the case of therapeutic IgG2s, we similarly monitor individual glycoforms of the TKPREEQFNSTRVVSVLTTVHQDWLNGK (N297) Lys-C generated peptide found only in human IgG2 Fc and covering the consensus IgG2 glycosylation site. We used ligand-based affinity chromatography to selectively purify µg/mL concentrations of therapeutic IgG Mab from human serum containing endogenous IgG1 and IgG2 at concentrations of ~5–12 and 2–6 mg/mL, respectively (French 1986). As endogenous IgGs, as well as some other serum proteins, are also glycosylated, glycan analysis could be compromised if these molecules were to copurify with Mab to a significant extent. The peptide map method reduces the potential pool of interfering impurities to only endogenous IgG of the same subclass as the therapeutic Mab, which contains the same N297 peptide, whereas methods relying on released glycans are potentially subject to interference from any co-purifying glycoproteins. The peptide map method therefore achieves a highly significant increase in specificity compared with methods relying on glycan release.

Clearance studies

Mab1, Mab2, Mab3 and Mab4, all fully human therapeutic IgGs, were injected into healthy adult subjects as part of separate phase I clinical trials. Mab1 was administered intravenously at doses of 100, 300 and 1000 mg and subcutaneously at 300 mg. Mab2 was administered intravenously at 1000 mg, and Mab3 and Mab4 were administered intravenously at 20 mg/kg (corresponding to 1250 mg for an average patient). Mabs were isolated from serum samples collected at various time points after dosing by affinity purification using each antibody’s specific ligand. These isolation procedures were capable of obtaining ≥70% of the Mab present in serum at over 90% purity, as judged by microchip capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) (Chen et al. 2009). Table I presents typical data from one clinical subject using the high-resolution Orbitrap mass spectrometer. Twenty-one glycoforms were identified and quantified. Close agreement between conventionally purified Mab standard and ligand affinity-purified samples recovered from serum at the earliest time point was observed. Peptide map analysis using the lower-resolution LTQ ion trap mass spectrometer, used for Mab3 and Mab4 samples, allowed quantification of only 6–10 of the most abundant glycoforms of IgG shown in Table I, but this did not affect the overall results.

Figure 1 plots the distribution over time of several of the major N297 glycopeptides for two different clinical subjects receiving Mab1 at a different dose than shown in Table I. Although there is some scatter in the levels of low-abundance glycopeptides, the only consistent trend observed among all Mabs studied, as well as also with different dosing regimens for Mab1, was a decrease in the level of the peptide containing M5 glycan, following an initial small increase over the first 2 days. The small increase in M5 between 0 and 2 days (in this example) can be attributed to serum α-1,2-mannosidase activity which has been shown to convert M6–M9 high-mannose glycans to M5 in a timeframe of ~24 h (Chen et al. 2009). We have confirmed the rapid loss of the larger high-mannose species in the first few days post-dosing (Table I). After the initial, rapid conversion of the majority of M6–M9 glycans to M5 is complete, M5 becomes a single, de facto marker to quantify the fate of the majority of high-mannose glycans in serum.
A peptide-mapping approach to glycan analysis also enables monitoring the impact of the absence of glycosylation on clearance. As shown in Figure 1, Mab1 also appears to exhibit a minor decrease in the relative abundance of non-glycosylated heavy chain over time. This was observed for multiple subjects and at various intravenous doses, but could not be confirmed with the other Mabs because levels of non-glycosylated heavy chain were too low to allow reliable quantification.

As the concentration of each Mab declines post-dosing in humans, and the ratio of therapeutic IgG to endogenous IgG in serum samples decreases, the purity of the Mab purified from corresponding serum samples might be expected to decrease due to relatively constant non-specific binding of non-glycosylated heavy chain.
endogenous IgG of the same subclass to the affinity resin. Any changes in the proportion of therapeutic to endogenous IgG in purified samples have the potential to compromise our clearance results. As endogenous human IgG contains very low or no high-mannose glycans (Flynn et al. 2010), it is important to exclude the possibility that our observed decrease in M5 content is simply the result of decreasing the purity of Mab (and corresponding N297 glycopeptide) over time during each PK study. Fortunately, recombinant monoclonal IgG2 and endogenous human IgG2 contain characteristic differences in the abundance of the major fucosylated glycoforms, which allows the levels of potential IgG2 impurities to be assessed. Similarly, large differences also exist between recombinant monoclonal IgG1 and endogenous human IgG1 (compare Table I with Table II). To illustrate, Mab1 contains G0F, G1F and G2F at levels of 57, 18 and 2%, respectively. Using this same analytical methodology, endogenous human IgG2 purified from five individual normal donors by protein A affinity chromatography has been found to contain the same glycans at levels of 23–33, 32–43 and 11–21%, respectively (Flynn et al. 2010). Because of their high absolute abundance and the resultant confidence in their measurement, as well as the large differences in abundance between therapeutic and endogenous IgG2, these glycopeptides are especially useful to monitor Mab purity. Dilution of monoclonal IgG2 recovered from serum samples by increasing levels of endogenous IgG2 impurities would be expected to result in decreasing levels of G0F and increasing levels of G1F and G2F over time. Figure 1 shows that the levels of these diagnostic N297 glycopeptides recovered from human sera at different time points remained constant within experimental error, demonstrating that the purity of recovered Mab did not change measurably over this time window. Furthermore, the distribution of these glycoforms was similar to that of the corresponding Mab standard at the initial time point and distinctly different from that of endogenous IgG2 (Table I), demonstrating no measurable contribution to the analysis from endogenous human IgG2. Table IV shows the calculated levels of G0F, G1F and G2F expected for Mab1 containing different impurity levels of endogenous IgG2. G0F is the most abundant of these glycans and is quantified with the highest degree of confidence. Comparison of the G0F levels measured in PK samples at different times post-dosing for Mab1 (Table I, Figure 1) to the calculated levels of this Mab1 glycan in the presence of varying levels of endogenous human IgG2 (Table IV) leads to the conclusion that the purity of Mab1 glycopeptides being monitored was ≥90% over the studied time range. Although the absolute levels of the less abundant G1F and G2F glycopeptides do not always match the theoretical value as well as G0F does, poorer precision would be expected for these less abundant forms. The trending of the levels with time is more important than the absolute amount of the glycan. Stable levels of the glycan forms post-dosing can be used to infer that Mab purity is relatively constant over the time points followed. Similar results were obtained for Mab2 and Mab3 as well as for Mab4, an IgG1 (data not shown).

An orthogonal approach to assess the purity of the IgG2 glycopeptide being monitored is to use mass spectrometry (MS) peak areas of peptides specific to the constant regions of IgG1, compared with similar peptides from the same regions of IgG2, to measure the abundance of IgG1 impurities. The abundance of IgG1 calculated this way ranged from 0.4 to 18% of total IgG2 (Mab + endogenous IgG2 impurity) for the samples shown in Figure 1 and from 1.6 to 8.3% of total IgG2 for the samples shown in Table I. Because there is typically less IgG2 than IgG1 in human serum, it is expected that the abundance of human IgG2 impurities is proportionately less, consistent with Mab glycopeptide purities of >90%, as estimated above. These findings are also consistent with the Mab purity estimated independently by microchip CE (data not shown).

The concentration of each glycoform as a function of time was calculated by multiplying the fraction of each glycoform by the total Mab concentration, as determined by enzyme-linked immunosorbent assay (ELISA). An apparent elimination rate was approximated for each glycoform assuming first-order clearance kinetics. Only data from the post-absorption phase (subcutaneous injections) or post-M6–M9 to M5 conversion phase (for M5) were used. It is recognized that experimental data do not strictly follow first-order kinetics, but the data fit to such idealized behavior were close enough, over the time range studied, to allow useful comparisons among glycoforms. The relative elimination rate for Mabs containing different glycan forms was then calculated by dividing the elimination rate of different glycoforms by that of the total Mab. Results for the major glycoforms are compiled in Table III. Mabs containing the most abundant G0F and G1F glycoforms were calculated, and demonstrated, to possess elimination rates very similar to those of the total Mab. G2F and G0 glycoforms are much less abundant (Table II) and consequently are quantified with lower precision. However, based on the data in Table III, antibodies containing either of these two forms are expected to clear from serum at rates similar to the total Mab. In contrast, antibody containing the M5 glycoform is calculated to clear faster than its parent Mab, a consistent finding for all Mabs, independent of dose or route of administration (Table III). Over the time range that data were obtained, M5 generally decreased by 50% or more and, as explained in the preceding two paragraphs, dilution by increasing amounts of endogenous IgG not containing M5 could account for at most a 10% decrease. We conclude that IgG Mabs bearing M5 are cleared at an increased rate in humans. It is likely that the relative elimination rates of the other Mab4 glycans shown in Table III are lower than bulk Mab because the amount of M5 for this antibody is higher (Table II).
random pairing preference (PP) is defined as the experimental 

\[
[M5:G0F]_\text{exp} \div [M5:G0F]_\text{r} = \text{PP}
\]


Denote that this equation reduces to \( \text{fr}(M5:X) = [M5] \) when all M5 residues are paired only with each other and PP approaches infinity and to \( \text{fr}(M5:X) = 2[M5] - [M5]^2 \) when totally random pairing occurs and PP = 1. Table V summarizes these calculations for Mab1, Mab2, Mab3 and Mab4 and shows that there is a strong, but not exclusive, preference for M5:M5 pairing, the degree of which may vary among molecules. Because of the strong PP, the fraction of M5:X IgG2 is only slightly larger than the fraction of M5-containing heavy chains among these four IgGs, but whether this is more generally true will require further study. In this study, however, the impact of M5 on antibody clearance is not significantly greater than that calculated based on M5 fraction per heavy chain.

In Figure 2, it is also possible to monitor intact Mab1 species containing non-glycosylated heavy chain. The mass region where Mab1 with two paired non-glycosylated heavy chains are expected shows at most a tiny peak, but much larger peaks are found corresponding to Mab1 with non-glycosylated heavy chain paired with heavy chains containing the most abundant glycan species. It is clear that non-
glycosylated heavy chains pair randomly with glycosylated heavy chains, which stands in clear contrast to the strong PP observed for M5 glycans.

Impact on systemic exposure
The faster clearance of M5-containing IgGs has implications for systemic exposure of therapeutic IgG Mabs. Specifically, the total systemic exposure, as measured by the area under the curve (AUC) of a concentration vs time plot, will be reduced in proportion to the M5:X content of a given antibody preparation. Figure 3 shows the PK data for Mab1 in a single patient dosed at 300 mg subcutaneously; M5 exhibits an ~2-fold increased elimination rate constant compared with Mab1 resulting in an elimination half-life that is approximately half that of Mab1. Using only our experimental data, the quantitative contribution to reduced systemic exposure was estimated for each therapeutic Mab at each dose as follows: the fraction of M5-containing Mab at each PK time point was first calculated as described in the preceding paragraphs. The hypothetical Mab PK curve, in the absence of faster M5 clearance, was obtained by adding to the experimental concentration, the extra Mab loss due to the presence of M5-containing Mab:

$$[\text{Mab}]_{\text{rec}} = [\text{Mab}]_{\text{expt}} + (f_{\text{M5}} - f_{\text{M5}}) \times [\text{Mab}]_{\text{expt}}$$

where $[\text{Mab}]_{\text{rec}}$ is the reconstructed Mab concentration, $[\text{Mab}]_{\text{expt}}$ the experimentally measured Mab concentration, $f_{\text{M5}}$ the fraction of M5-containing Mab at $t = 0$ and $f_{\text{M5}}$ the fraction of M5 containing Mab at each time point. The difference in AUC between the experimental and the reconstructed PK curves represents an estimate of the decrease in systemic exposure due to faster clearance of high-mannose glycoforms. These results are shown in Table VI and the effect on AUC is shown graphically in Figure 4. Note that the parameters used in the generation of Figure 4B are rather similar to those actually measured for Mab4 and consequently both cases result in a similar quantitative impact on AUC. This method of estimating the quantitative effect on systemic exposure has the advantage of relying only on experimental data.

Table V. Summary results for the calculation of percent of each Mab containing at least one Fc M5 glycan

<table>
<thead>
<tr>
<th>Mab</th>
<th>Mab1</th>
<th>Mab2</th>
<th>Mab3</th>
<th>Mab4</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5 by peptide map (%)</td>
<td>5.0</td>
<td>12.2</td>
<td>4.0</td>
<td>17.0</td>
</tr>
<tr>
<td>M5:M5 PP</td>
<td>~50</td>
<td>8.8</td>
<td>6.4</td>
<td>8.8</td>
</tr>
<tr>
<td>Calculated % of Mab containing M5 (%)</td>
<td>5.1</td>
<td>13.4</td>
<td>4.6</td>
<td>18.6</td>
</tr>
</tbody>
</table>

M5:M5 PP is defined as the experimental (M5:M5)/(M5:G0F) ratio divided by that expected from random pairing of heavy chains (see text for further details).

glycosylated heavy chains pair randomly with glycosylated heavy chains, which stands in clear contrast to the strong PP observed for M5 glycans.

Table VI. Calculated decrease in total PK AUC attributable to faster clearance of therapeutic Mabs containing at least one Fc M5 glycan at different dosing regimens (see text for details)

<table>
<thead>
<tr>
<th>Mab</th>
<th>Dose</th>
<th>% decrease in AUC due to faster M5 clearance, average of 2 (range)</th>
<th>PK time range (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab1</td>
<td>1000 mg intravenously</td>
<td>1.06 (0.84–1.27)</td>
<td>1–816</td>
</tr>
<tr>
<td></td>
<td>300 mg intravenously</td>
<td>0.90 (0.85–0.94)</td>
<td>2–816</td>
</tr>
<tr>
<td></td>
<td>100 mg intravenously</td>
<td>1.09 (0.95–1.22)</td>
<td>1–312</td>
</tr>
<tr>
<td></td>
<td>300 mg subcutaneously</td>
<td>1.70 (1.63–1.77)</td>
<td>24–648</td>
</tr>
<tr>
<td>Mab2</td>
<td>1000 mg intravenously</td>
<td>2.77 (2.58–2.96)</td>
<td>1–168</td>
</tr>
<tr>
<td>Mab3</td>
<td>20 mg/kg intravenously</td>
<td>1.31 (1.02–1.59)</td>
<td>1–696</td>
</tr>
<tr>
<td>Mab4</td>
<td>20 mg/kg intravenously</td>
<td>5.83 (5.73–5.92)</td>
<td>0.5–336</td>
</tr>
</tbody>
</table>
data and does not require assumptions of ideal, first-order kinetic behavior. Implicit in these calculations, however, is the assumption that the presence of M5 on only one of the two heavy chains of a Mab is sufficient to ensure faster clearance via mannose receptor.

The apparent elimination rate of therapeutic Mabs can be influenced by the dose. Figure 5A shows the pharmacokinetic clearance curves for Mab1, as measured by ELISA, as a function of dose and route of administration. Figure 5B plots these data using a logarithmic concentration scale. A linear trend line was fitted to these data on the assumption that the terminal elimination phase was first order, and elimination half lives were estimated. For intravenous dosing, half lives ranged from 15.8 days at 100 mg intravenously to 26.0 days at 1000 mg intravenously.

The M5-containing population was calculated to clear faster than bulk Mab1 at every dose (Figure 5C). The elimination half lives vary from 7.0 days to 10.1 days to 14.2 days at intravenous doses of 100, 300 and 1000 mg, respectively, and are reduced ~2-fold compared with those for Mab1.

Discussion

In this study, we provide clear evidence for increased serum clearance of therapeutic IgG-containing high-mannose glycans, but not other glycan forms, in humans. A previous study from this group (Chen et al. 2009) discovered
conversion of larger (M6–M9) high-mannose glycans to M5 in vivo, but concluded that none of the glycans, including high mannose, impacted antibody clearance over the time range studied. The apparent discrepancy between our current findings and this prior study using a different analytical methodology, but otherwise similar conditions, is instructive and worth examining in more detail.

The present study utilizes high-resolution mass spectrometry to quantify the glycovariants of the N297 Fc glycopeptide of human IgG. When used for therapeutic IgG2, this method is subject to interference primarily by endogenous human IgG2 that may have copurified because the corresponding IgG1 peptide has a different amino acid sequence. IgG2 is present in human serum at an average concentration of 4 mg/mL (French 1986), whereas the concentrations of Mab in our PK studies approached 6 µg/mL at the latest time points studied. The fact that the purity of Mab recovered from serum samples remained >90%, as inferred from the constancy of the major Mab glycoforms G0F and G1F (Figure 1, Table III) speaks to both the stringency of our ligand-based affinity purification procedure and the specificity resulting from using high-resolution MS to monitor IgG2-specific glycopeptides. In contrast, any method that monitors released glycans, such as the reversed-phase analytical method employed previously (Chen et al. 2009), is subject to interference from glycans released from any glycoprotein impurities that might be present as well as from glycans released from other sites on the therapeutic antibody. Such glycans might arise from co-purifying endogenous IgG of any class or subclass or non-consensus Fab glycans from the therapeutic antibody. The improved specificity of the present study results in greater sensitivity and allowed Mab1 clearance studies at a dose of 1000 mg intravenously to be followed for up to 34 days as opposed to 13 days previously (Chen et al. 2009). This is important because the rapid conversion of M6–M9 glycans to M5 glycan during the first few days after dosing results in a temporary increase in M5 (Chen et al. 2009), which counteracts the subsequent downward trend in M5 due to enhanced clearance and effectively prevents the establishment of a clear trend with time to be established by day 13 (Figure 1, arrow). Especially at the high dose of 1000 mg intravenously, it can take 7–10 days of dosing for M5 percentages to return to those of the dosed Mab. Data from longer time points (as long as 34 days in the present study) are required to establish the clear downward trend in M5 percentage. Such data collection is facilitated by the high specificity, and resultant sensitivity, of the present analytical methodology. It is possible that if the short-lived M6–M9 glycoform levels had been mathematically added to M5 in the earlier study, the faster clearance of high-mannose glycoforms would have been apparent even in that 13 days study.

When used to study Mab4, an IgG1, the current methodology, is subject to interference from endogenous IgG1 which is more abundant than endogenous IgG2 and, therefore, might be expected to result in lower purity of glycopeptides. There is no indication in our data (not shown) that this compromises our results as the levels of the major G0F, G1F and G2F glycans, which also differ diagnostically between recombinant monoclonal IgG1 and endogenous human IgG1, show no evidence for dilution by endogenous human IgG1 as the sample concentration decreases with time in circulation.

The faster clearance of M5, relative to other, glycoforms is an important consideration for the use of IgG as therapeutics. The proportion of Mab, which contains at least one M5 glycan and is presumably subject to faster clearance, can be calculated from the heavy-chain M5 glycan content and the PP of M5-containing heavy chains. The former can be most specifically determined by peptide mapping and the latter can be estimated by intact mass analysis of Mab. In the present study, we observe heavy-chain M5 content ranging from 4 to 17% and a strong tendency of M5 heavy chains to pair with each other (PP of 6–50, Table V). It is not known at present how general such a strong relative PP is among other therapeutic Mabs.

The difference in elimination half-life between Mab1 and the M5-containing Mab1 population increases with decreasing dose (Table VII), indicating that M5-containing IgGs are cleared “relatively” faster at lower intravenous doses. We presume that mannose receptor contributes to the faster clearance of the M5 IgG population and the slower relative clearance at higher doses may reflect saturation of this receptor. Although the half-life of serum IgG is generally mediated by FcRn and that of therapeutic IgGs may additionally be modulated by target-based clearance, the mannose receptor apparently contributes to more rapid clearance of non-natural (high-mannose) glycan variants of therapeutic IgGs. This is consistent with the role played by mannose receptor in the clearance of exogenous pathogens as well as unwanted endogenous molecules (Allavena et al. 2004) and is supported by earlier studies demonstrating faster clearance of M5-containing IgG1 in mice (Wright and Morrison 1994; Kanda et al. 2007). Our study suggests that, in spite of generally being regarded as sequestered inside the C1 domain, IgG1 and IgG2 Fc M5 glycans appear sufficiently accessible to interact with mannose receptors.

Conflicting conclusions have been drawn on the impact of glycan removal on antibody clearance (Tao and Morrison 1989; Wawrzynczak et al. 1992). Lack of Fc glycans has previously been shown to compromise the stability of the molecule (Mimura et al. 2000), perhaps translating into faster clearance via proteolysis. In the present studies, changes in the relative amount of the non-glycosylated heavy chain in vivo were minor, which would result in little impact on clearance overall.

### Table VII. Faster relative elimination of M5-containing population of Mab1 with decreasing dose demonstrated using data from Figure 5

<table>
<thead>
<tr>
<th>Dosing regimen</th>
<th>Mab t1/2 (d)</th>
<th>M5 t1/2 (d)</th>
<th>Mab t1/2/M5 t1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg intravenously</td>
<td>26.0</td>
<td>14.2</td>
<td>1.83</td>
</tr>
<tr>
<td>300 mg intravenously</td>
<td>17.8</td>
<td>10.1</td>
<td>1.76</td>
</tr>
<tr>
<td>1000 mg intravenously</td>
<td>15.8</td>
<td>7.0</td>
<td>2.26</td>
</tr>
<tr>
<td>300 mg subcutaneously</td>
<td>24.8</td>
<td>12.2</td>
<td>2.03</td>
</tr>
</tbody>
</table>

The elimination half-life for Mab increases relative to that of M5-containing Mab1 with decreasing dose, indicating that the M5-containing population is being eliminated relatively faster.
AUC, and therefore potentially efficacy, of a therapeutic IgG can be affected by faster clearance of M5 glycoforms. The impact will be greater when M5 elimination rates are relatively faster and when bulk IgG elimination rates are slower (Goetze et al. 2010). In practice, as Figure 5 shows, these factors tend to counteract each other to a large degree, as the therapeutic dosages are varied. For example, as the intravenous dose is increased from 100 to 1000 mg, the overall half-life of circulating Mab1 increases (Figure 5B), which would normally allow the decreasing M5 content to exert a larger influence on total AUC. However, in parallel, the relative rate of M5 clearance declines (Table VII), which decreases the relative influence this has on total AUC. As a result of these counteracting and largely offsetting influences, relatively modest impacts on AUC of 1–6% are estimated for the therapeutic IgGs in this study (Table VI). However, an even larger impact on efficacy may be possible in other circumstances (Figure 4).

Quantitatively, the degree to which high-mannose glycans contribute to increased clearance of therapeutic antibodies is determined by the inherent half-life of the antibody, the heavy-chain high-mannose content, the heavy-chain PP and the dosing regimen. We have observed, under some conditions, recombinant Mabs with up to 20% high-mannose content. Such levels, in combination with the factors mentioned in the first sentence of this paragraph, have the potential to alter the systemic exposure of therapeutic Mabs. Although the presence of high-mannose glycans does appear to impact the systemic exposure of therapeutic Mabs, the impact is relatively minor within the range of high mannose observed in the antibodies studied here, such that the effect would likely be difficult to quantify in a typical pharmacokinetic study in animals or humans. Mab4, with a high-mannose content of 17%, and a resultant 6% decrease in AUC between 0.5 h and 14 days when dosed at 20 mg/kg intravenously, represents an example on the high end of what was studied here. If high-mannose content were to vary beyond these ranges, the impact would become more significant.

Under the quality by design paradigm (Kozlowski and Swann 2009), high-mannose content of therapeutic Mabs should be considered an important product quality attribute that can impact the efficacy of the molecule.

Materials and methods

Materials

The recombinant human IgG Mabs used in this study were produced at Amgen (Thousand Oaks, CA) and consist of two gamma heavy chains and two kappa light chains. Mab1 (IgG2), Mab 2 (IgG2) and Mab4 (IgG1) target cell surface receptors, whereas Mab3 (IgG2) targets a soluble growth factor. Mabs were expressed in CHO cells and highly purified using protein A affinity and other chromatographic modalities that may include hydrophobic interaction and ion exchange using well-established protocols (Shukla et al. 2007). Acetonitrile and trifluoroacetic acid (TFA) were HPLC-reagent grade. Actigel ALS Superflow was from Sterogene Bioseparations (Carlsbad, CA). Endoproteinase Lys-C was from Wako (Osaka, Japan) and TPCK-treated trypsin was from Thermo Scientific (Rockford, IL). RapiGest™ was from Waters Corp (Milford, MA) and tris-[2-carboxyethylphosphine]hydrochloride (TCEP) was from Calbiochem (San Diego, CA). All other chemicals were reagent grade.

Human PK studies

Mab1 was administered to adult human subjects as either a single 100, 300 or 1000 mg intravenous injection or as a single subcutaneous injection of 300 mg. Mab2 was similarly administered as a single 1000 mg intravenous injection. Mab3 and Mab4 were administered to adult human subjects as single intravenous doses of 20 mg/kg. Blood samples were collected over several weeks at the time points indicated, allowed to clot and the clot was separated from serum by centrifugation (2000 × g for 15 min). Serum was stored in cryotubes at −20°C or colder until use. Concentrations of Mabs in serum were determined with appropriate sandwich ELISAs using two in-house anti-idiotype antibodies against each Mab.

Mab ligand affinity purifications

Ligand-based affinity purification of Mabs was carried out essentially as described previously. Briefly, a 0.5 mL aliquot of freshly clarified human serum-containing Mab was diluted with 4.5 mL of phosphate-buffered saline (PBS) and incubated with 0.2 mL of Mab-ligand resin. Soluble forms of the appropriate receptor were used as the ligand when the Mab target was a cellular receptor. After 4 h incubation at room temperature, the resin was washed with PBS containing 0.5 M NaCl and eluted with 10 mM glycine, pH 1.5. The eluate pH was immediately adjusted to 7 with 1 M Tris–HCl, pH 8 (Liu et al. 2008).

Enzymatic digestion

One of following two alternate procedures was employed. (i) To a microfuge tube containing 360 mg of urea, 1–2 mg of methionine, 100 µL of 1 M NaH2PO4 and 100 µL of 0.4 M NH2OH–HCl, 0.5 mL of ligand affinity-purified Mab (6–300 µg of Mab) was added. After vortexing to dissolve the urea, 10 µL of 40% TFA was added and the sample was denatured by incubating in this low pH buffer at 37°C for 1 h. The sample was neutralized by the addition of 144 µL of 1 N NaOH, followed by sequential addition of 268 µL water and 12 µL of 100 mM ethylenediaminetetraacetic acid. Finally, 20 µg of Lys-C was added, the sample was overlaid with nitrogen gas and digested for 20 h at 37°C. This protocol was employed only for Mab3. (ii) Mab sample eluted from ligand affinity chromatography by low pH was brought to pH 6 by the addition of 2-(N-morpholino)ethanesulfonic acid (MES), pH 6, to 20 mM and MES base to 80 mM. One percent of RapiGest (Yu et al. 2003) was added to achieve a final concentration of 0.05% and then the sample was denatured and reduced by adding TCEP to 136 µM followed by heating at 60°C for 30 min. Samples were then digested with 1:20 (w/w) trypsin for 18 h at 37°C. RapiGest was removed by the addition of TFA to 0.5%, followed by 40 min incubation at 37°C and centrifugation (Huang et al. 2009). This procedure was employed only for Mab3.
LC-MS/MS analysis

Two alternative procedures were employed as follows. (i) LC-MS/MS analyses were performed on an Agilent 1200SL HPLC system directly connected to a Thermo-Scientific LTQ-Orbitrap high-resolution mass spectrometer. About 1–10 μg of each digested Mab was injected. A Waters BEH300 C18, 1.7 μ, 2.1 × 100 mm column was used at 50°C at a flow rate of 0.3 mL/min. A gradient from 0.5–20%B in 40 min, then 20–40%B in 80 min, then 40–99%B in 6 min was used, where solvent A is 0.04% TFA and solvent B is 0.94% TFA in acetonitrile. MS data were collected using full MS scan in centroid mode with 60,000 resolution followed by three data-dependent MS/MS scans in centroid mode with dynamic exclusion, using collision-induced dissociation. All data analyses, including peptide identification and quantification, were performed using a custom-written program, MassAnalyzer (Zhang 2009). Glycopeptides were identified automatically by MassAnalyzer based on their accurately determined masses, their fragmentation pattern when compared with accurately predicted patterns (Zhang and Shah 2010) and biosynthetic restrictions of glycan structures. This approach was used for Mab1, Mab2 and Mab3. (ii) LC-MS/MS analyses were performed on an Agilent 1100 HPLC system directly connected to a Thermo-Scientific LTQ electrospray ion trap mass spectrometer. About 15 μg of each sample was injected onto a Jupiter C5, 5 μ, 2 × 150 mm column flowing at 0.2 mL/min and 50°C. A gradient from 2–22%B over 42 min followed by 22–42%B over 80 min was used, where solvent A is 0.05% TFA and solvent B is 0.045% TFA in acetonitrile. MS data were acquired using alternating “triple-play” mode: a full scan in positive mode followed by data-dependent ultra zoom scan followed by data-dependent MS/MS scan in the centroid mode. MassAnalyzer software developed in-house (Zhang 2009) was used for peptide identification and quantification. This protocol was used for Mab4.

Quantification of Fc glycopeptides

For Mab1 and Mab2 samples (both IgG2s), the MS peak areas of the glycovariants of the Lys-C generated TKPREEQNSTFRVSVLTVHQDWLNGK IgG2 heavy-chain peptide (EU #289-317) containing the consensus Fc glycosylation site were compared and the amount of each glycovariant as a percent of the total was calculated automatically with MassAnalyzer (Zhang 2009). For Mab4, an IgG1, the homologous TKPREEQVNSTYRVSVLTVHQDWLNGK peptide, was similarly analyzed. For Mab3, an IgG2, the trypsin generated EEQFNSTFR peptide (EU #293-301), was analyzed. Previously, a good correlation was established between UV and MS detectors for quantification of Fc glycopeptides, including M5 (Rehder et al. 2006). Therefore, only MS-based quantification was used in this study. Glycan nomenclature is as described previously (Chen et al. 2009).

Intact mass analysis of Mabs

Purified Mabs from stock solutions were diluted to 1 mg/mL with 50 mM Tris–Cl, pH 8, followed by mixing with an equal volume of 50% acetonitrile, 0.1% TFA; 25 μg of samples was injected onto a Poros R1/10, 4.6 × 50 mm column flowing at 0.2 mL/min at 75°C. A gradient of 30–50%B over 28 min was used where solvent A is 0.1% TFA and solvent B is 0.1% TFA in 90% acetonitrile. MS analysis was conducted on a Waters LCT Premier TOF instrument operating in positive ion and “V” mode. The capillary and cone voltages were set at 2500 and 150 V, respectively. The desolvation and source temperatures were set at 300 and 100°C, respectively. All other voltages were optimized to provide maximal signal intensity. The instrument was calibrated with Na TFA. Raw data were processed using Waters MassLynx MaxEnt 1 software to obtain deconvoluted mass spectra containing molecular weight values.

Estimation of M5-containing Mab population

Integrated MS ion intensities of the deconvoluted peaks corresponding to intact Mab with M5:M5 glycoforms and with M5:G0F glycoforms were compared with the ratio expected from the glycopeptide analysis assuming random pairing of heavy chain glycoforms to estimate the percentage of each Mab that contained at least one M5 glycan. Further details are described in Results.

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Conflict of interest

None declared.

Abbreviations

AUC, area under the curve; CE-SDS, capillary electrophoresis sodium dodecyl sulfate; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; ESI-TOF, electrospray ionization time-of-flight; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; LC, liquid chromatography; M5, mannose 5; Mab, monoclonal antibody; MES, 2-(N-morpholino)ethanesulfonic acid; MS, mass spectrometry; PBS, phosphate-buffered saline; PK, pharmacokinetic; PP, pairing preference; TCEP, tris[2-carboxyethylphosphine]hydrochloride; TFA, trifluoroacetic acid.

References


