

Impact of variable domain glycosylation on antibody clearance: An LC/MS characterization

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Abstract

Variable (Fv) domain N-glycosylation sites are found in approximately 20% of human immunoglobulin Gs (IgGs) in addition to the conserved N-glycosylation sites in the C_H2 domains. The carbohydrate structures of the Fv glycans and their impact on in vivo half-life are not well characterized. Oligosaccharide structures in a humanized anti-A β IgG1 monoclonal antibody (Mab) with an N-glycosylation site in the complementary determining region (CDR2) of the heavy chain variable region were elucidated by LC/MS analysis following sequential exoglycosidase treatments of the endoproteinase Lys-C digest. Results showed that the major N-linked oligosaccharide structures in the Fv region have three characteristics (core-fucosylated biantennary oligosaccharides with one or two *N*-glycolylneuraminic acid [NeuGc] residues, zero or one α -linked Gal residue, and zero or one β -linked GalNAc residue), whereas N-linked oligosaccharides in the Fc region contained typical Fc glycans (core-fucosylated, biantennary oligosaccharides with zero to two Gal residues). To elucidate the contribution of Fv glycans to the half-life of the antibody, a method that allows capture of the Mab and determination of its glycan structures at various time points after administration to mice was developed. Anti-A β antibody in mouse serum was immunocaptured by immobilized goat anti-human immunoglobulin Fc(γ) antibody resin, and the captured material was treated with papain to generate Fab and Fc for LC/MS analysis. Different glycans in the Fc region showed the same clearance rate as demonstrated previously. In contrast to many other non-antibody glycosylated therapeutics, there is no strong correlation between oligosaccharide structures in the Fv region and their clearance rates in vivo. Our data indicated that biantennary oligosaccharides lacking galactosylation had slightly faster clearance rates than other structures in the Fv domain.

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Glycosylation of an Asn residue or a Ser/Thr residue represents an important posttranslational modification of proteins. Immunoglobulin G (IgG)¹ antibodies are glycoproteins with an N-linked carbohydrate in the crystalliz-

able (Fc) domain, and the role of this carbohydrate in biological activities has been studied extensively. Although the Fc glycan has an important effect on antibody effector functions, it has a minimal impact on the half-life of the

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¹ Abbreviations used: IgG, immunoglobulin G; Fc, crystallizable domain; Fv, variable domain; Mab, monoclonal antibody; CDR2, complementary determining region 2; Gal, galactose; HEXase I, β -*N*-acetylhexosaminidase; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; TMB, tetramethylbenzidine; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GlcNAc, *N*-acetylglucosamine; NeuGc, *N*-glycolylneuraminic acid; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; CHO, Chinese hamster ovary; GalNAc, *N*-acetylgalactosamine; HexNAc, *N*-acetylhexosamine; Man, mannose; Fab, antigen-binding fragment; MALDI-MS, matrix-assisted laser desorption/ionization MS; CE, capillary electrophoresis; ASGP-R, hepatic asialoglycoprotein receptor; PNGase F, endoglycosidase F; FcRn, neonatal Fc receptor.

antibody or its antigen binding [1]. Approximately 20% of human IgGs also have the N-glycosylation motif Asn-Xxx-Thr/Ser in the variable (Fv) region [2], which has been proposed to arise from somatic mutation because the motif is absent in the germline [3]. There have been only a few reports describing the occupancy of this site with an oligosaccharide and its role on the function of the antibody, with most of the reports focusing on impact of the glycan on affinity or its compositional characteristics [4–8]. Coloma and coworkers [9] examined a series of anti-(1–6) dextran antibodies with site-specific glycosylation and their effects on half-life and organ targeting, but the carbohydrate structures have not been investigated in detail.

In the current work, we have characterized the carbohydrate structures in the Fc and Fv domains of an anti-A β humanized monoclonal antibody (Mab) using LC/MS techniques. As has been seen for other IgGs [10,11], the Fc glycan is a core-fucosylated, biantennary complex oligosaccharide with minimal sialic acid content. The glycosylation site in complementary determining region 2 (CDR2) of the heavy chain is fully occupied with core-fucosylated biantennary oligosaccharides and heterogeneous terminal sialylation (1.6–2.5 mol sialic acid residues/mol antibody).

For many of the non-antibody glycoproteins, it has been well established that a highly sialylated structure with minimally exposed galactose (Gal) residues is critical for long half-life due to asialoglycoprotein receptor-mediated clearance [12]. In the case of erythropoietin, it has been further demonstrated that a heterogeneous glycoform mixture can be fractionated to separate variably sialylated glycoforms that have different in vivo half-lives, with the more completely sialylated species having longer exposure [13].

Given the importance of sialylation to clearance properties of non-antibody glycoproteins, the impact of variable sialic acid content in the Fv glycan on the antibody half-life was investigated in the current study through carbohydrate profiling of the antibody isolated at various time points after administration to rodents by LC/MS analysis following papain treatments. If Fv glycans contributed to the half-life, it would be expected that samples isolated at later time points would show oligosaccharide profiles different from those of earlier time point samples.

Materials and methods

Materials

Humanized anti-A β Mab expressed in SP2/0 myeloma cell lines was generated at Eli Lilly (Indianapolis, IN, USA). Endoproteinase Lys-C, papain, and neuraminidase (*Atherobacter ureafaciens*) were obtained from Roche Diagnostics (Indianapolis, IN, USA). Exoglycosidases, β -galactosidase (*Streptococcus pneumoniae*), recombinant β -N-acetylhexosaminidase (*S. pneumoniae*) (or HEXase I), α -galactosidase (green coffee bean), and α -mannosidase (jack bean) were products of Prozyme (San Leandro, CA, USA). CNBr-activated Sepharose 4 Fast Flow was

obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). AffiniPure goat anti-human IgG Fc γ and horseradish peroxidase (HRP)-conjugated anti-human Fc γ were products of Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The immobilized goat anti-human IgG Fc γ Sepharose resin (10 mg antibody/ml resin) was prepared according to standard procedures provided by the supplier. A β (1–40) was purchased from Biosource International (Camarillo, CA, USA). Lyophilized powder was dissolved in purified water to a final concentration of 1 mg/ml, and single-use aliquots were stored at –70 °C. All other chemicals were commercially available analytical-grade reagents.

Methods

Rodent study: Mab administration and sample collection

Anti-A β Mab (100 μ g/mouse) was intraperitoneally administered to 3-month-old APP V717F transgenic mice. At various times after antibody administration, whole blood was collected via the retroorbital sinus method and plasma was stored at –20 °C until ready for use.

ELISA for Mab quantitation

An antigen capture enzyme-linked immunosorbent assay (ELISA) method was used for estimation of Mab concentrations in plasma samples. Greiner polystyrene microtiter plates were coated with 1 μ g/ml A β (1–40) in 0.05 M sodium carbonate (pH 9.6) and incubated overnight at 37 °C. Plates were washed with wash buffer (0.02 M Tris [pH 7.4], 0.15 M NaCl, and 0.1% Tween 20) and incubated with blocking buffer (10 mg/ml bovine serum albumin [BSA] in wash buffer) for 1 h. Blocking buffer was used to prepare serial dilutions of standards (0.03–500 μ g/ml) or plasma samples. After 1 h incubation with antibody samples, plates were washed and incubated with goat anti-human Fc HRP. After another hour of incubation at 25 °C, plates were developed with freshly prepared tetramethylbenzidine (TMB)/enzyme substrate and OD₆₅₀ nm read after 20 min using a microplate spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Results were analyzed with SOFTmax software (Molecular Devices).

Anti-A β Mab capture from mouse plasma

Approximately 50 μ l (or 300 μ l in the case of the 48-h time point) of each plasma sample was mixed with approximately 2 μ l of immobilized goat anti-human immunoglobulin Fc γ antibody resin, and the solution was incubated at ambient temperature with gentle shaking for 2 h. The resin was separated by centrifugation, and the supernatant was discarded. The resin was washed with phosphate-buffered saline (PBS) buffer containing 0.2% Tween 20, followed by three additional washes with PBS buffer. The antibody was released from the resin by treatment with 20 μ l of 0.15% aqueous formic acid solution at ambient temperature for 15 min. The supernatant containing the captured antibody was collected for papain digestion.

Papain digestion of captured anti-A β Mab

Each supernatant (20 μ l) containing anti-A β Mab was mixed with 20 μ l of 0.1 M Tris, 2 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM cysteine buffer (pH 7.5), to which 2 μ l of 0.1 mg/ml papain solution was added. The mixture was incubated at 37 °C for 1 h before LC/MS analysis.

Lys-C digestion of anti-A β Mab

Anti-A β Mab (40 μ g) was reconstituted in 5 μ l of 6 M guanidine HCl–0.5 M Tris buffer (pH 8.0) with 30 mM dithiothreitol (DTT). The solution was incubated at 37 °C for 30 min to denature and reduce the antibody molecule. The reduced Mab was alkylated by adding 10 μ l of 10 mM ammonium bicarbonate buffer containing 55 mM iodoacetamide, followed by incubation at ambient temperature in the dark for 10 min. The solution was diluted with 10 mM ammonium bicarbonate buffer to a final volume of 200 μ l. The Mab solution was treated with 10 μ l of 0.1 mg/ml Lys-C solution at 37 °C overnight (~15 h). The digest was heated at 100 °C for 5 min to eliminate the remaining enzymatic activity.

Exoglycosidase digestions of the Mab Lys-C digest

Multiple 80 μ l-aliquots of the Lys-C digest of anti-A β Mab were mixed with 20 μ l of 250 mM sodium phosphate buffer (pH 6.0) and treated with 1–2 μ l each of one or more of the following enzymes at 37 °C for 18 h to sequentially release the various carbohydrates: neuraminidase, α -galactosidase, β -galactosidase, HEXase I, and α -mannosidase.

Reversed-phase HPLC chromatography

An Agilent 1100 capillary HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used to analyze the Lys-C digest of anti-A β antibody as well as the digest treated with exoglycosidases. The HPLC column was a Zorbax 300SB C8 (1 $\times$ 50 mm, 3.5 μ m particle size, Agilent Technologies). The flow rate was 20 μ l/min, and the column was at ambient temperature. The solvents were as follows: solvent A, 0.15% aqueous formic acid solution; solvent B, 0.12% formic acid in acetonitrile. The column was equilibrated in 0% B. After the sample was injected, the gradient was linearly increased to 20% B over 5 min, to 25% B over 25 min, and to 90% B over 2 min and then was subsequently held at 90% B for 3 min before being rapidly returned to initial conditions.

A Waters Alliance HPLC system (model 2690, Waters, Milford, MA, USA) was used to separate the papain digests of the captured material. The HPLC column was a Zorbax 300SB C18 (2.1 $\times$ 150 mm, 5 μ m particle size, Agilent Technologies). The flow rate was 200 μ l/min, and the column was at ambient temperature. Prior to sample injection, the column was equilibrated with 20% B for 10 min. After the injection of sample, a gradient elution was performed from 20 to 35% B over 40 min and then to 90% B over 3 min before being held at 90% B for 1 min and then rapidly returned to initial conditions.

Mass spectrometry

LC/MS analysis of the exoglycosidase-treated Lys-C digests was performed on a Micromass Q-TOF spectrometer (Micromass, Manchester, UK) with the following settings: capillary voltage, 3.0 kV; cone voltage, 25 V; collision energy, 5 eV; mass range, 300–2000 Da; desolvation temperature, 250 °C; desolvation gas flow, 300 L/h. The HPLC effluent was introduced directly into the mass spectrometer without splitting the stream.

The papain digest of immunocaptured anti-A β Mab was analyzed using a Micromass Q-TOF Ultima Global mass spectrometer equipped with a nebulizer-assisted electrospray source. Q-TOF was operated with the following settings: capillary voltage, 3.15 kV; cone voltage, 100 V; collision energy, 10 eV; mass range, 800–2000 Da; desolvation temperature, 400 °C; desolvation gas flow, 600 L/h.

Results and discussion

Approximately 20% of human IgGs contain an additional N-glycosylation site in the Fv region besides the N-glycosylation in the Fc region [2], and the location of the glycosylation site has an impact on glycan structures in recombinant antibodies [9]. Anti-A β Mab contains a consensus sequence, Asn56-Ser-Thr58, in the V_H CDR2. LC/MS analysis indicated that this potential N-glycosylation site is fully occupied. No O-linked glycosylation was detected in this Mab. To elucidate the oligosaccharide structures at this site as well as the common glycosylation site in the Fc region, the Mab was denatured, S-carboxamidomethylated, and then treated with endoproteinase Lys-C. The Lys-C digest was analyzed by capillary HPLC/MS.

Figs. 1A and B show the deconvoluted mass spectra of glycopeptides 44–65 and 284–312, respectively. Glycan formulas, listed in Table 1, were calculated based on observed glycopeptide masses and expected mass of the peptide backbone. The relative distribution of these glycans was obtained according to each glycopeptide mass peak area, and these results are also listed in Table 1. Major oligosaccharide structures, listed in Table 2, were elucidated by mass spectrometric analysis following sequential exoglycosidase digestion of the respective glycopeptides.

Glycoforms from the Fc region of anti-A β Mab

Glycopeptide 284–312 has a simple mass profile. Only three major masses were detected—at 4907.1, 5069.1, and 5231.1 Da—corresponding to the peptide 284–312 plus oligosaccharide formulas [HexNAc]₄[Hex]_{3–5}[dHex]₁. These formulas could be assigned to biantennary oligosaccharides with zero to two β -Gal residues (Table 2) based on well-documented literature [1,9]. Sequential exoglycosidase digestion followed by LC/MS analysis also confirmed the assignments (Fig. 2). β (1–4)Galactosidase (*S. pneumoniae*) converted masses 5069 and 5231–4907 Da (i.e., removing one and two terminal β [1 $\rightarrow$ 4]Gal residues), and further HEXase I treatment removed two terminal β -linked

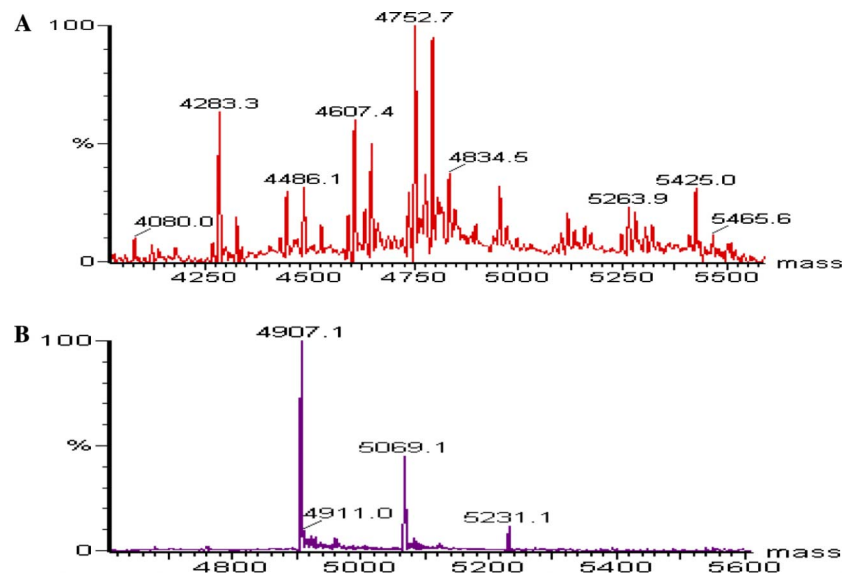


Fig. 1. Deconvoluted mass spectra of V_H glycopeptide 44–65 (A) and Fc glycopeptide 284–312 (B) of anti-Aβ Mab. The glycopeptides were obtained by treating with Lys-C for reduced and carboxamidomethylated anti-Aβ Mab.

Table 1
Glycan formulas in each N-glycosylation site of anti-Aβ Mab and their relative percentages

Mass found	Mass expected	Oligosaccharide formula					Relative percentage
		NeuGc	NeuAc	HexNAc	Hex	Fuc	
<i>V_H glycosylation, glycopeptide 44–65</i>							
3813.8	3814.0	0	0	4	3	1	2
4283.1	4283.4	1	0	4	4	1	9
4324.1	4324.4	1	0	5	3	1	3
4445.0	4445.5	1	0	4	5	1	4
4486.0	4486.6	1	0	5	4	1	4
4527.2	4527.6	1	0	6	3	1	2
4591.6	4591.7	1	0	4	5	2	3
4607.4	4607.7	1	0	4	6	1	10
4632.0	4632.7	1	0	5	4	2	3
4647.9	4648.7	1	0	5	5	1	7
4736.4	4736.8	1	1	4	5	1	4
4752.5	4752.8	2	0	4	5	1	14
4776.9	4777.8	1	1	5	4	1	4
4793.5	4793.8	2	0	5	4	1	13
4833.9	4834.9	2	0	6	3	1	4
4955.3	4956.0	2	0	5	5	1	2
5263.7	5264.3	2	0	5	6	2	4
5279.7	5280.3	2	0	5	7	1	3
5304.2	5305.3	2	0	6	5	2	2
5320.6	5321.3	2	0	6	6	1	2
5424.7	5426.4	2	0	5	7	2	2
<i>Fc glycosylation, glycopeptide 284–312</i>							
4906.9	4907.2	0	0	4	3	1	60
5068.9	5069.4	0	0	4	4	1	29
5231.1	5231.5	0	0	4	5	1	10
5393.2	5393.7	0	0	4	6	1	1

N-acetylglucosamine (GlcNAc) residues, shifting mass from 4907 to 4501 Da. Besides these three major glycans, glycosylation at the Fc region also contains a very low amount of α-linked Gal residue, determined by glycopeptide mass at 5393.2 Da, which disappeared after treatment

with α-galactosidase. However, this site does not contain any significant sialylated glycans, as confirmed by neuraminidase treatment. The oligosaccharide formulas, along with the relative distribution of each glycan, are shown in Table 1.

Glycoforms in Fab of anti-Aβ Mab

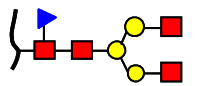
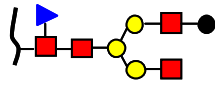
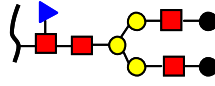
The mass profile (Fig. 1A) of glycopeptide 44–65 is much more complex than that of glycopeptide 284–312. Glycan formulas, listed in Table 1, were calculated based on obtained glycopeptide masses and expected mass of the peptide backbone. Besides the glycan structures, their relative distribution was also obtained according to the intensity of each glycopeptide mass peak (Table 1). Glycan structures at Asn56 have several specific characteristics. First, unlike the glycans found in the Fc region, most of the glycans are sialylated with N-glycolylneuraminic acid (NeuGc). Second, several sialylated major glycans have a ratio of [Hex]/[HexNAc] that is either higher (1.5 for [NeuGc]₁[HexNAc]₄[Hex]₆[Fuc]₁, mass at 4607 Da) or lower (0.8 for [NeuGc]₂[HexNAc]₅[Hex]₄[Fuc]₁, mass at 4794 Da) than that for normal complex-type oligosaccharides (ratios of 1.25, 1.20, and 1.17 for bi-, tri- and tetraantennary oligosaccharides, respectively). Third, a few minor glycans contain more than one fucose (Fuc) residue. The major oligosaccharide structures listed in Table 1 were elucidated by mass spectrometric analysis following sequential exoglycosidase digestion of glycopeptide 44–65. An example of these experimental results is shown in Fig. 3. After treatment with neuraminidase, most of the oligosaccharide masses shifted to lower mass, with a decrease of 307 or 614 Da, corresponding to the mass of one or two NeuGc residues, respectively. These results indicated that sialic acid residues are NeuGc and not normal N-acetylneuram-

Table 2
Major oligosaccharide structures in each N-glycosylation site of anti-A β Mab

Mass found	Mass expected	Peptide 44–65 + structure	Relative percentage
4283.1	4283.4		9
4324.1	4324.4		3
4445.0	4445.5		4
4486.0	4486.6		4
4591.6	4591.7		3
4607.4	4607.7		10
4632.0	4632.7		3
4647.9	4648.7		7
4736.4	4736.8		4
4752.5	4752.8		14
4776.9	4777.8		4
4793.5	4793.8		13
4833.9	4834.9		4
5263.7	5264.3		4
5279.7	5280.3		3

(continued on next page)

Table 2 (continued)

Mass found	Mass expected	Peptide 284–312 + structure	Relative percentage
4907.1	4907.2		60
5069.1	5069.4		29
5231.1	5231.5		10

Note. For interpretation of the references to color in the table legend below, the reader is referred to the Web version of this article:
● Gal ■ GlcNAc ▲ Fuc ◆ NeuGc
● Man □ GalNAc ◆ NeuAc ○ Galα(1→3).

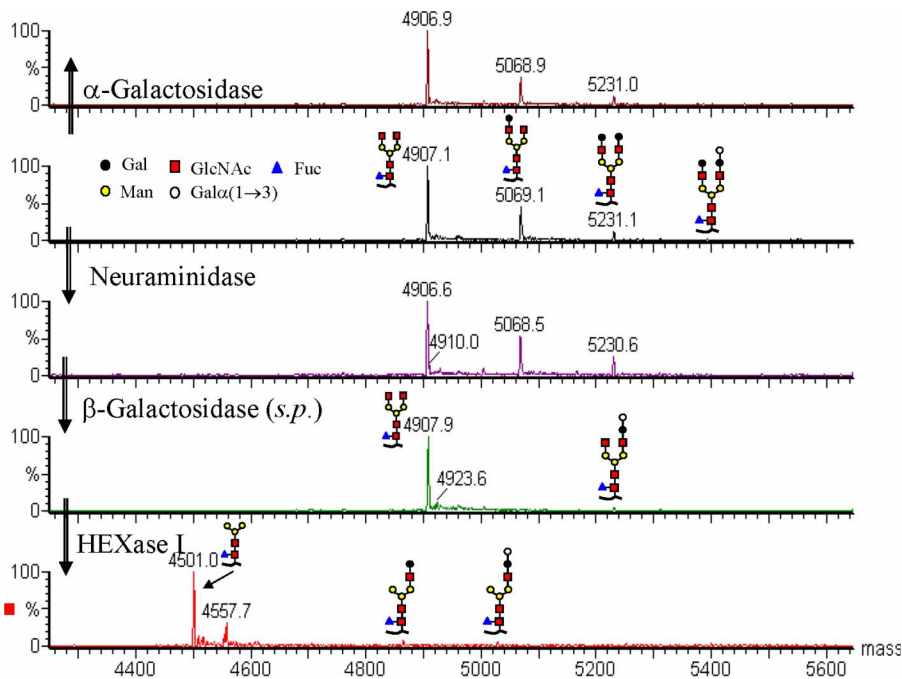


Fig. 2. Deconvoluted mass spectra of Fc glycopeptide 284–312 prior to and after treatment with exoglycosidases. Glycan formulas are depicted using symbols to represent the different monosaccharides attached to the peptide backbone depicted by a blue wriggle. The deconvoluted mass spectrum for the starting glycopeptide is the second panel from above. Spectra are marked with various exoglycosidases to allow easy interpretation of the peak masses and changes in glycan structure. Mass spectrum indicated with β -galactosidase was obtained following treatment of starting material with neuraminidase and β -galactosidase, and mass spectrum indicated with HEXase I was obtained following treatment with the three enzymes. The spectrum resulting from α -galactosidase treatment of starting glycopeptide is shown at the top. s.p., *S. pneumoniae*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inic acid (NeuAc), which has a residue mass of 291 Da. The type of sialic acid residues, whether NeuGc or NeuAc, depends on the cell line used for expression. Glycoproteins expressed in Chinese hamster ovary (CHO) cells predominantly contain NeuAc with only a few percentage NeuGc; however, glycoproteins from murine cell lines, such as SP2/0 and NS0, predominantly contain NeuGc [14].

Two major glycans, [NeuGc]₁[HexNAc]₄[Hex]₆[Fuc]₁ (4607 Da) and [NeuGc]₁[HexNAc]₅[Hex]₅[Fuc]₁ (4648 Da), were completely converted to [NeuGc]₁[HexNAc]₄[Hex]₅[Fuc]₁ (4445 Da) and [NeuGc]₁[HexNAc]₅[Hex]₄[Fuc]₁

(4486 Da) after treatment with α -galactosidase (i.e., one α -linked Gal was cleaved by the α -galactosidase). Because Gal α (1 $\rightarrow$ 3) linked to β -Gal will terminate antenna elongation of oligosaccharides [15], glycans containing α -linked Gal residue usually have a higher ratio of [Hex]/[HexNAc] than that of normal complex-type oligosaccharides. Glycans with a lower ratio (<1.1) of [Hex]/[HexNAc] may be due to a lack of galactosylation. However, glycan [NeuGc]₂[HexNAc]₅[Hex]₄[Fuc]₁ (mass at 4794 Da in Figs. 1A and 3) could not be explained by lack of galactosylation because it contains two sialic acid residues (i.e., it requires two Gal and/

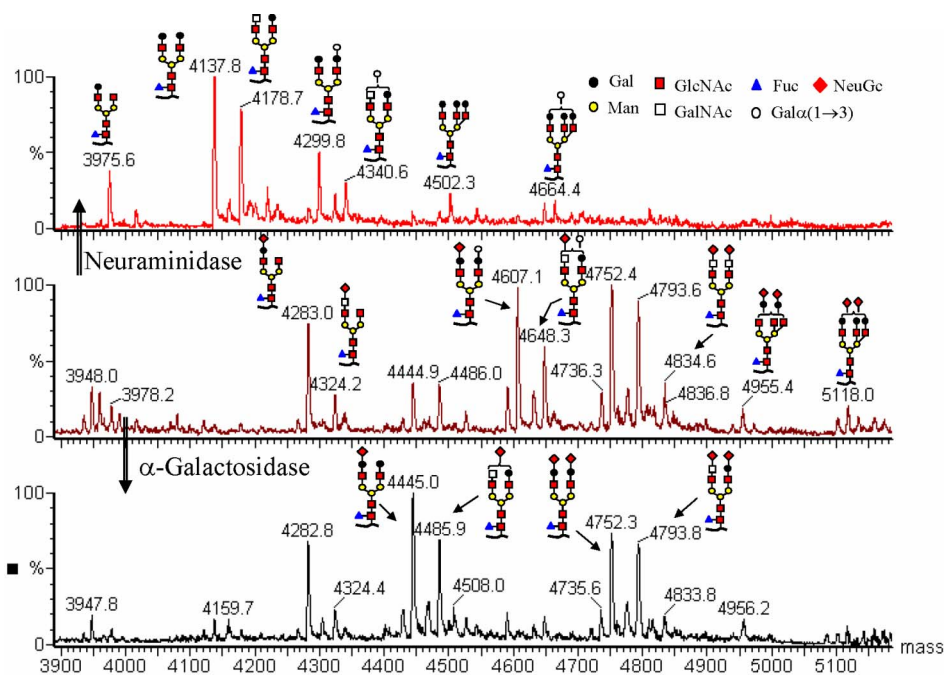


Fig. 3. Deconvoluted mass spectra of V_H glycopeptide 44–65 prior to and after treatment with neuraminidase and α -galactosidase. Only major peaks are labeled with oligosaccharide symbols. The mass spectrum of the starting glycopeptide, prior to exoglycosidase treatment, is shown in the middle.

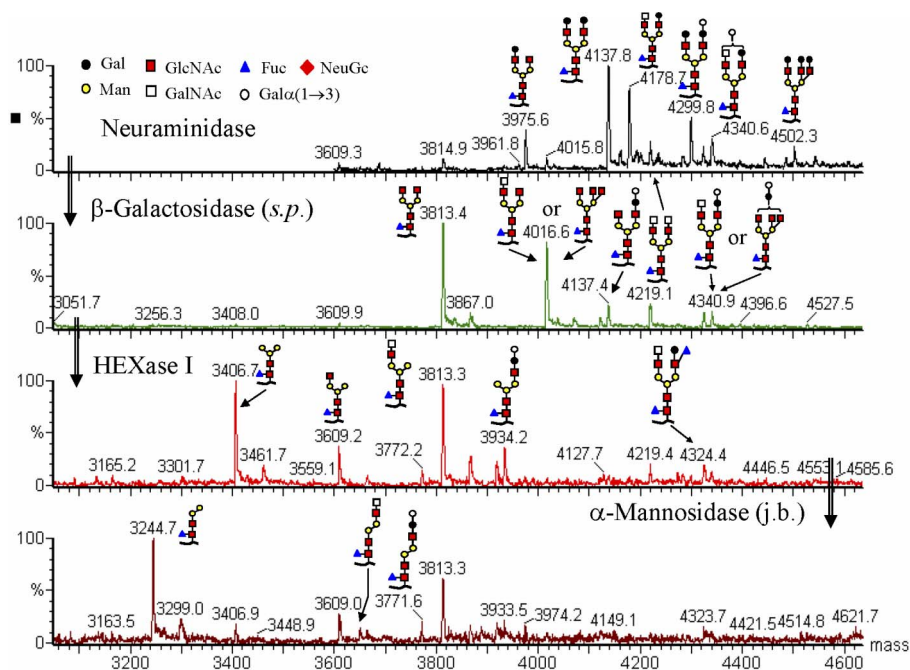


Fig. 4. Deconvoluted mass spectra of V_H glycopeptide 44–65 treated with neuraminidase followed by treatment with β -galactosidase, HEXase I, and α -mannosidase. The spectrum at the top of the figure is the same one shown at the top of Fig. 3 with additional exoglycosidase treatments indicated, as in Fig. 2. s.p., *S. pneumoniae*.

or *N*-acetylgalactosamine [GalNAc] residues). After subtracting the [GlcNAc]₂[Man]₃ pentasaccharide core structure of an *N*-glycan, glycan [NeuGc]₂[HexNAc]₅[Hex]₄[Fuc]₁ has only one hexose residue, which must be a Gal residue. Hence, one of the five *N*-acetylhexosamine (HexNAc) residues of this glycan is a GalNAc; that is, this glycan is a disialylated biantennary oligosaccharide with one GalNAc

and one Gal residue instead of two Gal residues. LC/MS analysis for the glycopeptide 44–65 after treatment with β -galactosidase, HEXase I, and α -mannosidase confirmed the assignment (Fig. 4). In Fig. 3, neuraminidase treatment shifted glycopeptide mass of 4794 Da ([NeuGc]₂[HexNAc]₅[Hex]₄[Fuc]₁) to 4179 Da (difference of 615 Da = 2[NeuGc]), which was further reduced by 162 Da (=one

Gal β [1 $\rightarrow$ 4]) to 4017 Da after β -galactosidase digestion (Fig. 4). Additional treatment with HEXase I removed only one GlcNAc residue from this glycan. These results indicate that only one GlcNAc is linked to Gal. Another GlcNAc must be linked to only available HexNAc (i.e., GalNAc, which is inert for HEXase I). α -Mannosidase treatment of the resulting glycan partially removed one mannose (Man) residue, indicating that most GalNAc was linked to GlcNAc β (1 $\rightarrow$ 2) Man α (1 $\rightarrow$ 3) Man antenna. Because no specific exoglycosidase for GalNAc is available, its linkage to GlcNAc β (1 $\rightarrow$ 2)Man could not be confirmed by this analysis. A number of glycoproteins have been found to contain N-linked oligosaccharides with GalNAc residues [16]. Whether a recombinant glycoprotein contains GalNAc residues depends on the cell lines used. Cell lines such as HEK293, SP2/0, and NS0 can synthesize N-linked oligosaccharides with GalNAc ([17] and our unpublished data). Other major oligosaccharide structures were also elucidated in the same way. Table 2 summarizes major glycan structures at both glycosylation sites.

Characterization of anti-A β Mab samples after in vivo exposure in mice

The relative levels of the anti-A β Mab in plasma at 3, 6, 12, 24, and 48 h following intraperitoneal administration of the Mab (100 μ g/mouse) were determined using an antigen capture ELISA, and the data are shown in Fig. 5. The concentration of the Mab in plasma reached a maximum at 6 h ($\sim$ 24 μ g/ml) and was approximately 5 μ g/ml at 48 h.

Mouse plasma containing approximately 1 μ g anti-A β Mab for each sample was used for immunocapture, followed by LC/MS analysis. Anti-A β Mab was captured using immobilized goat anti-human Fc γ antibody. To elucidate the glycan structures at each glycosylation site, the captured anti-A β Mab was treated with papain to cleave into antigen-binding fragment (Fab) and Fc, each of which contains a different glycosylation site, and then was

analyzed by LC/MS. After this analysis, the remaining sample was reduced, alkylated, and treated with Lys-C. The Lys-C digests were again analyzed by LC/MS to confirm whether the right molecule was captured. An example of the LC/MS analysis results for the papain digest of the captured anti-A β Mab is shown in Fig. 6, where each time point sample gave a clear deconvoluted mass spectrum. Because the expected backbone peptide masses for the Fab and Fc are known, glycan structures could be assigned based on the Fab and Fc masses obtained and the previously elucidated oligosaccharides for this Mab. The method described here has two advantages over other commonly used methods such as matrix-assisted laser desorption/ionization MS (MALDI-MS) analysis for releasing oligosaccharides and HPLC or capillary electrophoresis (CE) for fluorescent tag-labeled oligosaccharides [18,19]. First, this method does not require very pure material because the LC/MS system (LC-coupled mass spectrometer) could be used for further purification steps. Furthermore, glycoprotein or glycopeptide masses are specific, and the contaminating protein or peptide impurities do not normally have the same masses. Hence, this method is specifically suited for characterization of samples recovered from in vivo studies. In general, for very low-titer serum samples, one-step immunocapture might not be sufficient to obtain very pure material. When the impurity is a glycoprotein, MALDI-MS, HPLC, and CE with fluorescence detection might give incorrect information. Second, the method could be used to elucidate oligosaccharide structures at each glycosylation site in the same assay. This could be a disadvantage of the method, potentially decreasing sensitivity of the method. Another disadvantage is that the data analysis is more complicated than that with the other methods. The method selected for oligosaccharide characterization of samples isolated from in vivo studies depends on the purpose of the study and instrument availability.

Effect of oligosaccharide structures on anti-A β Mab clearance in mice

To quantitatively compare the clearance rate of each glycan in vivo, its mass peak area was also obtained from the deconvoluted mass spectrum of each sample, and then the relative ratio (vs. disialylated biantennary oligosaccharides for Fab or vs. G0 for Fc) was calculated. These results are depicted in Figs. 7 and 8. As shown in Fig. 7, for the Fc glycosylation site, the relative ratio of biantennary oligosaccharides with zero (G0), one (G1), or two (G2) Gal residues is the same for the different time points. The results show that N-glycosylation at the conserved site in the Fc does not have any effect on clearance (i.e., all three glycoforms in this site [G0, G1, and G2] show the same clearance rate). This is consistent with the literature [1] and other unpublished data from our laboratories.

As shown in Fig. 8, 9 of the 11 major oligosaccharides at the V_H site Asn56 are quantitatively similar for all of the time points in this study, suggesting that these glycoforms

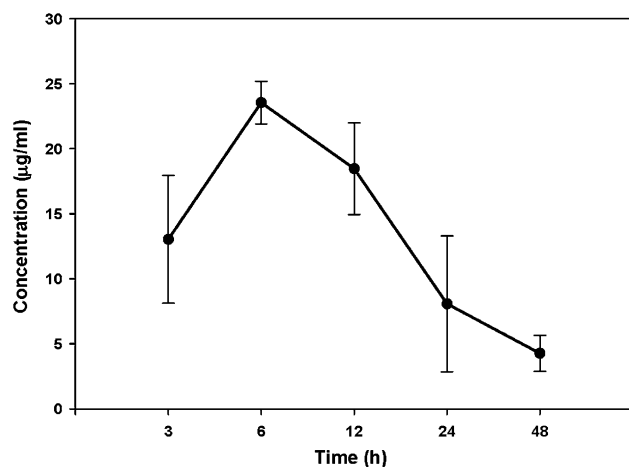


Fig. 5. Plasma concentration of anti-A β Mab determined by antigen capture ELISA at different time points after intraperitoneal administration to mice. Plasma samples from four mice per time point were analyzed.

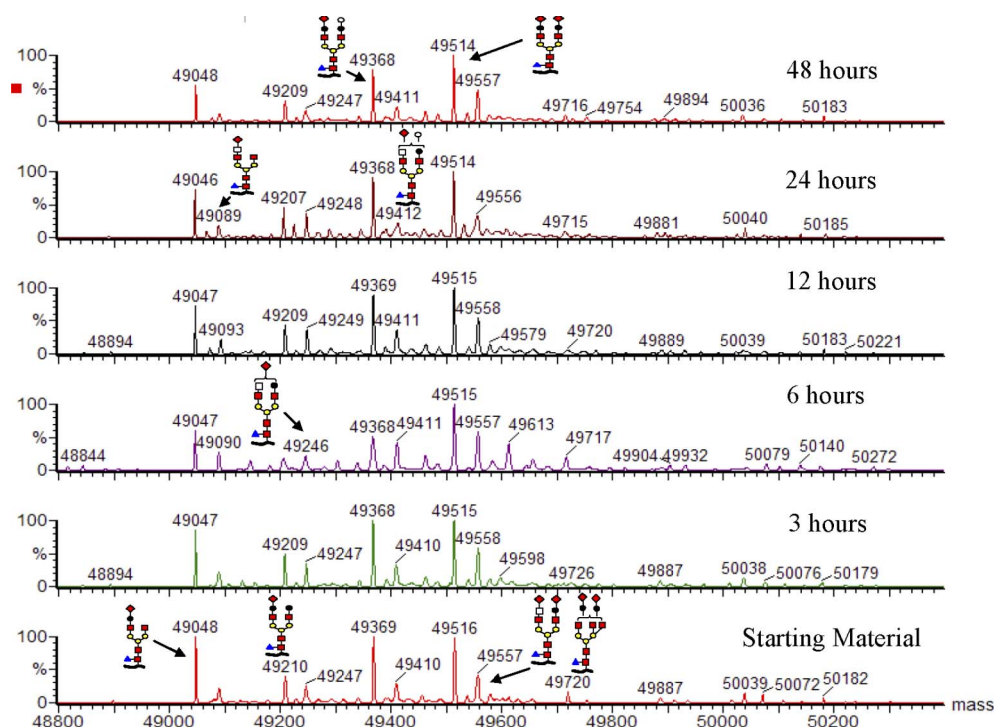


Fig. 6. Deconvoluted mass spectra for the Fab of anti-A β Mab captured from mouse plasma. The Fab was obtained by treating the captured anti-A β Mab with papain. The spectrum for the starting material is in the bottom panel. The spectra for the time point samples are indicated in the figure.

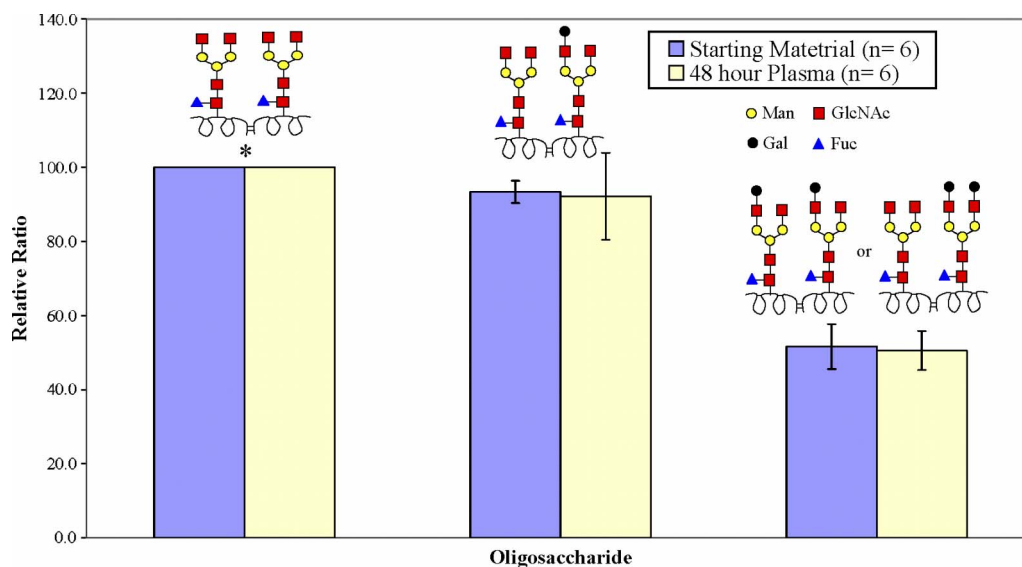


Fig. 7. Semiquantitative results for characterization of Fc N-glycosylation site Asn292 after recovery of Mab from plasma at 48 h relative to starting material. Oligosaccharide with an asterisk (*) was set 100%. Relative ratios of the other oligosaccharides versus this oligosaccharide were calculated by comparing their peak areas. The number of mice used for each time point is indicated in the figure. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article.)

have similar rates of clearance. The other two glycans, terminating in GlcNAc, showed a slight trend to lower relative ratios with increasing anti-A β Mab circulation time. Of the nine major oligosaccharides identified at Asn56, three glycans contain a terminal β -linked Gal or GalNAc residue and were expected to have faster clearance rates. However, their relative ratios versus fully sialylated biantennary oligosaccharides did not show any significant dif-

ference among the various time points (i.e., their clearance rates were similar). It has been well demonstrated that the hepatic asialoglycoprotein receptor (ASGP-R), identified by van den Hamer and coworkers [20], is able to rapidly remove glycoproteins from the circulation through specific binding to terminal β -linked Gal and GalNAc residues on glycoproteins [21]. Thus, these results are different from what would be predicted based on other

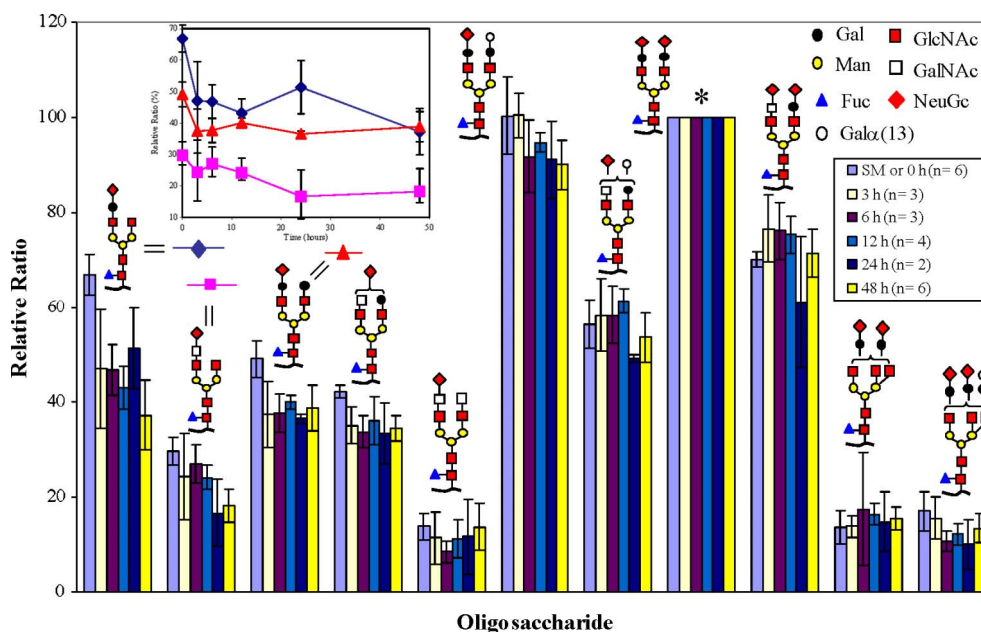


Fig. 8. Semiquantitative results for characterization of V_H N-glycosylation site Asn56 after recovery of Mab from plasma at various time points. Oligosaccharide with an asterisk (*) was set 100%. Relative ratios of the other oligosaccharides versus this oligosaccharide were calculated by comparing their peak areas. The number of mice used for each time point are indicated in the figure. (Inset) Relative ratios of oligosaccharides ending in GlcNAc are plotted as a function of time to show their clearance profiles versus the clearance profile of a non-GlcNAc-ending oligosaccharide. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article.)

glycoproteins, where terminal Gal or GalNAc residues lead to rapid clearance and the sialylation content has no impact on clearance in the case of this antibody.

Two possible reasons could explain the difference between this antibody and other glycoproteins with respect to the impact of glycosylation on exposure. The protein conformation around the N-glycosylation site might not be favorable for receptor binding to specific oligosaccharides. This could be a reason why endoglycosidase F (PNGase F) could not remove N-glycosylation from the V_H site, whereas the Fc carbohydrate was completely cleaved (data not shown). Similar results have been noted with other Fv glycans ([1] and our unpublished work). A second reason is that oligosaccharides with a terminal β -linked Gal residue contain only a single residue for recognition by the ASGP-R. The dissociation constant K_d of ASGP-R for such an oligosaccharide is only approximately 10^{-3} M [22] and might not be sufficient to allow the receptor to bind oligosaccharides and target the protein for degradation. Two oligosaccharides with slightly faster clearance rates have a terminal β -linked GlcNAc residue, which is recognized by Man/GlcNAc receptor [23], and the binding interaction in this case might be sufficiently strong to allow slightly greater clearance.

Conclusions

The glycosylation profile of a therapeutic glycoprotein is an important parameter that needs to be determined and controlled for maintaining the pharmacokinetics

and therapeutic efficacy. Although there are several publications documenting the role of the Fc oligosaccharides in therapeutic antibody function, and in particular effector properties of the antibodies, much less is known about the variable domain glycosylation, especially its impact on antibody half-life. A detailed LC/MS characterization of a humanized anti-A β Mab with a V_H CDR2 glycosylation described here has shown that whereas the Fc glycan is the typical biantennary, poorly sialylated structure with variable Gal content, the Fv glycan is occupied by biantennary carbohydrates with variable sialic acid content. In the case of this antibody, no differences were observed with respect to affinity of sialylated versus desialylated species for the ligand (unpublished data). In addition, the current study demonstrated that the sialic acid content of the V_H CDR2 has minimal impact on the antibody's clearance properties. Thus, it appears that the Fc–neonatal Fc receptor (FcRn) interactions that determine the half-lives of antibodies [24,25] are also the primary determinants of the half-life of this antibody. It should be noted, however, that in the case of other Fc fusion molecules, the glycosylation status of the non-Fc component has been shown to have an impact on the molecule's clearance properties [26]. Case-by-case determination of the contribution of the Fv domain carbohydrates to understand the structures of the glycoforms and their relative contributions to affinity, clearance, and other biochemical/biophysical properties will be important in defining the control strategies for antibodies with variable domain glycosylation.

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