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The glycosylation pattern of a humanized IgGl antibody (D1.3) expressed in CHO cells

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A humanized IgG antibody (D1.3) which retains murine complementarity determining regions specific for the antigen lysozyme has been expressed in CHO-DUKX cells. Heavy and light chain containing plasmids were co-transfected into CHO-DUKX cells and stable clones were grown in DMEM/F12 medium supplemented with 5% foetal calf serum. D1.3 antibody was purified from culture supernatants by Protein G chromatography. With the recombinant D1.3 antibody as a model, this cell culture system was shown to glycosylate the IgG Fc region in a similar manner to IgG isolated from serum. The neutral, core fucosylated biantennary oligosaccharides found are present in serum IgG and no novel carbohydrate sequences were detected. The degree of terminal agalactosylation was also similar to normal serum, in contrast to the increased levels found in rheumatoid serum. Furthermore, those oligosaccharides which lack only one terminal Gal are exclusively galactosylated on the GlcNAc(β1,2) Man(α1,6) Man(β1,4) antenna. Unambiguous identification of the exact glycosylation pattern of the antibody was carried out by a combination of specific exoglycosidase digestions, gel permeation chromatography of 2-aminobenzamide derivatives, high pH anion exchange chromatography and methylation analysis followed by gas–liquid chromatography-mass spectrometry.

Keywords: **humanized IgG antibody, immunotherapy, recombinant glycoprotein, CHO cell glycosylation, HPAEC**

Abbreviations: **CDR, complementarity determining region; CHO, chinese hamster ovary; GPC, gel permeation chromatography; 2-AB, 2-aminobenzamide; HPAEC-PAD, high pH anion exchange chromatography with pulsed amperometric detection; GC-MS, gas chromatography with mass spectrometry analysis; PNGase F, peptide-*N*-glycosidase F; PGC, porous graphitized carbon column; RAAM, reagent array analysis method; NeuAc: *N*-acetylneuraminic acid; NeuGc: *N*-glycolylneuraminic acid**

Introduction

Humanized IgG antibodies (also called reshaped CDR-grafted antibodies) which retain only the murine CDRs (complementarity determining regions) have largely replaced murine antibodies [1] for therapy. There have been several clinical antibody therapy studies of lymphomas and leukemias (reviewed in [2]). The efficacy of a humanized anti-CDw52 antibody (CAMPATH 1-H) in the treatment of B cell lymphoma [3] has led to the humanization of the anti-CD25 antibody anti-TAC [4] as a potential treatment for T-cell leukemias and to the humanization of several other antibodies [5]. Compared to murine antibodies, the humanized antibodies show a reduced immunogenicity, an improved effector function and a longer circulating half life in patients (reviewed in [6]), properties which have been shown previously [7–9] to be dependent on the glycosylation of the Fc region of the antibody.

The glycosylation profile of IgG was originally characterized from pooled normal and pathological serum IgG [10, 11]. In general there is one conserved NXS/T glycosylation site per heavy chain of IgG in the CH_2 domain of the Fc region. Glycosylation of the Fab region has also been reported in approximately 30% of serum antibodies [12]. The oligosaccharides are biantennary chains with or without bisecting GlcNAc and core Fuc, and a low level of sialylation within the Fc region (possibly due to the sterically hindered location of oligosaccharides). More than 70% of the chains are neutral oligosaccharides [10, 12]. In addition oligosaccharides lacking terminal Gal residues have been isolated [12] and termed G_1 or G_0 oligosaccharides (lacking either one or two Gal residues respectively), their presence having been suggested to play a role in the pathogenesis of rheumatoid arthritis, tuberculosis, systemic lupus erythremetosis and Sjörgens syndrome [13, 14].

From consideration of the original X-ray crystallographic co-ordinates for an IgG_1 anti-lysozyme antibody [15], it can be speculated that the absence of Gal on the GlcNAc (β1,2)Man (α1,6)Man(β1,4) antenna (α1,6 arm) will reveal

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peptide motifs which are normally masked and may contribute to immune complex formation [16]. The absence of Gal on either the (α1,6) arm or the (α1,3) arm could also generate ligands or endogenous carbohydrate binding proteins [17]. It is therefore important to assess the precise glycosylation status of immunoglobulins produced by cell constructs and used as immunotherapeutic antibodies.

The glycosylation pattern of a recombinant protein is affected by different criteria such as the cell type and the culture conditions [18]. CHO cells are becoming increasingly popular as an expression model for the production of recombinant proteins. Studies so far suggest that they lack the glycosyltransferases to generate bisecting GlcNAc and α2,6 linked sialic acid containing oligosaccharides [19, 20] which are usually found in serum IgG. In this paper we describe the detailed oligosaccharide profile of a humanized anti-lysozyme antibody (D1.3) [21, 22] expressed in CHO cells. The expressed protein has only one concensus N-linked glycosylation site at Asn 297 thus all the oligosaccharides isolated are located in the CH_2 domain. We used a combination of gel permeation chromatography (GPC) of 2-aminobenzamide derivatives (2-AB), exoglycosidase digestions, high pH anion exchange chromatography (HPAEC) and methylation analysis followed by gas–liquid chromatography-mass spectrometry (GC-MS).

Materials and methods

Cloning and expression of D1.3 antibody

The vector used was that described by Maeda *et al.* [21] based on the HuV_HLys construct of Verhoeyen *et al.* [22], and was a kind gift from Dr M. Bendig (NIMR Collaborative Centre, UK). D1.3 is a humanized antibody of the human IgG_1K isotype that retains the original mouse specificity for lysozyme. The antibody was expressed in CHO-DUKX cells [23]. Briefly, light and heavy chain containing plasmids were mixed and co-transfected into CHO-DUKX cells by electroporation using Gen Pulser apparatus, (BioRad, Munich, Germany). Stably transformed cells were selected in G418 containing medium and pooled. Supernatants of cultured pools were assayed for expression of Dl.3 by ELISA using goat anti-human IgG (Fc-specific) antibody for coating and peroxidase-conjugated goat anti-human IgG (Fab-specific) antibody for quantification. Human IgG was used as a standard. The most productive cell pools were transferred to multitray dishes and cultivated in DMEM/Fl2 medium supplemented with 5% foetal calf serum. Confluent dishes contained $\sim 2 \times 10^9$ cells in a total of 2.5 l of medium. Cell culture fluid was harvested at 3-day intervals (2 l per harvest) and cells were removed by microfiltration. Dl.3 antibody was purified by Protein G-Sepharose FF chromatography and characterized by SDS-PAGE and Western blotting. In Western blot experiments chicken lysozyme and a number of marker proteins were separated on SDS-PAGE, blotted on nylon membranes and incubated with purified D1.3. Incubation of these membranes with either peroxidase-conjugated goat anti-human IgG (Fab-specific) or sequentially with goat anti-human IgG (Fc-specific) and peroxidase-conjugated rabbit anti-goat IgG gave rise to a strong signal associated with the lysozyme band while the other proteins did not show any cross reaction demonstrating that the D1.3 antibody produced is highly specific for lysozyme.

Monosaccharide analysis

Ten μg of glycoproteins were hydrolysed for monosaccharide analyses by HPAEC-PAD. Neutral and amino monosaccharides were released by hydrolysis with 100 μl 2 M HCl for 2 h at 10 °C. The samples were dried and washed three times with 100 μl H_2O prior to analysis by HPAEC-PAD. Sialic acids were released from the protein with 100 μl 0.1 M HCl for 1 h at 70 °C and washed three times with 100 μl H_2O prior to analysis by HPAEC-PAD. HPAEC-PAD of monosaccharides was carried out on a CarboPac PA-1 column (4 × 250 mm; Dionex, Camberley, UK) and a biocompatible gradient HPLC system (Gilson, Villiers-le-Bel, France) fitted with a PED-2 detector (Dionex). Potentials for the gold electrode were as follows: El = 0.1 V, E2 = 0.7 V, E3 = − 0.3 V. Neutral and amino monosaccharides were eluted in an isocratic gradient of 2% 50 mM NaOH, 1.5 mM sodium acetate and 98% H_2O for 30 min followed by regeneration in 100 mM NaOH for 10 min. 300 mM NaOH was added as a postcolumn reagent to increase sensitivity and baseline stability. Sialic acids were eluted in a gradient from 100 mM NaOH to 300 mM sodium acetate 100 mM NaOH in 30 min followed by regeneration in 100 mM NaOH for 10 min. All neutral and amino monosaccharide samples were analysed with 2-deoxyglucose added as a reference peak after hydrolysis and quantitated against hydrolysed monosaccharide mixtures. Qualitative sialic acid analyses were performed by spotting proteins onto glass TLC plates and spraying with resorcinol reagent (100 mg resorcinol, 20 ml conc. HCl, 125 μl copper II sulphate and 25 ml H_2O) before incubating at 100 °C for 10 min.

Oligosaccharide release

N-linked oligosaccharides were released with PNGase F (Boehringer Mannheim, Lewes, UK or Oxford Glycosystems, Abingdon, UK) (1 unit PNGaseF 500 μg^{-1} protein) in 200 μl 40 mM KH_2PO_4, 10 mM EDTA, (pH 7.4) for 72 h at 37 °C. Five μl toluene was added to inhibit bacterial contamination. The protein was precipitated with 400 μl ice-cold ethanol and the pellet washed three times in ice cold ethanol. The supernatant and washes were pooled, concentrated and purified on a Biogel P2 column (2 mL bed volume). Glycans were eluted with water in the void volume. The pellets were redissolved and analysed for monosaccharide content as described above to assess the efficiency of oligosaccharide release.

Oligosaccharide profiling by HPAEC-PAD

HPAEC-PAD oligosaccharide profiles of released oligosaccharides were performed on a CarboPac PA-100 column (4 × 250 mm, Dionex, Camberley, UK) with a biocompatible gradient HPLC system as described above. The sodium acetate gradient used started with 98% solvent A (100 mM NaOH) and 2% solvent B (100 mM NaOH, 500 mM sodium acetate) for 4 min and increased to 10% solvent B in 21 min and to 60% solvent B over 20 min (elution programme 1). A similar gradient was also used with 250 mM NaOH as solvent A and 500 mM sodium acetate in 250 mM NaOH as solvent B [24] starting with 98% solvent A and 2% solvent B, held for 4 min and increased to 10% solvent B over 21 min (elution programme 2). Fetuin sialylated and neutral N-glycans used as standards were from Dionex, Camberley, UK.

Oligosaccharide profiling by PGC-HPLC

PGC-HPLC profiles were obtained on a Hypersil Hypercarb S column (4.6 mm × 100 mm, Hypersil, Runcorn, UK) run on a biocompatible HPLC with UV detection at 206 nm (Gilson). Oligosaccharides were eluted in a gradient of 100% 0.05% aqueous TFA to 40% 0.05% TFA/acetonitrile, 60% 0.05% aqueous TFA over 40 min.

Oligosaccharide labelling

Oligosaccharides released from 500 μg–1 mg of glycoprotein were labelled with 2-aminobenzamide (2-AB) (Oxford Glycosystems) according to the method described by Bigge [25]. Briefly, the oligosaccharides were suspended in 5 μl of 0.35 M 2-AB/1.0 M $NaCNBH_4$/30% v/v acetic acid in DMSO and incubated at 60 °C for 2 h. The reaction mixture was then purified by paper chromatography in a solvent system of 4:1:1, v/v/v, butanol:ethanol:water. The oligosaccharides eluted in water were further purified on a 300 μl mixed bed ion exchange resin (supplied with labelling kit) and filtered and dried prior to further analysis.

Oligosaccharide profiling on a Biogel P4 column

Analytical Biogel P4 chromatography was performed on the RAAM 2000 Glycosequencer (Oxford Glycosystems) at a temperature of 55 °C with water at a flow rate of 30 $\mu l\,min^{-1}$ for 11 ml ramped to 160 $\mu l\,min^{-1}$ over 27 ml and held for 7 ml. Detection was by fluorescence at λ max 330 nm, and λ max 420 nm for excitation and emission, respectively. Data were analysed by the Glyco-link software supplied with the RAAM 2000. Preparative Biogel P4 chromatography was carried out on the same instrument using a constant flow program in the absence of a dextran ladder.

RAAM oligosaccharide sequencing

The identity of the oligosaccharides was confirmed by RAAM sequencing [26] with a neutral enzyme array (Oxford Glycosystems) and analysis on the Biogel P4 column, the profiles being identified using the pattern matching software and database supplied with the RAAM 2000 glycosequencer (Eve software and New-Bio2 database, Oxford Glycosystems).

Exoglycosidase digestions

Oligosaccharides released from 1 mg antibody were dissolved in 100 μl 100 mM sodium citrate-phosphate buffer pH 5 and digested with 1U Jack Bean β-hexosaminidase (Oxford Glycosystems) for 21 h at 37 °C. The reaction was stopped by boiling for 2 min and cooling on ice and desalted by Biogel P2 chromatography as described above. Oligosaccharides eluted in the void volume were dried, redissolved in 100 μl 100 mM sodium acetate buffer and treated with 2U Jack Bean α-mannosidase (Oxford Glycosystems) for 21 h at 37 °C. The reaction was stopped by boiling for 2 min and cooling on ice. The glycans were desalted by Biogel P2 chromatography and separated on Biogel P4 as described above with refractometric detection.

Methylation analysis

Purified digested oligosaccharides were dissolved in 200 μl anhydrous DMSO under an argon atmosphere and sonicated for 30 min. They were permethylated by addition, in anhydrous conditions, of 2 mg (approximately) powdered NaOH and 200 μl methyl iodide according to the method of Ciucanu and Kerek [27]. The reaction was stopped by addition of water (5 ml) and the permethylated oligosaccharides extracted into chloroform (3 × 300 μl) . The chloroform phase was washed with 10 × 5 ml water, evaporated under N_2 and freeze dried. The permethylated oligosaccharides were then treated with anhydrous acidic methanol 0.5N (Supelco, Poole, UK) at 80 °C for 18 h and acetylated with a mixture of pyridine:acetic anhydride (1:1, v/v, at room temperature, overnight). The partially O-methylated O-acetylated methylglycosides obtained were characterized and quantified from peak area by GC-MS [28] (Hewlett Packard 5890 series II GC and HP5972A MSD operated in EI mode, with a Hewlett Packard Ultra-2 capillary column (25 m × 0.2 mm), a column temperature increasing from 60 °C to 265 °C with a gradient of 5 $°C\,min^{-1}$, helium pressure at 10 psi and cool on-column injection).

Results

Glycoprotein analysis

The D1.3 antibody was purified by protein G chromatography from CHO cells transfected with light and heavy chain plasmids as described in the experimental section. Dependent on the quantity of plasmids applied in the transfection, D1.3 concentrations of up to 1.4 $mg\,l^{-1}$ were obtained (Table 1).

Aliquots of the D1.3 antibody were hydrolysed under conditions optimized for the release of neutral and amino

Table 1. Expression of humanized anti-lysozyme antibody D1.3 in CHO-DUKX cells in seven different transfection studies.

Transfection number	*Weight of plasmids transfected per 1×10⁷ cells*		*IgG titre (μg l⁻¹)*
	D1.3 light chain	*D1.3 heavy chain*	
1	−	−	0
2	+	+	2
3	−	+	50
4	2 μg	2 μg	506
5	5 μg	5 μg	1240
6	10 μg	10 μg	1200
7	20 μg	20 μg	1463

sugars. The use of TFA for the hydrolysis of monosaccharides as described in the literature often results in poor recovery of monosaccharides and inaccurate molar ratios which are not observed with 2 M HCl. These analyses showed that 2% carbohydrate (w/w) are present in the recombinant D1.3 antibody. Analysis of the ethanol precipitated pellets showed that at least 80% of the total glycan pool has been recovered. Fuc, Gal, GlcNAc and Man were detected by HPAEC-PAD in the molar ratio 0.8:1.2:3.8:3.0 in both native D1.3 and the PNGaseF released oligosaccharides. This is consistent with the non-selective release of N-linked fucosylated complex type oligosaccharides. HPAEC-PAD of D1.3 hydrolysed under conditions used to release sialic acids indicated an absence of both NeuAc or NeuGc. The absence of sialylation was further confirmed by the negative staining of D1.3 aliquots with resorcinol.

Oligosaccharide profiling

The oligosaccharides released from the glycoprotein by PNGase F were analysed with three different chromatographic systems. Oligosaccharide profiling by HPAEC-PAD using two different gradients (Figure 1) shows that the oligosaccharides are contained within three fractions. Their early elution confirms the lack of sialylation. By comparison, the disialylated glycans from fetuin were eluted between 31 and 32 min with elution programme 1 (data not shown). The presence of three main N-glycans was confirmed by mapping on the porous graphitized column which showed two peaks for each of three α/β anomer pairs (data not shown). The mixture of oligosaccharides were fluorescently labelled with the fluorophore 2-aminobenzamide and separated using Biogel P4 chromatography (as part of a RAAM 2000 glycosequencer, Oxford GlycoSystems). This also revealed three fractions accounting for 43%, 36% and 21% of the total N-glycans from the integration of peak areas. A dextran hydrolysate run simultaneously allowed the calculation of the hydrodynamic volume of each fraction

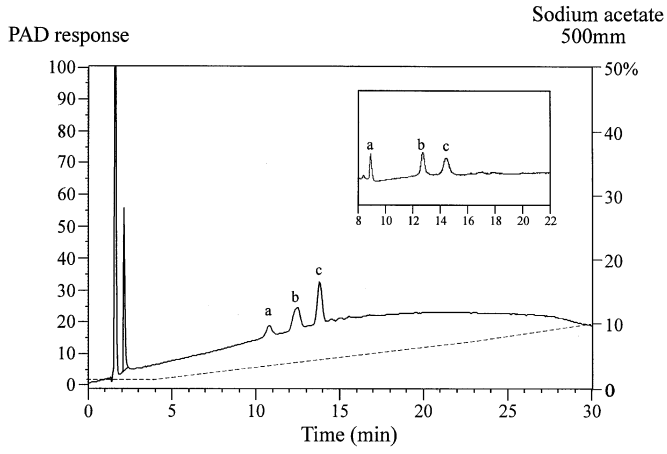

Figure 1. HPAEC profile of native D1.3 N-glycans released by PNGase F treatment. The dotted line indicates the sodium acetate gradient. The inset shows the separation with 250 mM NaOH and the same acetate gradient a : G_0, b : G_1, c : G_2. The peak area ratios are the same as those found in Figure 2.

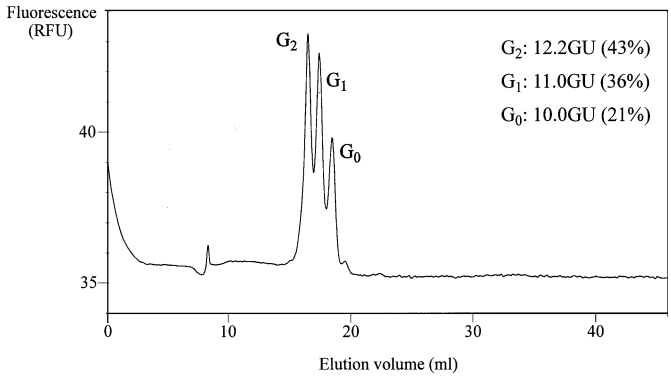

Figure 2. Profile of the D1.3 oligosaccharides labelled with 2-aminobenzamide separated on a Biogel P4 column. Flow rate: 30 μl min^{-1} for 11 ml and then ramped to 160 μl min^{-1} over 27 ml and held for 7 ml (High resolution programme). Eluant: water.

expressed in glucose units (GU). These are respectively 12.2 GU, 11.0 GU and 10.0 GU corresponding to G_2, G_1, and G_0 oligosaccharides (Figure 2). Allied to the monosaccharide composition data, these hydrodynamic volumes give structural information on the glycan by comparison with the database restricting the number of possible structures. These are mainly biantennary structures in which the number and position of Fuc, GlcNAc or Gal can vary. Finally, a preparative Biogel P4 chromatography of 2-AB labelled oligosaccharides run in the absence of a dextran hydrolysate, was used to isolate each oligosaccharide.

Sequencing of the oligosaccharide structures

The structure of oligosaccharide present in each peak was then investigated by RAAM sequencing with a neutral enzyme array and analysis by Biogel P4 chromatography

Figure 3. Structures of the N-glycans isolated from a humanized anti-lysozyme antibody (D1.3).

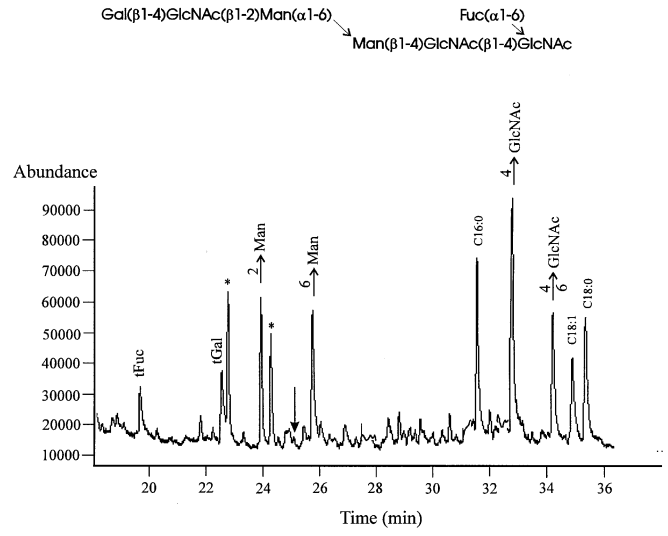

Figure 4. Total ion chromatogram of the partially methylated and acetylated methylglycosides from the G_1 oligosaccharide treated with Jack Bean *β*-hexosaminidase and *α*-mannosidase. Abbreviations and peak area ratios are as shown in Table 2; $C_{16:0}$, $C_{18:1}$, $C_{18:0}$: fatty acids; *: products from reagents; The arrow indicates the elution position of methyl 2,4,6 tri-*O*-methyl 3-*O*-acetyl mannoside (3 Man).

[26]. The isolated products were confirmed as biantennary core fucosylated N-linked oligosaccharides without any bisecting GlcNAc or sialic acid. They exhibit two, one or no terminal Gal (Fig. 3). However the location either on the (α1,3) arm or the (α1,6) arm of the single Gal residue present in G_1 cannot be determined by this method. This was unambiguously assigned by sequential *β*-hexosaminidase, α-mannosidase digestion and methylation analysis of oligosaccharides released by PNGaseF digestion of D1.3. G_0 oligosaccharides will be reduced to a tetrasaccharide by this treatment, whilst G_2 oligosaccharides will be unaffected. G_1 oligosaccharides will be reduced to a hexasaccharide, with the outer Gal-GlcNAc-Man antenna being linked to the inner mannose by either an (α1,3) or (α1,6) linkage depending on the starting structure. The products were separated by Biogel P4 chromatography with a constant flow programme and the fraction corresponding to the degradation product from G_1 analysed by methylation analysis. The methyl glycosides corresponding to terminal Fuc, terminal Gal, Man monosubstituted at C-2, Man monosubstituted at C-6, GlcNAc monosubstituted at C-4 and GlcNAc disubstituted at C-4 and C-6 (Figure 4) were obtained in the molar ratio: 0.6/0.7/1.0/0.9/1.7/0.9 (Table 2). No mannose monosubstituted at C-3 could be detected showing that the G_1 oligosaccharides are exclusively galactosylated on the (α1,6) arm.

N-Glycan heterogeneity

The lack of heterogeneity with respect to fucosylation and bisecting GlcNAc was further investigated by HPAEC-PAD. The use of 250 mM NaOH allows a better separation of neutral oligosaccharides [24]. The absence of small peaks between G_0, G_1 and G_2 or after G_2 with elution programme 2 (Figure 1, inset) suggests the absence

Table 2. Molar ratios of the methylglycosides from methylation analysis of the G_1 oligosaccharide treated with Jack Bean *β*-hexosaminidase and *α*-mannosidase.

*Abbreviation**	*Methylglycoside*	*Substitution*	*Molar ratio*
tFuc	methyl 2,3,4 tri-*O*-methyl fucoside	terminal Fuc	0.6
tGal	2,3,4,6 tetra-*O*-methyl galactoside	terminal Gal	0.7
→2Man	methyl 3,4,6 tri-*O*-methyl-2-*O*-acetyl mannoside	Man monosubstituted at C-2	1
→6Man	methyl 2,3,4 tri-*O*-methyl-6-*O*-acetyl mannoside	Man monosubstituted at C-6	0.9
→4GlcNAc	methyl 3,6 di-*O*-methyl-4-*O*-acetyl (*N*-methyl) glucosaminide	GlcNAc monosubstituted at C-4	1.7
→4,6GlcNAc	methyl 3 mono-*O*-methyl 4,6 di-*O*-acetyl (*N*-methyl) glucosaminide	GlcNAc disubstituted at C-4 and C-6	0.9

*Abbreviations are those used in Figure 4.

of heterogeneity due to the absence of fucose or the presence of bisecting GlcNAc. (With HPAEC-PAD a Fuc residue will decrease the retention time of an oligosaccharide whilst a bisecting GlcNAc will increase it and therefore heterogeneity due to the absence of core fucosylation or the presence of bisecting GlcNAc would result in closely eluting peaks.) However a small peak ($< 2\%$ of the total) was observed on Biogel P4 and could correspond, from its hydrodynamic volume, to the G_0 oligosaccharide without Fuc (Figure 2).

Discussion

Since its first success in 1988 [3], humanized antibody therapy has not seen the progress that was first predicted as unexpected immunogenicity or reduced effector functions of the antibodies have slowed down development. In addition, many challenges remain in the consistent production of humanized antibodies. As mentioned earlier, the Fc glycosylation of the antibody is crucial for functions mediated via the Fc receptor and half life in serum [9]. It is therefore important to choose cell lines and culture conditions that lead to the appropriate glycosylation of the recombinant protein designed for therapeutic use. Here we report a cell expression system which gives a glycosylation pattern of IgGl similar to that of serum IgG using a model humanized anti-lysozyme antibody (D1.3).

The structures of the N-glycans of D1.3 have been defined using RAAM sequencing. The structures are biantennary N-glycans with core Fuc but lack bisecting GlcNAc and sialic acid. They are heterogeneous with respect to the terminal galactosylation and therefore called G_2, G_1 and G_0 (Fig. 3). These three oligosaccharides comprise 67% of the total N-glycans in human IgG [10]. Compared to polyclonal IgG or paraproteins [11, 29], negligible heterogeneity due to the absence of core Fuc, presence of bisecting GlcNAc or sialylation is found in D1.3 expressed in CHO cells. These results were confirmed by HPAEC profiling of the N-linked glycans. PNGaseF was found to give reproducible and efficient release of oligosaccharides from D1.3, although it is worth considering that digestion conditions often have to be optimized for the glycoprotein being studied. The labelling of oligosaccharides with 2-AB by reductive amination has been studied and found to be highly reproducible and non-selective towards oligosaccharide structures [25].

The absence of bisecting GlcNAc or (α2,6) linked sialic acid is not surprising as CHO cells normally do not express *N*-acetylglucosaminyltransferase III or β-galactoside (α2,6) sialyltransferase [19, 20]. CHO cell expressed glycoproteins can contain (α2,3) linked sialic acids [30], however the sialylation of IgG is suggested to be (α2,6) only [10]. Moreover a study of Fc and Fab region glycosylation indicates that only a small proportion of Fc region oligosaccharides are sialylated whilst up to 73% of Fab region oligosaccharides are sialylated [31]. The absence of bisecting GlcNAc and sialic acid has also been reported in the glycans of a humanized anti-CD18 antibody expressed in NS/0 mouse myeloma cells [32]. The absence of heterogeneity in core fucosylation is more unusual and may be due to the cell culture conditions used. Twenty-one per cent of serum IgG biantennary glycans lack core Fuc and bisecting GlcNAc [11], as do at least 10% of IgG paraproteins [29]. Previous studies of humanized or reshaped antibodies expressed in mouse myeloma cells have also indicated the presence of oligomannose and truncated structures [32, 33]. CHO cell expressed D1.3 contains only complex biantennary oligosaccharides, though CHO cells are capable of synthesizing oligomannose and hybrid type N-linked oligosaccharides.

The relative concentrations of G_2, $G_1$and G_0, respectively 43%, 36% and 21%, are very similar to those found in IgG Fc from healthy individuals [11, 31]. The ratio between G_2, G_1, and G_0 may be important in pathologies where undergalactosylated forms of IgG predominate [11, 29]. A decrease in galactosylation of the N-glycans corresponding to high level of G_1, and G_0 has been directly correlated with the severity of rheumatoid arthritis [14]. The Gal residue present on the (α1,6) arm can interact with the aromatic group of Phe 243 on the protein which results in a loss of flexibility of this arm. An increase in the level of glycoforms lacking terminal Gal could lead to the exposure of certain Fc determinants at a much higher concentration and may elicit an immune response relevant to the disease [12]. Hence the precise location of the Gal in the G_1 oligosaccharide has been determined for the D1.3 antibody and found to be exclusively located on the (αl,6) arm. Studies carried out on human IgG revealed that the galactosylation of the (α1,6) arm is preferred in IgG_1 paraproteins, IgG_4 paraproteins and polyclonal IgG (between 60% and 84% of G_1 Gal is on the (αl,6) arm) [29], although, to our knowledge, the present study is the first report of exclusive galactosylation of the (αl,6) antenna. On the contrary, for IgG_2 paraproteins and some IgG_3 paraproteins it is the (αl,3) galactosylation that is preferred (on average 44% of G_1 Gal is on the (αl,6) arm [29]). This suggests that the length and flexibility of the hinge region influences which antenna is predominantly accesssed by the galactosyltransferase. In our construct, access of galactosyl transferase to the (α1-3) arm may be hindered until after galactosylation of the (α1-6) arm. On the other hand, a specific galactosidase found in CHO cells could result in cleavage of the (α1-3) arm galactose but this seems unlikely. Although terminal galactosylation may not be important for Fc receptor recognition mediated by the pentasaccharide core [34], the galactose residues may be essential for full Fc effector function [35]. Our results are therefore consistent with the observation that IgG produced in CHO cells retain the Fc receptor recognition and the effector functions [36].

In conclusion, the expression of this antibody in CHO cells leads to a glycosylation pattern similar to that of polyclonal IgG from healthy individuals. Our results are in

agreement with a recent study of the glycosylation of the CAMPATH-1H antibody expressed in CHO cells [37].

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