

Glycosylation pattern of humanized IgG-like bispecific antibody produced by recombinant CHO cells

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Abstract The glycosylation pattern of a humanized anti-EGFR×anti-CD3 bispecific single-chain diabody with an Fc portion (hEx3-scDb-Fc) produced by recombinant Chinese hamster ovary cells was evaluated and compared with those of a recombinant humanized anti-IL-8 antibody (IgG1) and human serum IgG. *N*-Linked oligosaccharide structures were estimated by two-dimensional high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. No sialylation was observed with purified hEx3-scDb-Fc and the anti-IL-8 antibody. From the analysis of neutral oligosaccharides, approximately more than 90% of the *N*-linked oligosaccharides of hEx3-scDb-Fc and the anti-IL-8 antibody were alpha-1,6-fucosylated. The galactosylated biantennary oligosaccharides comprise over 40% of the total *N*-linked oligosaccharides in both

hEx3-scDb-Fc and the anti-IL-8 antibody. The fully galactosylated biantennary oligosaccharides from hEx3-scDb-Fc and the anti-IL-8 antibody accounted for only 10% of the *N*-linked; however, more than 20% of the *N*-linked oligosaccharides were fully galactosylated biantennary oligosaccharides in human serum IgG. The glycosylation pattern of hEx3-scDb-Fc was quite similar to that of the anti-IL-8 antibody.

Keywords Bispecific diabody · Bispecific IgG-like antibody · Glycosylation · Immunoglobulin G · Chinese hamster ovary cells

Introduction

In the past decade, antibodies have been proven to be an excellent component for the design of a high-affinity, protein-based binding reagent. With several monoclonal antibody products currently on the market and more than 100 under clinical trials, it is clear that engineered antibodies have come of age as biopharmaceuticals (Wurm 2004; Butler 2005; Holliger and Hudson 2005). Immunoglobulin G (IgG) antibodies have one *N*-glycosylation site in the Fc region of their heavy (H) chain. This glycosylation is indispensable for their interactions with Fc receptors (FcRs) and for FcR-mediated effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC; Dwek 1995; Jefferis 2009). Although all IgG molecules have a common core oligosaccharide, they have marked heterogeneity owing to differences in their outer arm glycosylation, which results in differences in their ability to trigger an effector function (Lund et al. 1993; Helenius and Aebi 2001). The degalactosylation of RituxanTM has reduced

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CDC by approximately half, relative to an unmodified (variably galactosylated) control monoclonal antibody (Hodoniczky et al. 2005). The absence of fucose or the presence of a bisecting *N*-acetylglucosamine (GlcNAc) of Fc glycans has significantly increased binding to Fc γ R and has consequently shown enhanced ADCC (Shinkawa et al. 2003). The sialylation of the *N*-glycan structure of IgG results in reduced binding affinities to the subclass-restricted Fc γ Rs, thereby reducing their *in vivo* cytotoxicity (Kaneko et al. 2006). In general, the oligosaccharide heterogeneity of glycoproteins produced from recombinant Chinese hamster ovary (CHO) cells has been shown to change with culture conditions (Anderson and Goochee 1994). This implies that the product quality including structural analysis of sugar chains is an important issue in an establishment of manufacturing process for the production of new therapeutic proteins. Recently, bispecific antibodies with the Fc portion have been considered attractive molecules as new therapeutic reagents because they generally provide advantages of multivalent binding to two target antigens, prolonged half-life, utilization of protein A purification, and induction of ADCC and CDC (Carter 2001; Asano et al. 2007). Bispecific antibodies are attractive formats for recombinant antibodies because they can be designed to redirect T cells toward tumor cells by cross-linking the antigens on the tumor cells and the CD3–T cell receptor complex on the T cell. The advantage of this format is the availability of a simple industrial process for therapeutic antibody production. Affinity chromatography using protein A which is able to strongly bind to Fc portion is a powerful tool for effective purification. Bispecific antibodies with the Fc portion could be purified easily by protein A chromatography from supernatants of the culture medium. Furthermore, Fc portion plays an important role in the induction of ADCC and CDC and probably enhances the *in vitro* and *in vivo* activities of recombinant antibodies. The *N*-glycosylation of bispecific antibodies with the Fc portion might be indispensable for their activities, and their oligosaccharide structure may affect the product quality. However, the oligosaccharide structure of bispecific antibodies with the Fc portion has not been reported previously.

We report here for the first time a structural analysis of sugar chains on bispecific antibodies with the Fc portion produced by recombinant CHO cells. The refined structure was compared with those of intact and recombinant IgGs. The humanized anti-EGFR \times anti-CD3 bispecific diabody with the Fc portion (hEx3-scDb-Fc) was used as a bispecific antibody with the Fc portion (Asano et al. 2008). This bispecific antibody retargets lymphokine-activated killer cells with the T cell phenotype against epidermal growth factor receptor-positive cell lines and shows remarkable antitumor activity *in vitro* (Asano et al. 2008).

Materials and methods

Cell line, cultivation, and purification

The recombinant CHO cell line, which produces human Ex3-scDb-Fc (Asano et al. 2008), and the CHO-DP12 cell line (ATCC CRL12445; Shields et al. 2002), which produces a recombinant humanized anti-IL-8 IgG1 antibody, were used in this study. These cell lines are derivatives of the CHO-K1 cell line. The Fc portion of hEx3-scDb-Fc and anti-IL-8 IgG1 antibody is a human Fc region (subclass IgG1). Fetal calf serum-free cultivation is important for manufacturing processes for therapeutic protein production (Terada et al. 2002; Fujiwara et al. 2007). Serum-free adapted CHO-Top-H and CHO-DP12-SF cell lines were established from these two cell lines, respectively. Both cell lines were cultivated in a suspension culture with serum-free ExCD medium [a 1:1 mixture of ExCell302 (SAFC Biosciences, St. Louis, MO, USA) and IS CHO-CD (Irvine Scientific, Santa Ana, CA, USA) supplemented with 200 nM methotrexate and 1 mM G418]. The cells were cultivated in a 1-L glass bioreactor (Biott, Tokyo, Japan) containing 750 mL of the serum-free medium. Temperature was maintained at 37°C. Agitation speed was 70 rpm and the headspace of the vessel was aerated with air supplied at a flow rate of 100 mL/min. pH was controlled at 7.1. Dissolved oxygen (DO) concentration was measured by a DO sensor (InPro 6880, Mettler Toledo, Switzerland). DO concentration was always kept above 40% of air saturation. Human IgG derived from human plasma (Athens Research & Technology, Athens, GA, USA) was used as a control of *N*-linked oligosaccharide analysis.

Antibodies were purified from the serum-free culture medium or control IgG from human plasma by protein A affinity chromatography on a 5-mL HiTrap protein A column (GE Healthcare, Uppsala, Sweden) using single-step pH gradient elution from 50 mM sodium phosphate–0.1 M NaCl, pH 7.0 (buffer A) to 0.1 M glycine–HCl, pH 3.0 (buffer B). Final purity was determined to be greater than 95% by denaturing electrophoresis on 10% polyacrylamide gels and Coomassie brilliant blue staining. The purified proteins were stored in phosphate-buffered saline (pH 7.3) containing sodium azide (0.8 mM) at –20°C. Antibody concentration was determined using a sandwich enzyme-linked immunosorbent assay. A 96-well plate (Corning, Corning, NY, USA) was coated with a goat anti-human IgG-Fc polyclonal antibody (100 μ L/well, 10 μ g/mL; Bethyl Laboratories, Montgomery, TX, USA) in 0.1 M sodium hydrogen carbonate buffer. After overnight incubation at room temperature, the plate was washed three times with phosphate-buffered saline (PBS) containing 0.05% (*v/v*) Tween 20 (PBS-T). Unbound active

sites were blocked with 300 μL of 1% (*v/v*) bovine serum albumin (BSA)/PBS per well for 1 h. The plate was washed with PBS-T and human serum reference or samples were added to the plate. The plate was incubated for 2 h at room temperature. After being washed in PBS-T washing solution, the plates were incubated at room temperature for 1 h with 100 μL of horseradish peroxidase-conjugated goat anti-human IgG-Fc polyclonal antibody solution (1 $\mu\text{g}/\text{mL}$; Bethyl Laboratories) in 1% (*v/v*) BSA/PBS. Following washing of the plates in PBS-T washing solution three times, 100 μL of enhanced chemiluminescence solution (KPL, Geithersburg, MD, USA) was added to each well. The reaction was stopped after 30 min at room temperature by adding 100 μL of peroxidase stop solution (KPL), and absorbance was measured at 405 nm using a microplate reader.

Analysis of oligosaccharide structures

Sialic acids were released from the purified antibodies by incubation in 50 mM sulfuric acid at 80°C for 1 h. The released sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and analyzed by reverse-phase high-performance liquid chromatography (HPLC; Hara et al. 1987). The analysis was performed using an octa decyl silica (ODS) C18 column (4.6 \times 250 mm; Nacalai Tesque, Kyoto, Japan). The sialic acid derivatives were eluted using a linear gradient of 100% solvent A [methanol–acetonitrile–H₂O (7:4:89, *v/v/v*)] to 100% solvent B [methanol–acetonitrile–H₂O (35:20:45, *v/v/v*)] at a constant flow rate of 1.2 mL/min. Fluorescence was monitored at excitation and emission wavelengths of 310 and 448 nm, respectively. Separations were confirmed using standard sialyloligosaccharides (S1, S2, and S3) from a sialic acid fluorescence labeling kit (TAKARA Bio, Ohtsu, Japan) with an anion exchange chromatography [TSK-GEL DEAE 5PW (7.5 \times 75 mm) column; Tosoh, Tokyo, Japan]. In brief, *N*-linked oligosaccharides were released by digestion with peptide-*N*-glycosidase F (PNGase F; TAKARA Bio) and derivatized with 2-aminopyridine–acetic acid at the reducing terminus. The pyridylaminated *N*-linked oligosaccharides were eluted using a linear gradient of 100% solvent A (10% acetonitrile, pH 9.5) to 20% solvent B (10% acetonitrile in 3% acetic acid buffer, pH 7.3).

The core *N*-linked oligosaccharide structure of the antibodies was analyzed by the pyridylation method using 2D HPLC (Fujiyama et al. 2007; Omasa et al. 2008). The purified antibodies were hydrolyzed using PNGase F in 0.1 M Tris–HCl buffer (pH 8.6), 0.1% sodium dodecyl sulfate (SDS), and 0.2 M mercaptoethanol at 37°C for 48 h to release *N*-linked oligosaccharides from asparagine residues. The reducing ends of the released *N*-linked oligosaccharides were pyridylaminated in 2-aminopyridine–acetic

acid solution. Pyridylaminated oligosaccharide mixtures were analyzed by 2D HPLC mapping using a Shimadzu LC20AD HPLC system with two different columns: ODS C18 column (4.6 \times 250 mm; Tosoh) and TSK-GEL amido-80 (4.6 \times 250 mm; Tosoh). Variants were identified by comparing their retention times with those of pyridylaminated standards (Masuda Chemical Industries, Takamatsu, Japan). The integrated areas of peaks on each chromatogram therefore correspond to the molar ratios of these pyridylaminated oligosaccharides. The molecular masses of the major peaks were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using an autoflex mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Two undetermined peaks were further analyzed by MALDI-TOF tandem mass spectrometry MALDI-TOF MS/MS (Bruker Daltonics) and liquid chromatography tandem mass spectrometry [LC-MS/MS; Agilent Technologies 1200 series (Agilent Technologies, Wilmington, DE, USA) equipped with HCT plus (Bruker Daltonics)].

Results

Production and characterization

For the purpose of using them in the comparative experiments described in this study, the serum-free adapted CHO-Top-H and CHO-DP12-SF cell lines were established by stepwise adaptation to a decrease in the serum concentration of the medium for about 2 months. Both cell lines were cultured in a 1-L bioreactor. Cultures were seeded at 1.5 \times 10⁵ cells per milliliter from the cell culture in mid-exponential phase growth. The products were harvested at 120 h, corresponding to the middle-to-late phase exponential growth. Cell viability was routinely maintained at over 85% and final antibody concentration was set over 40 mg/L in both cultivations (data not shown). The antibodies were purified from the serum-free culture medium or control IgG from human serum by one-step protein A affinity chromatography. The purified samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 1). hEx3-scDb-Fc gave a single band with a molecular mass of 80 kDa on SDS-PAGE, whereas the purified human serum IgG and humanized anti-IL-8 antibody dissociated into heavy and light chains with apparent molecular masses of 52 and 28 kDa, respectively. There is a detectable shift in the molecular mass of human serum IgG, humanized anti-IL-8 antibody, and hEx3-scDb-Fc upon PNGase F digestion, suggesting that these antibodies are modified by *N*-linked glycosylation. The predicted molecular weights from the protein sequences were in agreement with the masses of aglycosylated proteins. hEx3-scDb-Fc has only two Asn-X-

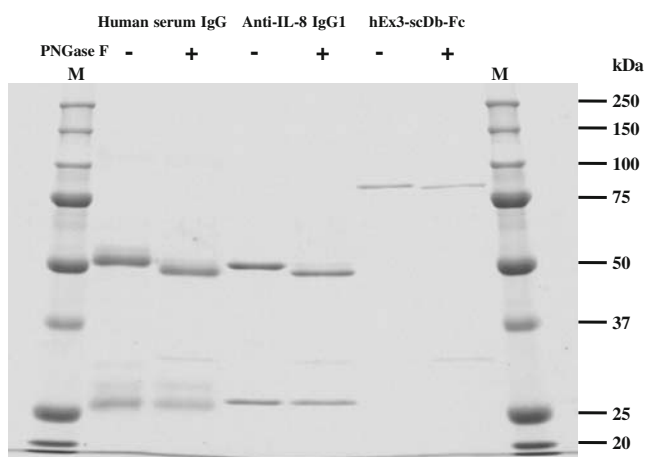


Fig. 1 Coomassie-stained SDS-PAGE gel with antibodies purified by protein A affinity chromatography. Purified antibodies were electrophoresed under reducing condition on a 10% gel

Thr/Ser (where X can be any amino acid except proline) sites (*N*-linked glycosylation sites) at the Fc portion. This result indicates that the *N*-linked sugar chain binds to the Fc domain of hEx3-scDb-Fc.

Analysis of *N*-linked oligosaccharides

Sialic acids were released from 0.5 mg of intact human serum IgG, purified humanized anti-IL-8, and purified hEx3-scDb-Fc antibodies and derivatized with DMB. *N*-Linked oligosaccharides were also released from 5.0 mg of the purified antibodies and derivatized with 2-aminopyridine. Chromatograms of the DMB and pyridylaminated derivatives are shown in Fig. 2a–d, e–h, respectively. As shown in Fig. 2c, d, no DMB derivative was detected in the humanized anti-IL-8 antibody and hEx3-scDb-Fc, respectively. On the other hand, human serum IgG contains di-sialyl (S2) and tri-sialyl (S3) oligosaccharides (Fig. 2f). The estimated molar ratio of sialyl oligosaccharides to total oligosaccharides in human serum IgG was approximately 30%, calculated from the peak area (Fig. 2f). The neutral oligosaccharide fraction (S0) of purified human serum IgG, hEx3-scDb-Fc, and the humanized anti-IL-8 antibody were analyzed by reverse-phase HPLC (RP-HPLC; Fig. 3). The eluted peaks were further analyzed by normal-phase HPLC with an amide-80 column and compared with those of pyridylaminated standards by 2D HPLC mapping (data not shown). The *N*-glycan structures of the neutral oligosaccharides estimated by 2D HPLC mapping were confirmed by MALDI-TOF MS. For example, the molecular mass of the PA-derivatized oligosaccharide corresponding to peak G (*m/z* 1,887) agreed well with the calculated mass of Gal₂GlcNAc₂Man₃FucGlc-

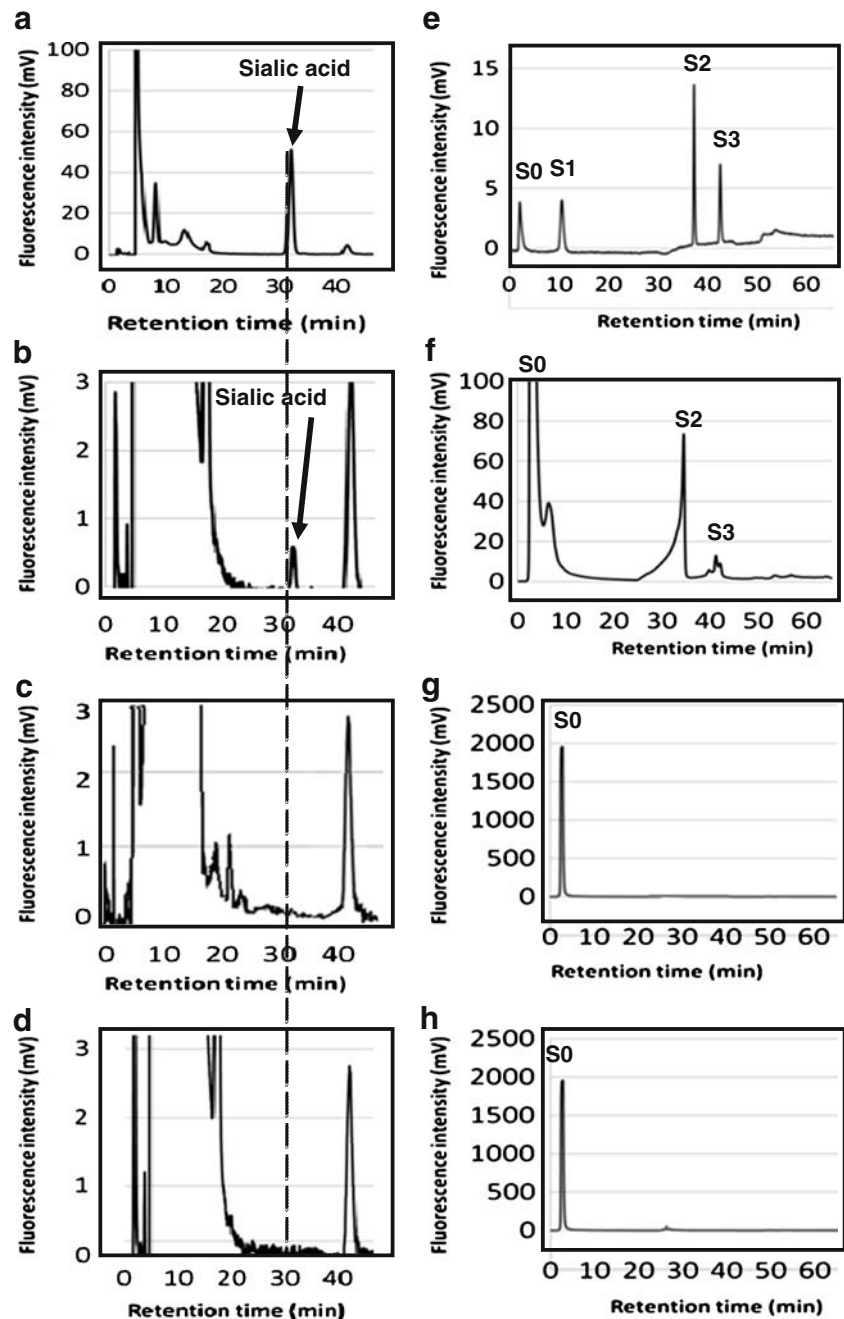
NAc₂-PA (Gal₂GN₂M₃F; 1,887.77). The fraction G in the RP-HPLC profile was identical to that of standard Gal(β1-4)GlcNAc(β1-2)Man(α1-6)[Gal(β1-4)GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-PA. These results suggest that the oligosaccharide in peak G was Gal(β1-4)GlcNAc(β1-2)Man(α1-6)[Gal(β1-4)GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-PA. The *N*-linked oligosaccharide structures in peaks C (*m/z* 1417), D (*m/z* 1,742), E (*m/z* 1,563), and F (*m/z* 1,726) were also confirmed by the same method as above. The *N*-linked oligosaccharide structures in peaks A (*m/z* 1,563) and B (*m/z* 1,725) were not identified by the same method because the pyridylaminated oligosaccharide standards corresponded to these peaks were not commercially available. These structures were estimated by the analysis of MALDI-TOF MS/MS and LC-ESI-MS/MS and from previous reports of recombinant erythropoietin (Cointe et al. 2000) and IgG (Chen and Flynn 2007) produced by CHO cells (data not shown). Table 1 shows a summary of the identified or estimated *N*-linked oligosaccharide structures, which are more than 5% of the total oligosaccharides. The molar ratio of each oligosaccharide to total oligosaccharide was calculated on the basis of the RP-HPLC profile.

The above results revealed that hEx3-scDb-Fc had a similar *N*-linked oligosaccharide structure to humanized anti-IL-8 antibody. In contrast, the *N*-linked oligosaccharide structure of human serum IgG was more complicated than those of the recombinant antibodies expressed in CHO cells. Approximately more than 90% of the *N*-linked oligosaccharides of hEx3-scDb-Fc and the anti-IL-8 antibody were α-1,6-fucosylated. The galactosylated biantennary oligosaccharides comprise over 40% of the total *N*-linked oligosaccharides in both hEx3-scDb-Fc and the anti-IL-8 antibody. However, the fully galactosylated biantennary oligosaccharides from hEx3-scDb-Fc and the anti-IL-8 antibody accounted for only 10% of the *N*-linked oligosaccharides, whereas more than 20% of *N*-linked oligosaccharides were fully galactosylated biantennary oligosaccharides in human serum IgG. The triantennary oligosaccharides (peaks A and B) were detected in all the samples.

Discussion

The activation of effector mechanisms is dependent on the structural characteristics of antibody molecules that result from posttranslational modifications, particularly, glycosylation (Jefferis 2005). The production of therapeutic antibodies having a glycoform profile consistent with that of human antibodies has remained a considerable challenge for the biopharmaceutical industry. It is therefore important to select cell lines and culture conditions that lead to a suitable glycosylation of the recombinant protein designed

Fig. 2 a–d Typical HPLC profile of DMB derivatives released from antibodies. Sialylated *N*-linked oligosaccharides were released by incubation in 50 mM sulfuric acid, labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) at the released sialic acid and separated by reverse-phase HPLC (RF-HPLC). The arrow indicates the elution position of sialic acid. **a** Fluorescence-labeled standard. **b** Intact human serum IgG. **c** Humanized anti-IL-8 IgG1. **d** Recombinant hEx3-scDb-Fc. **e–h** Anion exchange separation of pyridylaminated *N*-glycans released from antibodies. *N*-Linked oligosaccharides were released by digestion with peptide-*N*-glycosidase F, derivatized with 2-aminopyridine-acetic acid at the reducing terminus and separated by anion-exchange HPLC. The pyridylaminated *N*-linked oligosaccharides were eluted using a linear gradient of 100% solvent A (10% acetonitrile, pH 9.5) to 20% solvent B (10% acetonitrile in 3% acetic acid buffer, pH 7.3) as follows. *S*0 neutral oligosaccharides, *S*1 mono-sialyl oligosaccharides, *S*2 di-sialyl oligosaccharides; *S*3, tri-sialyl oligosaccharides. **e** Fluorescence-labeled standard. **f** Intact human serum IgG. **g** Humanized anti-IL-8 IgG1. **h** Recombinant hEx3-scDb-Fc



for therapeutic use. In particular, it remains controversial as to whether the sialylation of the Fc glycan can enhance the function of the antibodies (Raju 2008). It has been suggested that the lack of sialic acid from a carbohydrate has no effect on the activities of the tested IgGs (Boyd et al. 1995).

Here, we report a structural analysis of oligosaccharides released from Fc domains using 2D HPLC and MALDI-TOF MS. Our data reveal that the most significant differences among human serum IgG, hEx3-scDb-Fc, and the anti-IL-8 antibody are the high agalactosyl (G0) oligosac-

charide level (Table 1) and the deficiency in terminal sialic acid in hEx3-scDb-Fc and the anti-IL-8 antibody (Fig. 2).

The sialylation percent of human serum IgG1 was approximately 30% (S1, S2, and S3), which is in agreement with the result of a previous human serum glycosylation study (Kobata 2008). Sialic acid could attach to the terminal galactose residues of sugar chains. It was considered that the weak galactosylation of the *N*-linked glycan interferes with sialic acid binding to galactose, thereby causing a deficiency in terminal sialic acid in hEx3-scDb-Fc and the anti-IL-8 antibody. These differences are

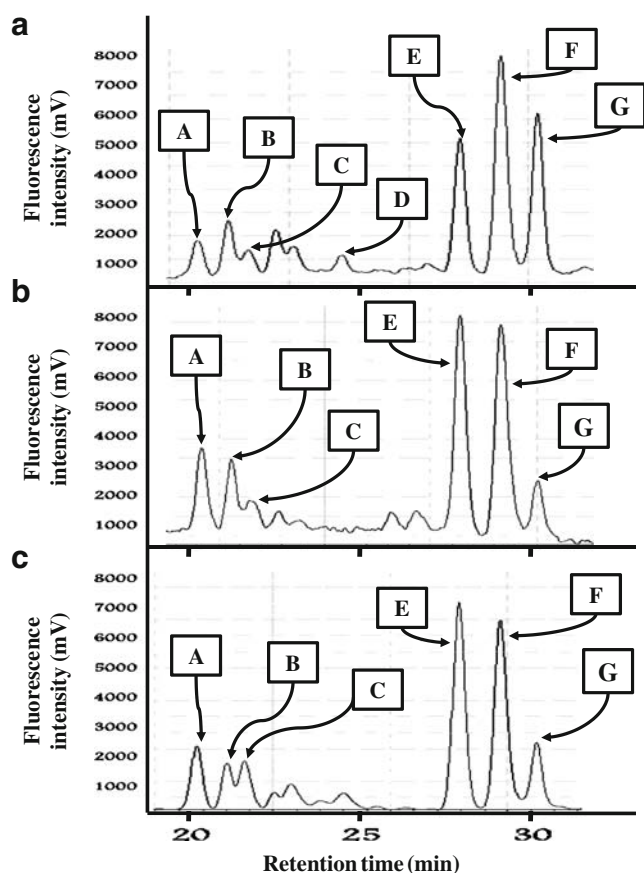


Fig. 3 Separation of pyridylaminated *N*-glycans released from human serum IgG, hEx3-scDb-Fc, and humanized anti-IL-8 IgG1 by RP-HPLC. The neutral oligosaccharide (*S*₀) separated on the anion exchange column is analyzed by RP-HPLC on an ODS C18 column. Pyridylaminated neutral oligosaccharides eluted using a linear gradient of 100% solvent A (0.02% trifluoroacetic acid) to 20% solvent B (20% acetonitrile in solvent A). The portion of these major peaks (A–G) is more than 90% of the total oligosaccharides. Monosaccharide compositions and putative glycan structure deduced by MALDI-TOF MS. **a** Human serum IgG. **b** Humanized anti-IL-8 IgG1. **c** hEx3-scDb-Fc

most probably because of the presence of CMP-*N*-acetylneuraminic acid hydroxylase in CHO cells or limited accessibility for the β -galactosyltransferase enzyme and terminal galactose residues being unavailable to the sialyltransferase (Chenu et al. 2003). The other possibility is that all the sialic acid was removed from glycoprotein during purification process. Since we do not know yet whether the humanized anti-IL-8 IgG1 and hEx3-scDb-Fc contain no sialic acid, despite the CHO-derived erythropoietin generally containing α -2,3-sialic acid (Watson et al. 1994), further study of this issue is important to produce recombinant pharmaceutical proteins with a glycosylation as close as possible to that of the native protein.

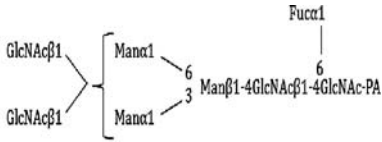
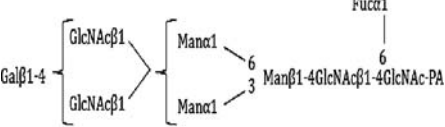
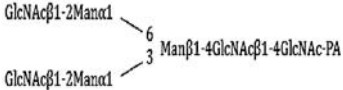
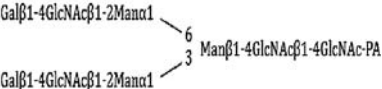
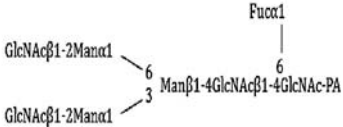
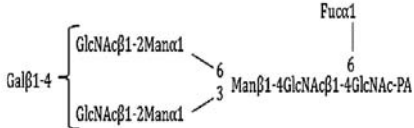
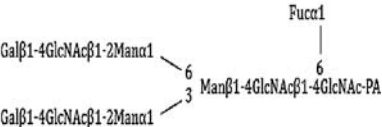
As shown in Fig. 3 and Table 1, the mixture of neutral oligosaccharides released from antibodies was separated

into approximately seven major *N*-glycan structures. The major peaks (E, F, and G) are biantennary *N*-glycans with a core fucose but have no bisecting GlcNAc residues (Table 1). These three oligosaccharides, called digalactosyl (G2), monogalactosyl (G1), and agalactosyl (G0) oligosaccharides, comprise over 70% of the total *N*-glycans in our samples. Our results support a previous result showing that the percentages of G0, G1, and G2 are over 67% (Parekh et al. 1985). The aggregations of hEx3-scDb-Fc and the anti-IL-8 antibody were frequently observed during the experiments compared with that of human serum IgG (data not shown). Leader et al. (1996) reported that the aggregation of IgG derived from the serum of a rheumatoid arthritis patient is due to the lack of terminal galactose residues at the Fc glycosylation site. In our study, the fully galactosylated biantennary oligosaccharide from hEx3-scDb-Fc and the anti-IL-8 antibody accounted for only 10% of the *N*-linked oligosaccharides. It is indicated that agalactosyl antibodies might be more prone to the aggregation or formation of insoluble immune complexes.

The glycosylation pattern of hEx3-scDb-Fc was quite similar to that of the anti-IL-8 antibody. In contrast, the *N*-glycan structure of human serum IgG was more complicated than those of hEx3-scDb-Fc and the anti-IL-8 antibody expressed in CHO cells. Glycosylation is a highly complex cellular process dependent on the 3D structure of a protein, the enzyme repertoire of the host cell, the transit time in the Golgi, and the availability of intracellular sugar–nucleotide donors (Butler 2006). The anti-IL-8 antibody is a molecule of about 160 kDa composed of four peptide chains. In contrast, the bispecific antibodies with the Fc portion (hEx3-scDb-Fc) is a molecule of about 160 kDa composed of two peptide chains. *N*-glycosylation is dependent on the 3D structure of a protein. Similar glycosylation pattern of hEx3-scDb-Fc means that the larger size of bispecific antibodies with the Fc portion may not affect Fc glycosylation. Our previous study showed that the growth inhibition effects of hEx3-scDb-Fc were considerably superior to those of the approved therapeutic antibody cetuximab in vitro (Asano et al. 2007). The right glycosylation of Fc portion may activate the interactions with FcRs and FcR-mediated effector functions.

In this study, we compared the oligosaccharide structures of hEx3-scDb-Fc and the anti-IL-8 antibody at the end of the same batch cultivation because large amounts of products were necessary for the analysis of oligosaccharide structure. However, the *N*-glycosylation pattern is also dependent on the cell culture conditions. In the industrial cultivation, several factors should be fed into the reactors during cultivation to enhance the antibody production. The detail time course of the glycosylation pattern should be investigated using large-scale fed-batch cultivation in future.

Table 1 Structures of *N*-linked glycans obtained from purified IgG from human serum and humanized anti-IL-8 IgG1 and hEx3-scDb-Fc produced in recombinant CHO cells

Peak (Code No.) ^a	Oligosaccharide structure	Human Serum IgG	Anti-IL-8 IgG1	hEx3-scDb-Fc	Molecular mass ^b
A ^c (-)		5.0±1.4	11.9±0.9	10.3±0.1	1563
B ^c (-)		7.2±0.7	9.7±0.2	6.7±0.1	1725
C (200.1)		4.9±0.7	5.9±1.1	8.2±0.1	1417
D (200.4)		5.4±0.9	-	-	1742
E (210.1)		17.8±0.2	30.6±0.6	32.1±0.5	1563
F (210.2 or 210.3)		29.4±2.0	33.0±1.3	31.4±0.2	1726
G (210.4)		23.7±0.1	8.9±0.4	11.2±0.8	1887

Means ± standard deviation

^aNumbering of oligosaccharides is described according to previous report (Tomiyama et al. 1988)

^bThe molecular masses of compounds were analyzed by MALDI-TOF MS

^cEstimated structures were shown in peaks A and B

In conclusion, the glycosylation pattern of a hEx3-scDb-Fc produced by recombinant CHO cells is quite similar to that of a recombinant humanized anti-IL-8 antibody (IgG1). However, the fully galactosylated biantennary oligosaccharide from hEx3-scDb-Fc and the anti-IL-8 antibody accounted for only 10% of the *N*-linked oligosaccharides, whereas more than 20% of the *N*-linked oligosaccharides were fully galactosylated biantennary oligosaccharides in human serum

IgG. The improvement of galactosylation is of practical importance for bispecific antibodies with the Fc portion produced by CHO cells.

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