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# Pairing of oligosaccharides in the Fc region of immunoglobulin G

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**Abstract The Fc portion of immunoglobulin G (IgG) expresses paired oligosaccharides with microheterogeneities, which are associated with efficiencies of effector functions and with pathological states. A comparison of electrospray ionization mass spectrometry data obtained using a variety of Fc fragments derived from human and mouse IgG that do and do not retain the inter-chain disulfide bridge(s) revealed that (1) the Fc portion can be asymmetric as well as symmetric with respect to glycosylation and (2) the ratios of the individual glycoforms are different from what is expected from the random pairing.**



*Key words:* Immunoglobulin G; Fc; *N*-glycan; Glycosylation; Electrospray ionization mass spectrometry; Galactosylation; High-performance liquid chromatography

## 1. Introduction

Immunoglobulin G (IgG), which is a multifunctional glycoprotein with an approximate molecular mass of 150 kDa, consists of two heavy chains (with identical amino acid sequences) and two light chains (with identical amino acid sequences). The heavy chains are composed of four homologous domains, $V_H$, $C_H1$, $C_H2$ and $C_H3$, whereas the light chains are divided into two homologous domains, $V_L$ and $C_L$. The $C_H1$ and $C_H2$ domains are separated by the hinge region, where the two heavy chains are disulfide-linked to each other. The hinge region is generally susceptible to the attack of proteases, e.g. papain, giving rise to the Fab and the Fc fragments. The Fab portions carry antigen recognition, whereas the Fc portion promotes a variety of effector functions.

IgG molecules possess one conserved glycosylation site at Asn-297[1] in the $C_H2$ domain of each of the heavy chains, where complex biantennary type oligosaccharides are expressed (Fig. 1). It has been reported that aglycosyl IgG molecules, which have been prepared by treating hybridoma cells with tunicamycin or by site-directed mutagenesis at position 297, retain little ability in activating complements and in binding to Fcγ receptors (FcγR) [1,2]. These results indicate that the oligosaccharide linked to Asn-297 is essential for the expression of the proper effector functions of IgG.

It is widely known that the carbohydrate chains exhibit various microheterogeneities resulting from the presence or absence of fucose (Fuc), bisecting *N*-acetylglucosamine (GlcNAc), galactose (Gal) and sialic acid, e.g. *N*-acetylneuraminic acid (NeuAc) residues (Fig. 1), depending on species, aging, pathological states and culture conditions of IgG-producing cells (for review, see [3–5]). In the case of mouse IgG antibodies, the N-linked oligosaccharides lack bisecting GlcNAc and are almost fully fucosylated [6].

The neutral oligosaccharides linked to IgG are classified into three types, i.e. G0, G1 and G2, which possess zero, one and two terminal Gal residues, respectively. The proportion of carbohydrate chains of IgG bearing G0 is increased in sera of patients with rheumatoid arthritis (RA) [7,8], juvenile chronic arthritis [9] active Crohn's disease [10,11], infection due to *Mycobacterium tuberculosis* [11] or spondyloarthropathy [12]. The decrease in the galactosylation of IgG oligosaccharides has also been found in autoimmune MRL/Mp-lpr/lpr mice [13]. It has been reported that agalactosyl IgG glycoforms are directly associated with pathogenicity in murine collagen-induced arthritis [14]. Certain rheumatoid factors (RFs) bind better to IgG that is deficient in Gal [15]. It has been recently proposed that agalactosyl IgG activates complements through interaction with mannose-binding protein (MBP), an endogenous lectin, resulting in the inflammation of RA [16]. By contrast, Gal content in IgG carbohydrate chains is significantly increased during pregnancy, when remission of RA symptoms occurs [17]. In addition, galactosylation of fetal IgG is higher than that of maternal IgG, which suggests that there exists a placental selective transport with preference for highly galactosylated IgG molecules [18,19].

It has been reported that agalactosyl IgG exhibits a significant degree of reduction of the ability to bind to complement component C1q and FcγRs resulting in impaired effector functions [10–23] and slower clearance of this molecule [24]. Although the observed effects of agalactosylation on FcγR-mediated activity varies depending upon details of the analyses, and even contradictory data have been reported [25–28], the hypothesis that different glycoforms of IgG have different roles in vivo is worth addressing.

A variety of techniques have been used to obtain information concerning the glycoforms of IgG-Fc. The levels of galactosylation of IgG have been conventionally estimated by membrane blots and by enzyme-linked immunosorbent assay (ELISA)-based assays using lectins or anti-GlcNAc antibodies (for reviews, see [29,30]). Chromatographic methods have

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[1] The numbering system used in the present study is based on human myeloma protein Eu [76].


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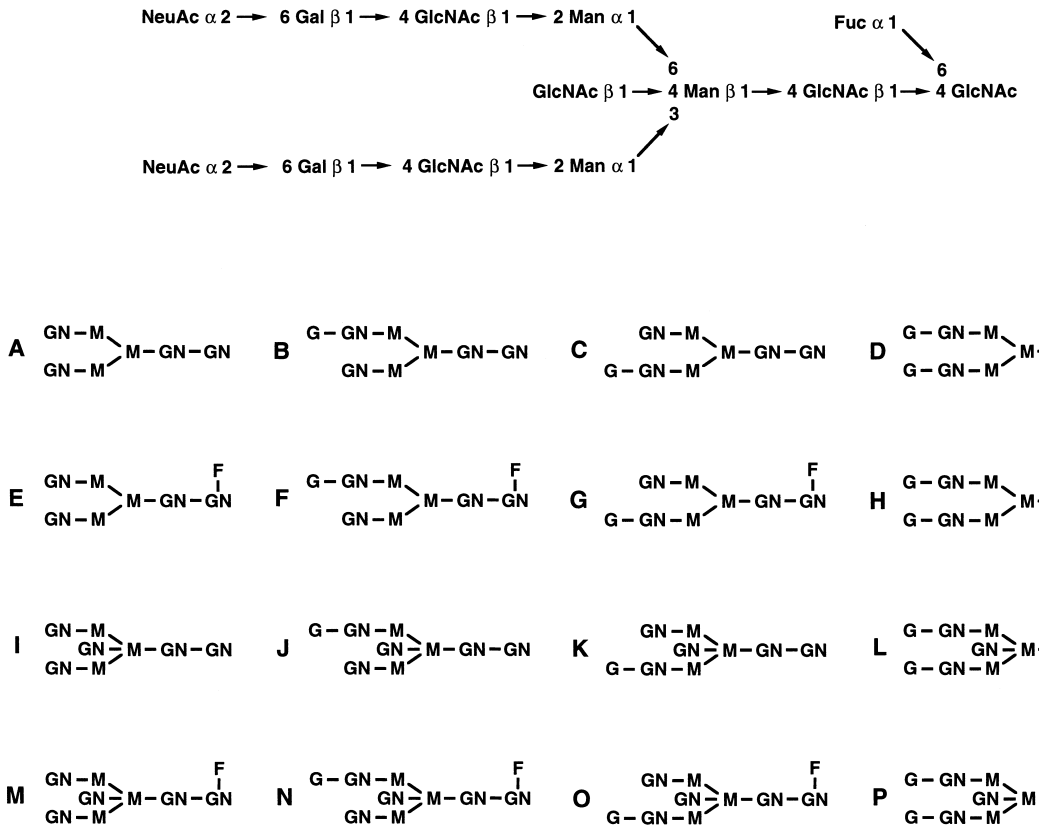


Fig. 1. Structure of the fully matured complex biantennary type oligosaccharide (top) and 16 possible structures of neutral oligosaccharide contained in IgG-Fc (A–P). Key: F, fucose; G, galactose; GN, *N*-acetylglucosamine; M, mannose.

greatly facilitated the determination of prevalence of oligosaccharides released from IgG [31]. Especially the high-performance liquid chromatography (HPLC) analyses of pyridylamino derivatives of oligosaccharides have made it possible to identify individual neutral and sialyl oligosaccharides rapidly [32–34]. Recently, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have greatly expanded the analytical utility of mass spectrometry (MS) for the structural characterization of biological macromolecules including IgG and its proteolytic fragments [35,36]. Information of carbohydrate heterogeneity has been provided by ESI-MS analyses of digested peptides [37–39], monomeric Fc subunits [40–43] or reduced heavy chains of IgG [37,41,44,45].

Although all of the above techniques have contributed greatly to the elucidation of IgG glycosylation profiles, an incidence of G0 determined by chemical sequencing does not always correlate with the level of agalactosyl IgG (IgG with no Gal residue) estimated by an ELISA-based assay using an anti-GlcNAc antibody [46]. As an explanation for this paradox, Williams and Rademacher have suggested that selective pairing of agalactosyl oligosaccharides in one IgG molecule is necessary for epitope formation and therefore enhances the binding to the anti-GlcNAc antibodies. Similar concepts can be applicable in considering the interactions of IgG with other ligands. Namely, it is possible that particular pairing of oligosaccharides is necessary for optimum binding of IgG to C1q, FcγRs, RFs and/or MBP, and the apparent contradiction among the data so far reported on the glycoform–function relationships of IgG is, at least partially, due to the lack of information of the mode of pairing of the oligosaccharides. White et al. have reported that a pair of dinitrophenyl-specific murine monoclonal IgG2a having similar monosaccharide content show difference in lectin-binding and complement activation [47]. Although the authors have suggested that orientation of the *N*-glycan is different between these antibodies resulting in differential abilities of lectin-binding and of interaction with the complement system, it is possible that the observed differences are due to the difference in incidence of pairing of particular oligosaccharides. Therefore, knowledge concerning pairing of oligosaccharides in Fc is of vital importance for our further understanding of biological and pathological significance of the heterogeneities of the IgG glycans.

Since two oligosaccharides are paired in the Fc region of one IgG molecule, complete glycoforms of IgG-Fc can be quite diverse. For example, the 16 kinds of neutral oligosaccharides, which have been identified in human IgGs, theoretically give rise to 136 glycoforms. X-ray crystallographic studies have shown that the pair of carbohydrate chains are packed between the two $C_H2$ domains: the core and Manα1-6 branch make contact with an inner surface of the $C_H2$ domain, whereas the Manα1-3 branch protrudes to the space between the $C_H2$ domains [48–51]. In the crystal structure of the rabbit IgG-Fc, the terminal Gal residue of the Manα1-3 branch of one sugar chain makes contact with the Manα1-3 branch of the opposing sugar chain, leaving no space to accommodate the two galactosylated Manα1-3 branches [3,49]. On inspection of this observation, it has been proposed that one of the paired oligosaccharides must always devoid Gal in the Manα1-3 branch, i.e. not all possible

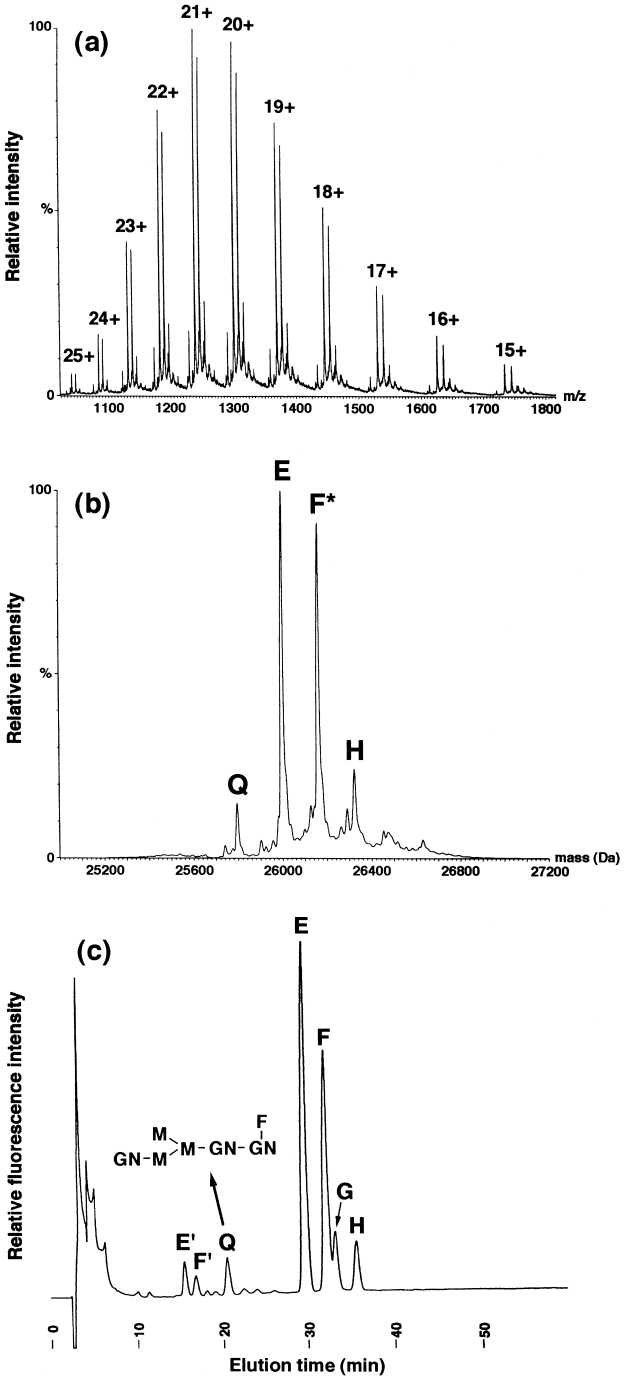


Fig. 2. Glycosylation profiles of Fc/2 fragment of mouse IgG2b: raw (a) and transformed (b) nano ESI-MS spectra of Fc/2 and an HPLC profile of pyridylamino derivatives of N-linked oligosaccharides liberated from Fc/2 on an ODS column (c). The alphabetical peak codes correspond to those of the oligosaccharide structures in Fig. 1. In (b), F* represents either F or G (see text). The structure of oligosaccharide Q is shown in (c). Peaks E′ and F′ in (c) are due to epimers of the pyridylamino derivatives corresponding to peaks E and F, respectively. The two very minor peaks observed at 26472.8 and 26634.9 Da were tentatively assigned to (Fuc)-$(Hex)_4(HexNAc)_4(NeuGc)$ and $(Fuc)(Hex)_5(HexNAc)_4(NeuGc)$, respectively, although these monosialyl glycoforms were barely detected in the HPLC analysis because of a low incidence (less than 1%).

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combination of the carbohydrate chains can occur. Since no experimental methodology that could determine oligosaccharide pairing in Fc has so far been established, the upper and lower estimates of IgG glycoform population have been computed on the basis of molar percentage of oligosaccharides liberated from IgG, postulating the pairing rules [52].

Mass spectrometry has a potential ability to elucidate pairing of oligosaccharides in IgG-Fc. Previous studies have shown that ESI-MS spectra of IgG and Fc fragments, which retain the inter-chain disulfide bridge, exhibit multiple series of peaks that are associated with the carbohydrate heterogeneities [41,44,45]. However, in these studies, identification of the unambiguous glycoforms of Fc has never been achieved mainly due to the lack of information of glycan structures with discrimination of isomeric carbohydrate moieties. Also, it has never been clarified whether the MS data thus obtained could provide quantitative information of IgG glycoforms.

Here, we performed structural analyses of IgG glycoforms by combined use of the HPLC and ESI-MS methods. The structure and prevalence of oligosaccharides contained in the Fc fragments of IgG used were unambiguously determined by HPLC analyses on their pyridylamino derivatives. It was demonstrated that Fc glycosylation profiles estimated by nano ESI-MS are in agreement in a quantitative way with those obtained by the HPLC analyses of the liberated oligosaccharides. By use of the Fc fragment retaining inter-chain disulfide bridge(s), pairing of oligosaccharides in Fc has been analyzed.

## 2. Materials and methods

### 2.1. Purification of IgGs

Mouse monoclonal IgG antibodies were prepared by cultivating IgG-producing cells. Cell lines 27-13.6 and 27-35.8, which produce mouse anti-dansyl IgG2a and IgG2b, respectively [39,53], were generously provided by Professor L.A. Herzenberg and Dr. V.T. Oi, and adopted to a serum-free medium (Nissui NYSF 404). The IgG-producing cells were cultivated in tissue culture flasks (Corning) at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. After cell growth, mouse IgGs were isolated from the supernatants by use of an Affi-Gel protein A column (Bio-Rad) as described previously [54].

Human IgG was isolated from venous samples collected from pregnant women or from the umbilical cord (fetus) by use of protein G affinity columns as described previously [19]. For desialylation, 20 mU of sialidase from *Arthrobacter ureafaciens* (Nacalai Tesque) was used to treat 500 pmol of substrate in 15 μl of 0.1 M citrate-phosphate buffer, pH 5.0, at 37°C for 16 h.

### 2.2. Preparation of Fc fragments

Fc fragments retaining the inter-chain disulfide bridge(s) were prepared by proteolysis as described with slight modifications [55,56]. The mouse IgG (10 mg/ml) was incubated in the presence of Lys-C endoprotease (Wako Pure Chemical Industries) at pH 8.5, 37°C in 50 mM Tris–HCl for 1 h. The enzyme/substrate molar ratio was 1:200. The reaction was terminated by the addition of 3 mM diisopropyl fluorophosphate. The human IgG (0.5–5 mg/ml) was incubated in the presence of papain (Sigma) at 37°C, in 75 mM sodium phosphate buffer, pH 7.0, containing 75 mM NaCl and 2 mM EDTA for 4.5–6 h. The enzyme/substrate molar ratio was 1:25. The reaction was terminated by the addition of 33 mM *N*-ethylmaleimide. The Fc fragments were purified by a combined use of protein A affinity chromatography and Superose 12 gel-filtration HPLC (Amersham Pharmacia Biotech) as described [56]. By amino acid sequence analyses on an Applied Biosystem protein sequenator model 477A/120A, the N-terminal residues of the Fc fragments of mouse IgG2a, IgG2b and human IgG1 was confirmed as Lys-228, 228D and Thr-225, respectively.

The Fc fragments (1–5 mg/ml) were reduced by 10 mM DTT at room temperature for 1 h in 1.5 M Tris–HCl, pH 8.5, containing

2 mM EDTA. For alkylation, 22 mM iodoacetic acid was added to the reaction mixture, which was incubated in the dark for 20 min at room temperature. The reaction was stopped by addition of 10 mM DTT and then dialyzed against water. Hereafter, the Fc fragment retaining the inter-chain disulfide bridge(s) and the reduced and alkylated Fc fragment, which is detected as monomeric subunit in ESI-MS, will be simply designated as Fc and Fc/2, respectively.

*2.3. Nanoflow ESI-MS analyses*

Nano ESI-MS was acquired using a Q-TOF instrument (Micromass, Manchester, UK) and MassLynx data acquisition. This instrument is a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, with a Z-spray nanoflow electrospray ion source. The mass spectrometer was operated in the positive-ion mode. Resolution of the mass spectrometer was 5000 at *m/z* 1500. The cone voltage was set to 50–70 V. Purified samples were extensively dialyzed against water, lyophilized and then dissolved at a protein concentration of 1–20 μM in a solution containing equal amounts of acetonitrile and 0.2% formic acid. Two μl of the sample solution was loaded into a nanoflow tip. A flow rate of about 50 nl/min into the analyzer was produced as a result of a potential of 1.0 kV applied to the nanoflow tip in the ion source. Ten to 20 spectra were averaged, baseline subtracted, smoothed, centroided and deconvoluted.

*2.4. HPLC analyses of oligosaccharide profile*

Neutral and sialyl oligosaccharide analyses of IgG were performed by HPLC, as previously described in detail [32–34]. The oligosaccharide moieties were released by enzymatic cleavage with glycoamidase A (Seikagaku Kogyo). The reducing ends of the oligosaccharides were reductively aminated with 2-aminopyridine by use of sodium cyanoborohydride [57]. The mixture of pyridylamino derivatives of the oligosaccharides thus obtained was applied onto a diethylaminoethyl (DEAE) HPLC column to separate neutral, monosialyl and disialyl oligosaccharide fractions, each of which was then subjected to HPLC analysis on a Shim-pack CLC-octadecylsilyl (ODS) column (Shimadzu) with fluorescence detection. The collected fraction was further subjected to an HPLC analysis using a TSK-GEL Amide-80 column (Tosoh) if necessary. The structure of each peak separated on the column was identified as described previously [32–34].

## 3. Results and discussion

*3.1. Oligosaccharide profiles by ESI-MS*

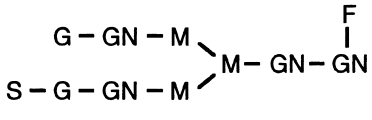
For obtaining information of the Fc-associated glycoforms by MS, the use of Fc fragments was essential, because the resolution of ESI-MS of intact IgG was not sufficient for quantitative analyses [41,44,45]. It is also possible that glycosylation in the Fab and/or the hinge regions can make the spectral analyses equivocal. We have established protocols to prepare Fc as well as Fc/2 fragments of mouse IgG2a, IgG2b and human IgG1 with the homogeneous N-terminal [55,56]. Firstly, by using the Fc/2 fragments, we compared glycosylation profiles obtained by nano ESI-MS with those determined by HPLC analyses of the liberated oligosaccharides.

Fig. 2a,b show nano ESI-MS and its transformed spectra of the Fc/2 fragment of a mouse IgG2b antibody. Two major (25999.5 and 26161.6 Da) and two minor (25796.3 and 26323.8 Da) ion peaks are observed in the spectra with mass differences corresponding to a hexose or *N*-acetylhexosamine residue. Molecular mass of the polypeptide chain of this Fc/2 calculated on the basis of the amino acid sequence [39] is 24555.9. This led us to conclude that the chemical structures of glycans corresponding to the observed ion peaks

Table 1
Major glycoforms of Fc/2 fragments

| Fc/2 | Glycoforms | Molecular mass (Da) | | Relative quantity | |
|---|---|---|---|---|---|
| | | calculated | observed | ESI-MS[a] | HPLC[b] |
| Mouse IgG2b (lot 1)[c] | Q | 25798.1 | 25796.3 | 6.2 | 8.1 |
| | E | 26001.3 | 25999.5 | 45.7 | 47.9 |
| | F* | 26163.4 | 26161.6 | 40.1 | 37.8 (30.5)[g] |
| | H | 26325.6 | 26323.8 | 8.0 | 6.2 |
| Mouse IgG2b (lot 2) | E | 26001.3 | 26000.7 | 15.6 | 14.8 |
| | F* | 26163.4 | 26163.0 | 57.4 | 56.7 (50.4) |
| | H | 26325.6 | 26325.2 | 27.0 | 28.5 |
| Mouse IgG2a (lot 1) | E | 26380.8 | 26382.1 | 41.7 | 39.4 |
| | F* | 26543.0 | 26543.8 | 52.8 | 56.4 (53.7) |
| | H | 26705.1 | 26706.3 | 5.5 | 4.2 |
| Mouse IgG2a (lot 2) | E | 26380.8 | 26380.7 | 54.2 | 52.8 |
| | F* | 26543.0 | 26543.1 | 44.1 | 44.8 (41.0) |
| | H | 26705.1 | 26705.7 | 1.7 | 2.4 |
| Maternal human IgG1[d] | E | 26495.8 | 26494.9 | 8.1 | 8.5 |
| | F* | 26657.9 | 26657.1 | 31.6 | 28.7 (25.8) |
| | H | 26820.1 | 26819.2 | 60.3 | 62.8 |
| Fetal human IgG1[e] | E | 26495.8 | 26493.9 | 8.8 | 10.3 |
| | F* | 26657.9 | 26656.3 | 22.9 | 24.6 (22.0) |
| | H | 26820.1 | 26818.3 | 43.8 | 46.6 |
| | Mc[f] | 27111.3 | 27109.6 | 24.5 | 18.5 |

[a]Relative ion intensities in transformed spectrum.
[b]Relative peak areas.
[c]ESI-MS spectrum is shown in Fig. 2.
[d]With sialidase treatment.
[e]ESI-MS spectrum is shown in Fig. 3.
[f]Structure of Mc:




[g]Relative quantities of F are indicated in parentheses.

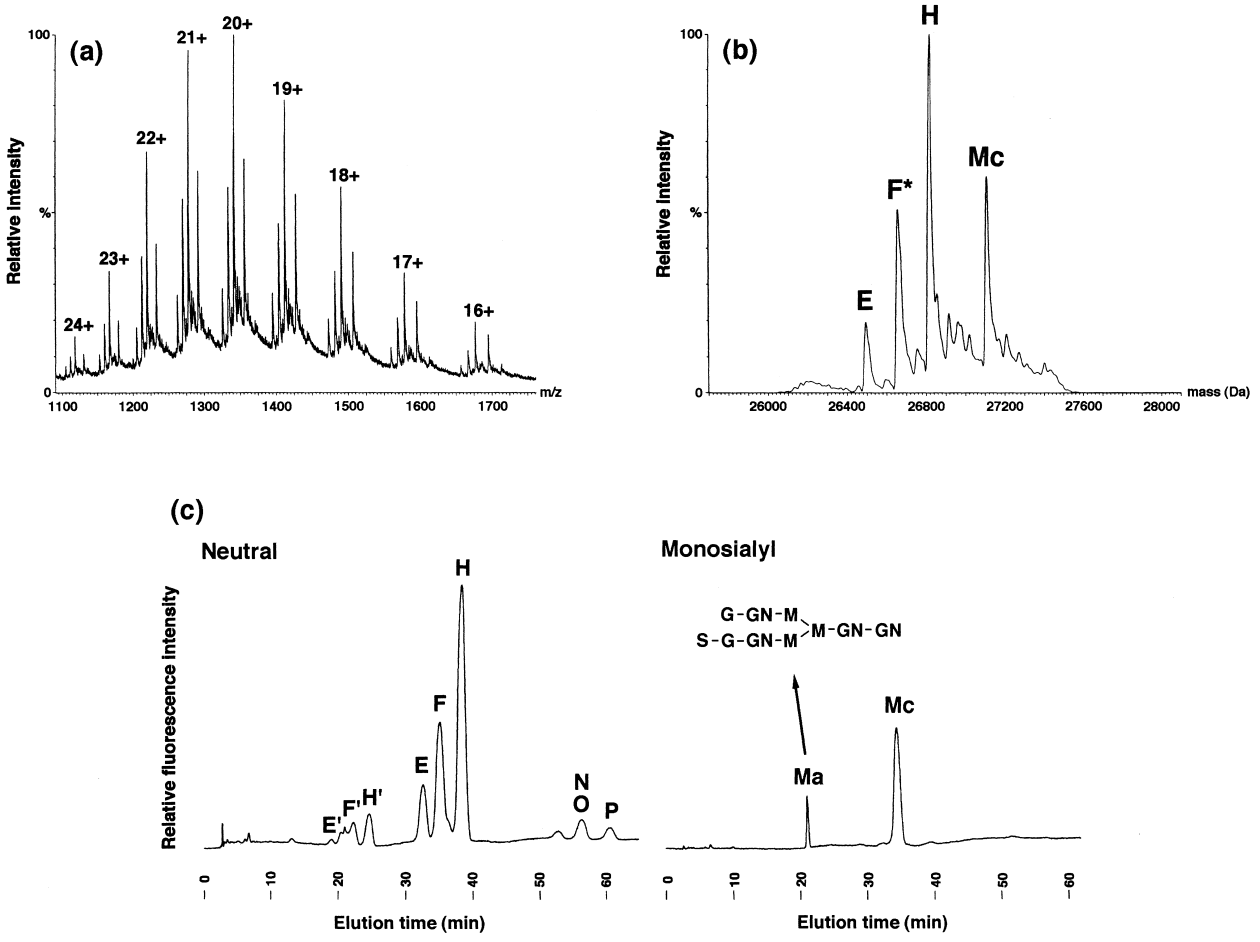



Fig. 3. Glycosylation profiles of Fc/2 fragment of human IgG1: raw (a) and transformed (b) nano ESI-MS spectra of Fc/2 and an HPLC profile of pyridylamino derivatives of N-linked oligosaccharides liberated from Fc/2 (c). In (c), elution profiles on an ODS column of pyridylamino derivatives of neutral and monosialyl oligosaccharides, which were previously separated on a DEAE column, are individually shown. Virtually no disialyl oligosaccharide was detected for the Fc/2 sample (data not shown). The structure of oligosaccharide Ma is shown in (c), where S represents *N*-acetylneuraminic acid. Peaks E′, F′ and H′ in (c) are due to epimers of the pyridylamino derivatives corresponding to peaks E, F and H, respectively. The minor peaks observed at 26858.9, 26963.2, 27020.8 and 27401.1 Da are due to N and/or O, Ma, P and disialyl glycoforms, respectively.

at 25796.3, 25999.5, 26161.6 and 26323.8 Da are $(Fuc)(Hex)_3(HexNAc)_3$, $(Fuc)(Hex)_3(HexNAc)_4$, $(Fuc)(Hex)_4(HexNAc)_4$ and $(Fuc)(Hex)_5(HexNAc)_4$, respectively. Fig. 2c shows the HPLC elution profiles of the pyridylamino derivatives of the oligosaccharides released from the same Fc/2 fragment used in the experiment. The oligosaccharides attached to the IgG2b-Fc used consist of two major components, E and F, and three minor components, G, H and Q. No sialyl oligosaccharides were detected for the Fc sample (data not shown). Oligosaccharide Q is monoantennary and lacks GlcNAc linked to the Manα1-6 residue. Monoantennary oligosaccharides have been detected in human IgG2 and IgG3 paraproteins [5] and human IgG1 and IgG2 secreted by human–mouse hybridoma [58]. As far as we know, this is the first example of a monoantennary oligosaccharide detected in mouse IgG.

On the basis of these data, we assigned the ESI-MS ion peaks to Q, E, F plus G, and H as indicated in Fig. 2b. Although oligosaccharides F and G are isomeric with each other and, therefore, cannot be distinguished solely on the basis of the ESI-MS data, the HPLC profile clearly indicates that F predominates G (Fig. 2c). Hereafter, an oligosaccharide that can be either F or G will be represented as F*. It should be noted that the relative intensities of the nano ESI-MS ion peaks are in good agreement with those of the corresponding neutral oligosaccharides detected in the HPLC profile.

We have also compared the glycosylation profiles obtained by nano ESI-MS with those determined on the basis of the HPLC elution profiles for Fc/2 fragments of various samples of mouse and human IgG antibodies, which contain only neutral oligosaccharides with different degrees of galactosylation. The results are summarized in Table 1. In each case, relative quantities of individual glycans determined by the nano ESI-MS analyses are in good agreement with those determined by the HPLC analyses.

We have further examined the glycoforms of the Fc/2 fragment of fetal human IgG1 by nano ESI-MS as well as HPLC methods (Fig. 3). As shown in Fig. 3b, the Fc/2 fragment of the fetal human IgG1 used is heavily galactosylated and contains NeuAc exclusively at the terminal of Manα1-3 branch. Preferential monosialylation on the Manα1-3 branch has been commonly observed for IgG-Fc [59]. Although the spectrum is complicated owing to the minor peaks from bisected or sialyl glycoforms, the relative intensities of the major ion peaks

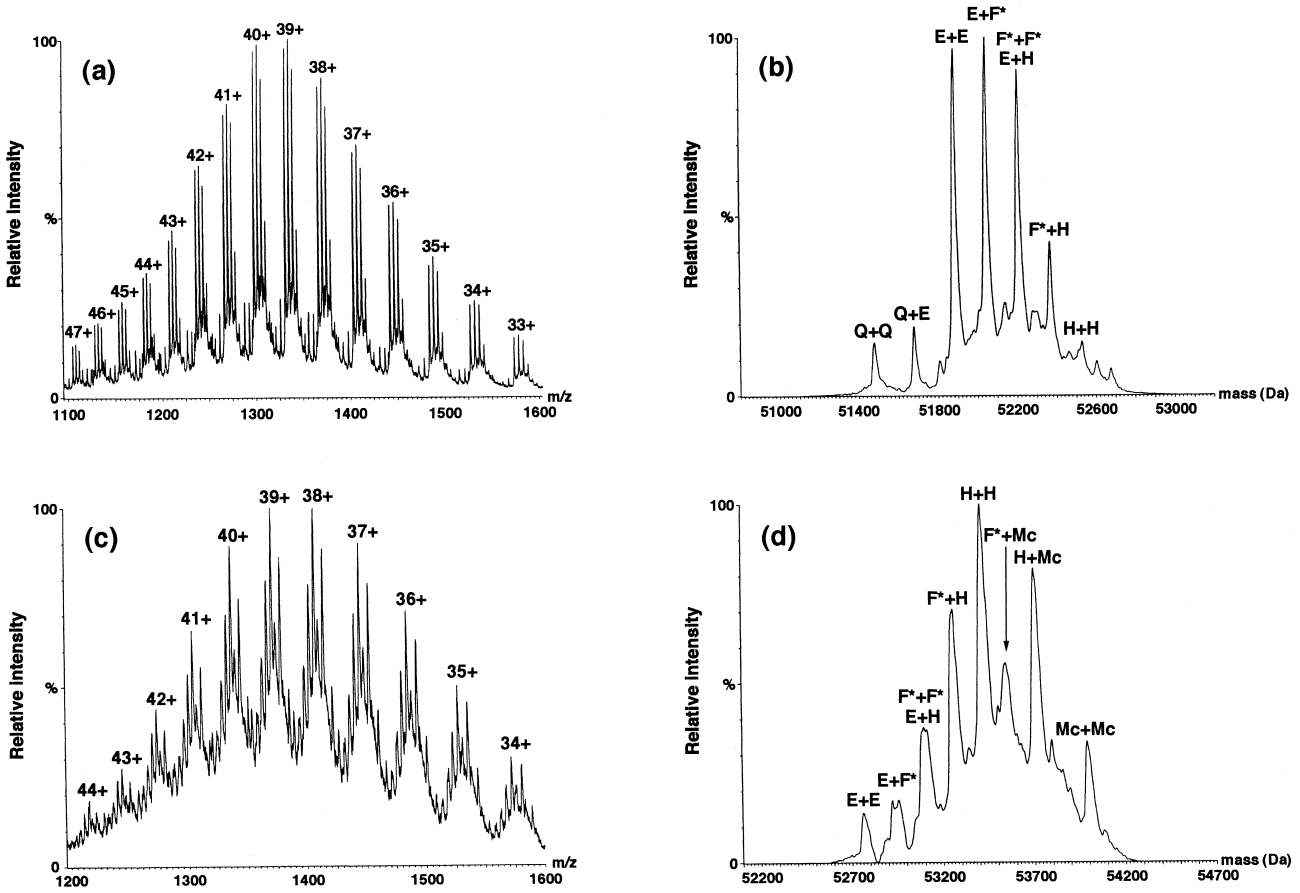



Fig. 4. Glycosylation profiles of Fc fragments of mouse IgG2b (a, b) and fetal human IgG1 (c, d): raw (a, c) and transformed (b, d) nano ESI-MS spectra of the Fc fragments. In (d), each peak exhibits a shoulder presumably due to a sodium adduct.

again well correlate with those of corresponding peaks in the HPLC elution profiles. On the basis of all of these data, we conclude that nano ESI-MS provides us with information of glycoforms of IgG-Fc involving neutral and sialyl oligosaccharides in a quantitative way.

Roberts et al. have shown that MALDI- and ESI-MS data of tryptic glycosylated non-apeptides originating from a reshaped human monoclonal IgG provide us with quantitative information on the carbohydrate chains [38]. They have argued that differences in charging of glycans in ESI-MS can affect the observed ion peak ratios among glycoforms and the lowest charge state reflect the relative solution concentrations most faithfully. In the present data, a modest difference in ion peak ratios was observed for different charge states (Figs. 2a and 3a), indicating that any effects resulting from the charging of glycans on apparent prevalence of individual glycoforms are negligible for larger glycoproteins such as Fc/2 as compared with small glycopeptides.

### 3.2. Pairing of the carbohydrate chains in Fc

In order to obtain information of pairing of oligosaccharides in the Fc region, Fc fragments, which retain the inter-chain disulfide bridge(s) in the hinge region, were subjected to mass spectrometry. Fig. 4a,b show nano ESI-MS spectrum of the Fc fragment of mouse IgG2b with the inter-chain disulfide bridge, which is the counterpart of the Fc/2 fragment shown in Fig. 2. In the transformed ESI-MS spectrum, ion peaks corresponding to the individual pairing were clearly observed showing that glycoforms with homologous (e.g. Q+Q and E+E) and heterologous (e.g. Q+E and E+F*) combinations of oligosaccharides co-exist. Ratios of individual glycoforms are shown in Table 2. It should be noted that the ratios of the individual glycoforms were significantly different from what is expected on the basis of the random pairing. Namely, pairing of the carbohydrate moieties tends to be homologous with the exception that the incidence of the F*+H pairing surpasses the expectation. For example, the incidence of the E+F* pairing, which would be protuberant if a random pairing is assumed, is just comparable with those of E+E and F*+F*. A similar tendency was observed for mouse IgG2a-Fc (Table 2).

It is possible that the glycosylation profiles that involve pairing of the oligosaccharides in Fc offer insights into intracellular trafficking of individual IgG molecules on the biosynthesis pathway. The high mannose (Man) carbohydrate moieties containing three glucose (Glc) residues ($Glc_3Man_9GlcNAc_2$) is transferred from dolichyldiphosphate to Asn-297 in a newly synthesized IgG heavy chain polypeptide as it emerges from the ribosome. After removal of the three terminal Glc, the glycoprotein moves to the Golgi, where the two heavy and two light chains have been already assembled [60] and enzymes involved in processing of glycan are compartmentalized [61]. In the *cis* Golgi, the glycans undergo trimming of the Man residues by α-mannosidases. Next, in the *medial* Golgi, the glycans are further trimmed and fucosylated giving rise to oligosaccharide Q and then converted to oligosaccharide E by *N*-acetylglucosaminyltrans-

ferase II (GnT-II). The glycoprotein then moves to the *trans* Golgi, where it undergoes galactosylation by β1,4 galactosyltransferase (β1,4-GTase) and sialylation by α2,6 sialyltransferase. The present nano ESI-MS data revealed that oligosaccharide Q is paired either with Q itself or E but not with galactosylated oligosaccharides, i.e. F* and H. This indicates that the IgG molecules carrying oligosaccharide Q, which is generated possibly as a result of deficiency of GnT-II in the *medial* Golgi, escape galactosylation in the *trans* Golgi. Various possibilities may explain the observed bias: (1) there exists a bypass from the *medial* Golgi that allows IgG to be secreted without moving to the *trans* Golgi, (2) oligosaccharide Q in one heavy chain precludes galactosylation of oligosaccharide E in the other heavy chain in the same IgG molecule and (3) a subset of hybridoma is deficient in β1,4-GTase as well as in GnT-II.

The present nano ESI-MS data also showed that oligosaccharide H is predominantly paired with F*, resulting in low incidences of the pairing H+H and E+H. Namely, selective digalactosylation of one of two heavy chains (giving rise to the G0+G2 pairing) or digalactosylation in both of the heavy chains (giving rise to the G2+G2 pairing) seldom occurred in the cells. The quite low incidence of the G2+G2 pairing seems to be consistent with the pairing rule proposed on the basis of the X-ray crystallographic data of rabbit IgG-Fc [3,49]. However, it has been reported that G2 contributes 62% of oligosaccharides of human IgG4 paraprotein A.S. [5]. This would indicate that at least 24% of IgG possesses the G2 type oligosaccharide moieties on both Fc subunits unless non-glycosylated Fc subunits exist. A high percentage of G2 oligosaccharides (around 75%) has been also reported for human monoclonal IgG2 produced by Epstein-Barr virus-transformed B-lymphoblastoid cell lines grown in serum-free medium in low density static cultures [21]. In order to address this issue more directly, we examined the pairing of the Fc fragment of fetal human IgG1, which is more heavily galactosylated and sialylated than the mouse IgGs used in the present study.

The nano ESI-MS spectrum of the Fc fragment of fetal human IgG1 with the inter-chain disulfide bridges, which is the counterpart of the Fc/2 fragment shown in Fig. 3, indicates that there absolutely exists a significant amount of the Fc molecules that exhibit the H+H pairing (Fig. 4c,d and Table 2). As far as we know, this is the first direct evidence for the existence of the G2+G2 pairing in IgG that occurs naturally. The present ESI-MS data indicate that the pairing rule is not so strict, if any. It is likely that the low incidence of the H+H pairing observed for the mouse IgG2b-Fc is presumably due to a low concentration of β1,4-GTase in the cells, which is not sufficient to achieve galactosylation of every potential substrate.

Furthermore, the present ESI-MS data clearly indicates the existence of the IgG molecules exhibiting the Mc+Mc pairing. Although little is known about the biological significance of the sialylation of IgG-Fc, it has been suggested that NeuAc residues occupy space in the vicinity of His residues involved in binding to the neonatal Fc receptor (FcRn), which is expressed on many tissues and involved in the control of transport and the catabolism of IgG, and can influence their p*K*a values [62]. Since the stoichiometry of the interaction between

Table 2
Major glycoforms of Fc fragments

| Fc | Glycoforms | Molecular mass (Da) | | Relative quantity | |
|---|---|---|---|---|---|
| | | calculated | observed | calculated[a] | observed[b] |
| Mouse IgG2b (lot 1) | Q+Q | 51478.1 | 51479.4 | 0.4 | 3.9 |
| | Q+E | 51681.3 | 51679.0 | 5.7 | 5.3 |
| | Q+F* | 51843.4 | nd[c] | 5.0 | nd |
| | E+E | 51884.5 | 51882.8 | 20.9 | 28.3 |
| | Q+H | 52005.6 | nd | 1.0 | nd |
| | E+F* | 52046.6 | 52045.0 | 36.7 | 27.9 |
| | F*+F* | 52208.8 | 52207.1 | 16.0 | 21.6[d] |
| | E+H | 52208.8 | 52207.1 | 7.3 | 2.1[d] |
| | F*+H | 52370.9 | 52368.8 | 6.4 | 9.1 |
| | H+H | 52533.0 | 52530.6 | 0.6 | 1.8 |
| Mouse IgG2a (lot 1) | E+E | 52643.5 | 52644.6 | 17.4 | 22.2 |
| | E+F* | 52805.7 | 52807.2 | 44.0 | 34.3 |
| | F*+F* | 52967.8 | 52969.7 | 27.9 | 31.9[d] |
| | E+H | 52967.8 | 52969.7 | 4.6 | 4.1[d] |
| | F*+H | 53130.0 | 53129.3 | 5.8 | 7.5 |
| | H+H | 53292.1 | nd | 0.3 | nd |
| Fetal human IgG1 | E+E | 52755.4 | 52757.6 | 0.8 | 4.9 |
| | E+F* | 52917.5 | 52918.4 | 4.0 | 5.3 |
| | F*+F* | 53079.7 | 53081.4 | 5.3 | 7.4[d] |
| | E+H | 53079.7 | 53081.4 | 7.7 | 2.1[d] |
| | F*+H | 53241.8 | 53246.1 | 20.0 | 18.2 |
| | E+Mc | 53370.9 | nd | 4.3 | nd |
| | H+H | 53404.0 | 53405.3 | 19.2 | 24.7 |
| | F*+Mc | 53533.1 | 53535.6 | 11.2 | 7.5 |
| | H+Mc | 53695.2 | 53696.5 | 21.5 | 20.4 |
| | Mc+Mc | 53986.5 | 53990.1 | 6.0 | 9.5 |

[a]Calculated from relative quantities of glycoforms Fc/2 (Table 1) assuming a random pairing.
[b]Relative ion intensities in transformed spectrum.
[c]Ion peak not detected.
[d]Relative quantities of isomeric glycoforms E+H and F*+F* could be individually calculated from those of the other glycoforms of Fc and E, F* and H of Fc/2.

IgG and FcRn is 1:2, the pH dependence of the interaction might be affected by pairing of the sialyl oligosaccharides, e.g. Mc+Mc, Mc+H and so on, resulting in a different efficiency of transport and catabolic half-life.

## 4. Concluding remarks

In the present paper, we have determined using nano ESI-MS the glycoforms of IgG-Fc involving the paired oligosaccharides in a quantitative way. It has been reported that parts of IgG are asymmetrically glycosylated in the Fab and/or the hinge regions [63–65]. Biological significances of the asymmetric glycosylation in the Fab portion have been extensively discussed [66]. The present nano ESI-MS data indicate that the Fc portion can be asymmetric as well as symmetric from the aspect of glycosylation. Since agalactosylation induces local conformational change in Fc [67], asymmetric glycoforms would result in asymmetric protein conformation of Fc. Since IgG-Fc can be divalent and monovalent depending on its ligands [48,68–72], contribution to the biological activities of the carbohydrate moiety in each of the heavy chains may be cooperative as well as additive. Therefore, it is obviously necessary to correlate the biological activities of IgG with direct glycoforms of Fc as revealed in the present study rather than simply with the overall compositions of liberated oligosaccharides. We also propose that the nano ESI-MS-derived glycosylation profiles of Fc are useful diagnostically and prognostically, because they can be obtained with small amounts of samples as compared to the chromatographic methods and may offer insights into disease pathogenesis by providing unique information of pairing of oligosaccharides. It has been suggested that IgG exhibiting particular combinations of the Fab- and Fc-associated glycoforms are preferentially involved in self-association and aggregation and therefore have pathological significance [73,74]. Current progress in MS would make it possible in the near future to obtain quantitative information at a high resolution of carbohydrate heterogeneities of intact IgG molecule in which the Fab as well as the Fc are glycosylated. Information on the Fc glycoforms involving the paired oligosaccharides as determined in the present study using the proteolytic fragments will contribute to the basis of knowledge of complete glycoforms of the multi-glycosylated IgG molecules. The evaluation of pairing of the oligosaccharides would be especially important for the antibody engineering that intends to utilize the most efficacious Fc glycoform for a particular therapeutic purpose [75].

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