The Glycosylation and Structure of Human Serum IgA1, Fab, and Fc Regions and the Role of N-Glycosylation on Fcα Receptor Interactions*

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The human serum immunoglobulins IgG and IgA1 are produced in bone marrow and both interact with specific cellular receptors that mediate biological events. In contrast to IgA1, the glycosylation of IgG has been well characterized, and its interaction with various Fc receptors (FcγRs) has been well studied. In this paper, we have analyzed the glycosylation of IgA1 and IgA1 Fab andFc as well as three recombinant IgA1 molecules, including two N-glycosylation mutants. Amino acid sequencing data of the IgA1 Fab O-glycosylated hinge region revealed that O-glycans are located at Thr238, Ser239, and Ser242, while O-glycan sites at Thr239 and Thr240 are partially occupied. Over 90% of the N-glycans in IgA1were sialylated, in contrast to IgG, where <10% contain sialic acid. This paper contains the first report of Fab glycosylation in IgA1, and (in contrast to IgG Fab, which contains only N-linked glycans) both N- and O-linked oligosaccharides were identified. Analysis of the N-glycans attached to recombinant IgA1 indicated that the Co2 N-glycosylation site contained mostly biantennary glycans, while the tailpiece site, absent in IgG, contained mostly triantennary structures. Further analysis of these data suggested that processing at one Fc N-glycosylation site affects the other. Neutrophil FcαR binding studies, using recombinant IgA1, indicated that neither the tailpiece region nor the N-glycans in the Co2 domain contribute to IgA1-neutrophil FcαR binding. This contrasts with IgG, where removal of the Fc N-glycans reduces binding to the FcγR. The primary sequence and disulfide bond pattern of IgA1, together with the crystal structures of IgG1 Fc and mouse IgA Fab and the glycan sequencing data, were used to generate a molecular model of IgA1. As a consequence of both the primary sequence and S-S bond pattern, the N-glycans in IgA1 Fc are not confined within the inter-chain space. The accessibility of the Co2 N-glycans provides an explanation for the increased sialylation and galactosylation of IgA1 Fc over that of IgG Fc N-glycans, which are confined in the space between the two Cγ2 domains. This also suggests why in contrast to IgG Fc, the IgA1 N-glycans are not undergalactosylated in rheumatoid arthritis.

Serum IgA, produced in bone marrow, consists predominantly of monomeric IgA1 (1). IgA interacts with specific Fcα receptors (FcαR) present on granulocytes, monocytes, and macrophages, which can mediate phagocytosis, superoxide generation, enzyme release, and the clearance of immune complexes (2). IgA antibody molecules lack the C1q binding site present on IgG (3) and do not activate the classical complement pathway (4, 5). The activation of the alternative complement pathway by IgA remains controversial. A study avoiding artificial aggregation and chemical modification has shown that chimeric IgA1 bound to C3, but no C3b was generated (6). In contrast, using chimeric IgA2, binding to C3 leads to C3b formation and the terminal complement complex (7). This is consistent with the reported anti-inflammatory activity of IgA1 antibodies (8–10).

Human IgA1 contains two conserved N-glycosylation sites in each α-chain (Asn363 and Asn459), while the IgA2 subclass contains an additional two (IgA2m(1)) or three (IgA2m(2)) conserved N-glycans. In addition, Putnam and colleagues identified an N-glycosylation acceptor sequon in the variable region of the Bur myeloma α-chain of IgA1 (11), which was not present in either the IgA2 subclass (12) or IgA1 isolated from other myeloma patients (13). The N-glycans of myeloma IgA1 have been reported as biantennary complex-type structures with sialic acids attached exclusively in the α2–6 linkage (13). The first detailed structural analysis of the glycans of normal serum IgA1 was published by Field et al. (14). However, as with other studies (15), the analysis was carried out on the desialylated glycan pool from intact IgA1 and sialylated glycans were not directly analyzed. The N-glycans identified by Field et al. (14) were predominantly of the biantennary complex type with a core Fuc, bisecting GlcNAc, or both, and were generally similar to

1 The abbreviations used are: FcαR, receptor for the Fc region of IgA; FcγR, receptor for the Fc region of IgG; CHO, Chinese hamster ovary; GF, gel filtration; HPLC, high performance liquid chromatography; NIP, 3-nitro-4-hydroxy-5-iodophenylacetate; NP-HPLC, normal phase high performance liquid chromatography; WAX, weak anion exchange; 2-AB, 2-aminobenzamide; gu, glucose unit; PNGase F, peptide N-glycosidase F. The nomenclature for describing oligosaccharide structures is as follows: An (where n = 1, 2, 3, or 4) indicates the number of antennae linked to the trimannosyl core; Gn (where n = 0–4) indicates the number of terminal galactose residues in the structure; F, core fucose; Fo, outer arm fucose attached α1–3 to GlcNAc; B, bisecting GlcNAc; S, sialic acid (indicated within brackets are the specific linkages (6 or 2), and subscript numbers after these parentheses indicate the number of these residues in the specific linkage; M or Man, mannos; GalNAc, reducing terminal N-acetyl galactosamine of mucin-like O-linked glycans.

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those reported for myeloma IgA1. In addition, approximately 13.6% of tri- and tetraantennary structures were reported.

IgA1 contains a proline-rich hinge sequence between the Fab and Fc regions of the glycoprotein (Fig. 1). Within this sequence, there are nine potential O-glycosylation sites/α-chain (18 sites/molecule). The occupancy of these sites in normal serum IgA1 is not known. In myeloma serum IgA1, the sites of O-glycosylation were located at the five serine residues (16). In contrast, both serine and threonine residues are O-glycosylated in the hinge region of serum IgD (17) and secretory IgA1 (18), although in the latter case no experimental data was presented. These differences in O-glycan site occupancy as well as the finding that the O-glycans of secretory IgA1 are larger and more heterogeneous than in myeloma IgA1 (18, 19) may reflect tissue-specific glycosylation.

IgA N-glycosylation is required for the intracellular stability and secretion of mouse IgA (20, 21). However, recent studies have shown that this is not the case for either human IgA1 (6) or human IgA2 (7). The IgA1 study also indicated a role for N-glycosylation in antigen binding, suggesting that the secretion of glycosidases by bacteria may aid their survival in the host. In addition, certain species of bacteria may avoid an immune response by secreting IgA1-specific proteases. These proteases cleave the highly stable O-glycosylated hinge region of IgA1, thereby separating the two monomeric antigen-binding regions from the effector region of the antibody. These IgA1 protease-producing bacteria include the three leading agents of bacterial meningitis as well as other clinically important bacterial infections of the genitourinary and respiratory tracts (reviewed in Ref. 22). Interestingly, the O-glycosylation status of the immunoglobulin is known to affect the activity of some of the proteases (23). IgA1 glycans have been implicated in the pathology of a number of diseases including IgA nephropathy (24–27).

In this study of pooled serum IgA1, we have (a) determined the sialylation of the N- and O-linked oligosaccharides; (b) defined the sites of O-glycosylation in the proline-rich hinge region of IgA1; (c) provided the first report that the Fab region of IgA1 is glycosylated, identifying both N- and O-glycans; (d) compared the N-glycosylation of wild-type recombinant IgA1 and N-glycan deletion mutants to determine whether occupancy of one Fe N-glycosylation site affects processing at the other; and (e) explored the location and orientation of the IgA1 N-glycans in a molecular model of IgA1, constructed from the crystal structures of human IgG1 Fe fragment (28), mouse Fab fragment (29), and the analytical data obtained in this study. This model suggested some possible explanations for the differences in the composition of the glycan populations in IgA1 and IgG and in their structural and functional roles. In IgG, the conserved Fe N-glycans are situated in the interstitial region between the CH2 domains and, as a consequence, are incompletely galactosylated and sialylated. Binding to C1q is reduced upon deglycosylation (30) or deglycosylation (31), and, although the glycan structures are not required for binding to protein A (32), they are necessary to maintain the conformation of the Fe region to allow binding to FcγR (30). Therefore, we tested the possibility that IgA1 Fe N-glycans may play a similar structural role to those in IgG Fe by comparing the binding of carbohydrate-deficient IgA1 mutants to the neutrophil FcγR with that of wild-type recombinant IgA1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Goat Fab(ab)2 anti-human IgA fluorescein isothiocyanate conjugate was purchased from Caltag (Bradbury, Biologicals, UK). ProSorb cartridges were obtained from Perkin-Elmer Ltd. (Applied Biosystems Division, Warrington, UK). Glycan sequencing HPLC columns (GlycoSep-C and GlycoSep-N) were purchased from Oxford GlycoSciences Ltd. Enzymes were obtained as follows: PNGase F from New England Biolabs (Hitchin, UK); Arthrobacter ureafaciens neuraminidase, almond meal α-fucosidase, Newcastle disease virus Hitchiner B1 strain neuraminidase, and bovine testes β-galactosidase from Oxford GlycoSciences Ltd.; jack bean N-acetyl β-hexosaminidase, Charonia lampas α-fucosidase, and jack bean α-mannosidase from Oxford Glyco-biology Institute.

**Purification of Monomeric Serum IgA1**—Pooled human serum IgA1 was prepared as previously reported (14).

**Digestion of IgA1 with Clostridium ramosum IgA Protease**—Affinity-purified IgA1 (2 mg/ml in phosphate-buffered saline, pH 7.4) was incubated for 36 h at 37 °C with the extracellular bacterial protease secreted by C. ramosum (33). The Fe and Fab fragments (Fig. 1) were purified by gel filtration (GF)-HPLC (TSK SW3000GW; 21.5 × 600 mm; 0.1 m sodium phosphate, 0.15 M sodium chloride, pH 7.2). Fractions containing Fe and Fab were identified by molecular weight (SDS-PAGE), pooled appropriately, and purified to homogeneity by repetitive GF-HPLC. The purity of the isolated fragments was confirmed by reducing SDS-PAGE (12.5%), GF-HPLC, and N-terminal sequencing.

**Peptide:N-Glycosidase F Digestion of IgA1 Fc**—The purified Fe fragment (15 µg) was denatured by boiling for 10 min in 4 µl of 50 mM sodium phosphate (pH 7.5) containing 0.5% SDS and 1% β-mercaptoethanol. After cooling, 2 µl of 5% Nonidet P-40 was added followed by the 6 µl of PNGase F (3000 units), and the mixture was incubated overnight at 37 °C. Samples were analyzed by SDS-PAGE.

**N-terminal Amino Acid Sequencing**—All samples were analyzed on an Applied Biosystems (ABI) 470A protein sequencer with on-line phenylthiohydantoin analyzer (Perkin-Elmer). The Fe fragment was analyzed using an elevated temperature (48 °C) to facilitate quantitative cleavage of the prolyl residues from the peptide chain during Edman reaction. Samples were transferred to polyvinylidene difluoride membrane prior to sequencing using ProSorb cartridges (Perkin-Elmer).

**Expression and Purification of Recombinant Human IgA Mole-
cules—A wild-type plasmid vector based on pE6.hCMV (34) and containing the human α1 gene downstream of a mouse Vnp domain has been described previously (4). Construction of an expression vector for the IgA1 mutant Perm455, in which the entire tailpiece was deleted, involved replacing the first tailpiece residue (proline) with a stop codon (35). The recombinant antibody (N263A, lacking the AsnN-glycan acceptor site) the wild-type IgA1 expression vector was used as a template for polymerase chain reaction-based mutagenesis. Mutagenesis was performed using a 3′-primer annealing to a unique restriction site (SnaI, as described by Atkin et al. (35)) and a 5′-match primer (5′-CTGCACCGACCGCCCTCTGAGACCCGTCATCAGACAGAGACCTGACCGTCATCAGACAGAGACCTGACCGTCGACCGTGACC-3′) annealed across a unique restriction site (XhoI) and in which the codon AAC (coding for Asn) was replaced with GCC (coding for Ala). Following digestion with XhoI and SnaI, polymerase chain reaction products were ligated into the XhoISnaI-digested α1 expression vector, replacing the wild-type sequence in this region. Restriction analysis confirmed that the insert was correctly oriented. DNA sequencing (36) confirmed that the predicted mutation had been incorporated and that no misincorporations had occurred during polymerase chain reaction amplification.

Transfections of IgA1 heavy chain expression vectors into CHO-K1 cells previously transfected with a compatible light chain were performed as described (4). Positive clones expressing high levels of IgA, as assessed by antibody-binding enzyme-linked immunosorbent assay, were selected and expanded in culture (4). The recombinant antibodies were purified from supernatants of CHO-K1 transfected by affinity chromatography on NIP-Sepharose (4).

Oligosaccharide Release and Labeling—N-Glycans were released from the purified glycoproteins (IgA1, 1065 μg; Fab fragment, 610 μg; Fc fragment, 250 μg; recombinant IgA1 wild type, 120 μg; N263A, 84 μg; and Perm455, 200 μg) by hydrazinolysis at 95 °C using a Glyco-Prep1000 (Oxford GlycoSciences) optimized for maximum recovery (~85%) of glycans (37).

IgA1, Fab, and Fc were cryogenically dried over activated charcoal at −196 °C (<0.1 pascal) and incubated with double vacuum-distilled hydrazine at 60 °C for 4 h according to standard procedures (37) to release O-glycans preferentially with minimal degradation. During peptide removal by descending chromatography, the retention of GalNAc was compared against authentic GalNAc visualized with alkaline silver nitrate (38). Glycans were recovered from the first 13 cm from the origin of the chromatography paper by elution with water through a 0.45-μm filter, evaporated to dryness, and stored at −20 °C.

Analysis of Oligosaccharides by Weak Anion Exchange (WAX) Chromatography—High performance anion exchange chromatography was carried out using a GlycoSep-C column (Oxford GlycoSciences Ltd.) according to the conditions described (39). The column was calibrated by the injection of 2-AB-labeled sialylated glycans from bovine fetuin.

Analysis of Neutral and Acidic Oligosaccharides by Normal Phase Chromatography (NP-HPLC)—Normal phase HPLC was carried out using the 50 mM ammonium formate, pH 4.4, buffer system on a GlycoSep-N column over 180 min, as described previously (40). The column was calibrated using 2-AB-labeled dextran hydrolasate, the elution positions of which were used to obtain the NP-HPLC glucose unit (gu) values for each glycan.

Exoglycosidase Digestion Conditions—Enzyme digests were performed at 37 °C for 16–24 h in 100 mM citrate/phosphate buffer, pH 4.5, 0.2 mM zinc acetate, 0.15 mM sodium chloride in 10-μl volumes. The enzymes were used either together in various combinations (termed an enzyme array) or alone, as specified in the appropriate figure legend. After incubation, proteins were removed by filtration through nitrocellulose (Pro-Spin, Radleys, UK) as described (41). An aliquot of the aqueous sample (~25 pmol) was mixed with acetonitrile in the ratio 30:70 and applied to the NP-HPLC column. Conditions for the digestions are as described previously (42). The cleavage positions for the exoglycosidases are shown in Fig. 2b.

Interaction of Recombinant IgA with Neutrophil Fce Receptors—Receptor assays were performed (43) using NIP-derivatized RBC (labeled with wild-type or mutant IgA1. Coating levels for each antibody were selected and expanded in culture (4). The recombinant antibodies were purified from supernatants of CHO-K1 transfected by affinity chromatography on NIP-Sepharose (4).

FIG. 2. The oligosaccharide sequencing strategy used in the investigation. a, the purified glycoproteins were subjected to hydrazinolysis, and the released oligosaccharides were labeled with 2-AB. The glycans were analyzed by NP-HPLC to obtain preliminary assignments, which were confirmed by exoglycosidase digests. Analysis of the NP-HPLC profiles of the products of exoglycosidase digestions of both the glycans and the WAX fractions confirmed the predicted structures. b, a sialylated biantennary complex glycan (A2G2FucS(6)(3)) with the cleavage positions of the exoglycosidases used in this study.

Molecular Modeling—Molecular modeling was carried out on a Silicon Graphics Indigo II computer using the Insight 95 and Discover software package (Biosys Tech, Inc., San Diego, CA). Initially, the Fab and Fc structures were modeled separately, linked to give a model of the entire antibody, and then minimized. The model of the human IgA1 Fab was based on the mouse IgA1 Fab crystal structure (29), while the human IgA1 Fab model was based on the human IgG1 Fab (28). Protein crystal coordinates were obtained from the Brookhaven data base (44). Both the sequence alignment (45) and inspection of the protein tertiary structure determined the location of sequence insertions and deletions. In all cases, insertions and deletions could be made in loops or linker regions between the domains; these regions were reconstructed using structural homology searches. The structure of the proline-rich hinge region (Val42 to Pro46) was modeled as an extended peptide sequence with all proline residues in the trans conformation (46). The tailpiece (residues 454–472) was also modeled as an extended peptide.

The modeling procedure required (a) construction of the alignment (45) sets, including positions of disulfide bonds (47–49), amino acid changes, and insertions/deletions (45); (b) fitting the amino acid changes to the IgG1 Fc structure and the mouse IgA Fab structure; (c) reconstruction of loop regions where insertions or deletions were required using structural homology searches; (d) altering the quaternary structure of the CH2 domain to conform to the disulfide pattern reported; and (e) introduction of the hinge and tailpiece polypeptides. After each step, the resulting model was optimized by energy minimi-
IgA1 Glycosylation and N-Glycan Function in FcaR Binding

RESULTS

Purification of Monomeric IgA1, Fab, and Fc

Affinity-purified IgA1 was fractionated by GF-HPLC to remove polymeric immunoglobulins and contaminating C1 esterase. The final yield was 0.6 mg/ml serum. The IgA1 was >98% pure as determined by SDS-PAGE, immunoprecipitation, and N-terminal sequencing. The preparation gave two bands on reducing SDS-PAGE at 58 kDa (heavy chain) and 25 kDa (light chain; Fig. 3a, lane 1). Immunoprecipitation confirmed the presence of IgA1, while other immunoglobulins (IgG, IgM, and IgA2) were not detected. No N-terminal sequence was detected apart from the sequence for IgA.

Exhaustive proteolysis of affinity-purified IgA1 by C. ramosum protease released the expected Fc- and Fd-light chain heterodimer (Fab) fragments. The digestion products were purified to homogeneity and shown to be >98% pure by N-terminal sequencing and reducing SDS-PAGE (Fig. 3a, lanes 2 and 3, respectively). On SDS-PAGE, the Fab migrated as a broad band at 28 kDa, while the Fc separated into two discrete bands migrating at 35 and 38 kDa (Fig. 3a, lane 5), and unglycosylated IgA1 Fc was not detected. PNGase F digestion of the IgA1 Fc yielded a single band on SDS-PAGE. This band migrated at a molecular mass (33 kDa) equivalent to the nonglycosylated IgA1 Fc, indicating that the Fc doublet was due to variable N-glycan site occupancy (Fig. 3a, lane 4).

Generation of Recombinant IgA1 Molecules

Following transfection of mutant heavy chain expression vectors into a light chain-producing CHO cell line, stable cell lines were established, and the secreted antibodies were purified by hapten affinity purification. Fig. 3b shows an SDS-PAGE gel of the Pterm455 mutant, in which the tailpiece has been deleted. Other antibodies displayed similar levels of purity. Under nonreducing conditions (lane 2), two major bands of 170 and 80–90 kDa were obtained for the Pterm455 mutant. These two bands have been shown previously to represent monomers (H2L2) and half-molecules (HL), respectively (35), suggesting that the tailpiece may play some role in bringing together the two α-chains in IgA assembly. There are minor bands above 250 kDa and at 60 and 25 kDa, which may result from aggregated IgA, heavy chains, and light chains, respectively, since under reducing conditions (lane 1), only heavy and light chains were detected.

Identification of IgA1 Hinge O-Glycosylation Sites

The Fc fragment from IgA1 was analyzed by N-terminal amino acid sequencing using an increased temperature sequencer cycle to maximize the release of proline residues. This enabled the identification of sites of O-glycosylation by comparison of the yield of hydroxyamino acids relative to expected yields of nonglycosylated hydroxyamino acid residues. Under the sequencing conditions used, the expected yields of unmodified serine and threonine, relative to other amino acids, were 30–40% and 70–80%, respectively. During sequencing cycles for Thr228, Ser230, and Ser232, no hydroxyamino acids were identified. Thr225 and Thr236 produced depressed signals (30 and 60% relative to the expected signal for threonine), while hydroxyamino acids at other positions gave the expected yield of an unmodified residue. Depressed yields of hydroxyamino acids are consistent with O-glycosylation, since glycosylated anilinothiozoline-amino acids are not extracted from the sequencer reaction chamber under the conditions used (51–53).

Strategy for Glycan Analysis

The overall scheme of intact IgA, Fab, and Fc glycan analysis is shown in Fig. 2a and described below.

A. Analysis of Pooled Human Serum IgA1, Fab, and Fc

N-Linked Oligosaccharides by WAX—The 2-AB-labeled glycan pool from serum IgA1 was separated according to charge by WAX chromatography (Fig. 4a). Five populations were assigned by comparison with an analysis of standard sugars from fetuin resolved on the same HPLC system. Fractions were pooled according to charge and analyzed by NP-HPLC (see step B).

B. Analysis of Neutral, Mono-, Di-, and Trisialylated Glycans from Human Serum IgA1, Fab, and Fc by NP-HPLC—NP-HPLC analysis of the neutral WAX fraction (Fig. 4b) identified
an oligomannose series representing less than 0.1% of the total N-glycan pool, and other neutral structures accounted for 1.8% of the total N-glycan population. All sialylated structures eluted as neutral glycans after digestion with A. ureafaciens neuraminidase, indicating that the charge was entirely due to sialic acid. Newcastle disease virus neuraminidase was used to investigate the linkage position of sialic acid to the glycan structures, since this exoglycosidase removes α2–3 but not α2–6-linked sialic acid residues. NP-HPLC analysis of each sialylated WAX fraction from IgA1 (Fig. 4, c–e), Fab (Fig. 4, f–h), and Fc (Fig. 4, i–k) indicated that the mono- and disialylated N-glycans contained only α2–6-linked sialic acids, while trisialylated oligosaccharides contained both α2–6- and α2–3-linked sialic acids.

C. Analysis of the Total N-Glycan Pools from Human Serum IgA1, Fab, and Fc by NP-HPLC—The heterogeneous mixtures of neutral and sialylated N-linked oligosaccharides from IgA1, Fab, and Fc were analyzed by NP-HPLC (Fig. 5, a–c) and resolved into at least 20 peaks. Each peak was assigned a NP-HPLC gu value by comparison with the elution position of a standard dextran hydrolysate mixture (shown at the top of each NP-HPLC panel). Individual peaks were then assigned preliminary structures from their gu values using the positions of standard glycans and predetermined incremental values for monosaccharide residues (Ref. 40; Table I). These predicted structures were confirmed as follows: (a) sialylation status was confirmed by WAX chromatography followed by NP-HPLC (see step B), and (b) analysis of digestions with exoglycosidase enzymes confirmed the bi- and triantennary complex glycans and the oligosaccharides containing core and/or outer arm fucose (see step D).

Consistent with earlier studies (14), over 80% of the N-glycans of intact IgA1 were digalactosylated biantennary complex type oligosaccharides (Fig. 6a, Table I). Less than 10% of triantennary and no tetraantennary or extended structures were detected, 64% of terminal galactose residues were sialylated, and more than 95% of sialic acids were linked in the α2–6 configuration (Table I).

30% of Fab fragments contained N-linked glycans. Fig. 5, a–c, compares the NP-HPLC profiles of the glycans associated with IgA1 with those of the Fab and Fc, which contained the entire hinge region and the conserved N-glycan sites at Asn263 and Asn459. Consistent with Fab glycosylation, the glycosylation profile of the Fc was not identical to that of the intact molecule. In particular, the sialylated structures A2G2FS(6) and A2G2FS(6)2 (peaks 7 and 10, respectively) and the trisialylated glycans identified in IgA were derived mainly from the Fab. The neutral glycans were associated mainly with the Fc. In contrast to the Fc, where the majority of the fucosylated oligosaccharides were disialylated, fucosylated structures in the Fab were more variably sialylated. These differences between Fc and Fab glycosylation are consistent with the NP-HPLC analysis of WAX fractions (Fig. 4, f–h and i–k, respectively).

D. Simultaneous Sequencing of the Total Glycan Pool Using
Exoglycosidase Arrays—The preliminary assignment of structures to the N-glycans from human serum IgA1 was made using the HPLC gu values as described (40–42). The digestion of aliquots of both individual WAX fractions (data not shown) or the total glycan pool of each sample confirmed these preliminary assignments. Fig. 6 shows the digestion of the total glycan pool from IgA1 with different exoglycosidase arrays monitored by NP-HPLC. The assignment of each of the main peaks in the IgA1 glycan pool was confirmed by following its predicted elution position through each of the enzyme digestions (Table II) as described (42). Predicted elution positions were based upon predetermined incremental values for the monosaccharide additions to standard glycan cores (40) and the known specificity of the enzymes (Fig. 2b).

**Table I**

| Peak | gu | Oligosaccharide structure | Percentage of N-glycan (%)
|------|----|---------------------------|-------------------------|
|      |    |                           | IgA | Fc | Fab |
| 1    | 7.2| A2G2                      | ND  | 6.0| 2.0 |
| 2    | 7.2| A2G1S(6)                  | 2.4 | 1.8| 2.9 |
| 3    | 7.5| A2G1S(3)                  | 4.2 | 2.4| 3.2 |
| 4    | 7.8| A2G2S(3)                  | <0.5| <0.5| <0.5|
| 5    | 7.9| A2G2S(6)                  | 20.4| 22.3| 11.5|
| 6    | 8.2| A2G2S8(6)                 | 8.3 | 10.7| 4.5 |
| 7    | 8.3| A2G2FS(6)                 | 7.7 | 2.0 | 8.11 |
| 8    | 8.4| A2G2FS(3)                 | <0.5| 2.5 | >0.5 |
| 9    | 8.5| A2G2FS(6)                 | 2.8 | 2.0 | 5.3 |
| 10   | 8.7| A2G2S8(6)                 | 24.4| 12.6| 25.1|
| 11   | 9.0| A2G2FS8(6)                | 11.3| 10.7| 8.1 |
| 12   | 9.15| A2G2FS8(3)               | 13.79| 12.6| 9.1 |
| 13   | 9.35| A3G3S2(6)                | 1.3 | 0   | 3.68 |
| 14   | 9.6| A3G3S2(6)                 | 1.7 | <0.2| 4.35 |
| 15   | 9.9| A3G3F3S3(6)               | <0.5| <0.2| <0.5 |
| 16   | 10.3| A3G3F0S3(6)               | 0.5 | <0.2| 3.7 |

*Fig. 5.* Comparison of the NP-HPLC profiles of released N-glycans from human pooled serum IgA1 (a), Fc (b), and Fab (c). Peaks were assigned gu values by comparison with a dextran hydrolysate ladder shown at the top. The glycan analysis is shown in Table I.

*Fig. 6.* Parallel sequential exoglycosidase digestions of the total N-linked glycan pool released from pooled human serum IgA1 analyzed by NP-HPLC. a, the total glycan pool from IgA1 digested with A. ureafaciens neuraminidase (sialidase). b, A. ureafaciens neuraminidase and bovine testes β-galactosidase. c, A. ureafaciens neuraminidase, bovine testes β-galactosidase, and jack bean N-acetyl β-hexosaminidase (β-hexosaminidase). d, A. ureafaciens neuraminidase, bovine testes β-galactosidase, jack bean N-acetyl β-hexosaminidase, and C. lampas α-fucosidase. The elution positions of the dextran hydrolysate ladder used to calculate the gu values are shown across the top. The analysis of the digestion products is shown in Table II. Peak assignments were made by comparison with glycan standards as described previously (40, 42).
The Analysis of the O-Linked Glycans from Human Serum IgA1, Fab, and Fc by NP-HPLC

The O-glycans from intact serum IgA1, Fab, and Fc were released by hydrazinolysis, under conditions that optimized the release and recovery of intact O-glycans, and analyzed by NP-HPLC (Fig. 7, a–c). The data were generally consistent with previous reports (14, 26, 54, 55). Table III shows the relative proportions of the glycan species. The most abundant was the core 1 monosialylated T antigen (NeuNAc2–3Gal1–4GlcNAc) confirmed by endo-β-N-acetylhexosaminidase; CL-Galβ1–4GlcNAcβ1–6Galβ1–3GalNAc) were also identified. Degradation of O-glycans by β-elimination (peeling) during hydrazinolysis gives rise to NeuNAcc2–3/6Gal and GalNAc. Quantitation of these structures during NP-HPLC analysis indicated that peeled products represented less than 2% of the total glycans in IgA and Fab and less than 10% in Fc. The O-glycans released from the Fc (Fig. 7b, Table III) were identical to those released from IgA1. Although the GalNAc neoantigen was detected, the abundance of this monosaccharide was comparable with that of the core 1 monosialylated T antigen; less than 2% of the total glycans in IgA and Fab. The deletion of the C2 domain N-glycosylation site (N263A, Fig. 8b) gave rise to an increase in A3G3 to 74% of the total N-glycan pool. In contrast, the Pterm455 mutant (Fig. 8c), where the whole tailpiece polypeptide was deleted including the N-glycan site at Asn455, gave a glycosylation profile dominated by the biantennary structure, A2G2 (82.9%, peak 22). All glycan assignments were confirmed by the sequencing strategy described above. The data suggest that the C2 glycosylation site contains less processed N-glycans than the tailpiece site.

The Analysis of the Glycans from Recombinant IgA1 and Two Carbohydrate-deficient Mutants

The glycosylation of the two conserved N-glycosylation sites (Asn263 and Asn455) was probed by comparing the glycosylation of carbohydrate-deficient mutant IgA1 molecules (N263A, Pterm455) with that of wild-type recombinant IgA1 (Fig. 8). Panel a shows the NP-HPLC desialylated N-glycan profile for the wild-type antibody. 37% of the glycans consist of the triantennary oligosaccharide, A3G3 (Fig. 8a, peak 25; Table IV).

TABLE II

| gu value | Asialo | Enzyme array components* |
|----------|--------|--------------------------|
|          |        | AB-sialidase  | AB-sialidase  | AB-sialidase  | AB-sialidase  |
|          |        | BTφ-gal-ase  | BTφ-gal-ase  | JBβ-hex-ase  | JBβ-hex-ase  |
|          |        |              |              |              |              |
| 4.41     | 2      | M3N2         | M3N2         |              |              |
| 4.90     |        | M3N2F        | M3N2         |              |              |
| 5.5      | 0.5    | A2G0B        | A2G0B        | A2G0B*       | A2G0B*       |
| 5.90     | 0.2    | A3G0        | A3G0         |              |              |
| 5.93     |        | A2G0F        | A2G0F        |              |              |
| 6.24     | 0.1    | A2G0FB       | A2G0FB       | A2G0FB*      | A2G0FB*      |
| 6.29     | 0.5    | A2G1(1,6)    | A2G1(1,6)    | A2G0F        | M3N3Fo       |
| 6.51     | 0.5    | A2G1(1,3)    | A2G1(1,3)    |              |              |
| 6.65     | 0.2    | A3G0Fo       | A3G0Fo       | A3G0Fo       |              |
| 7.15     | 0.8    | A2G2         | A2G2         |              |              |
| 7.30     | 0.9    | A2G2B        | A2G2B        |              |              |
| 7.57     | 0.5    | A2G2F        | A2G2F        |              |              |
| 7.67     | 0.5    | A2G2FB       | A2G2FB       |              |              |
| 8.32     | 0.5    | A3G3         | A3G3         |              |              |
| 8.66     | 0.5    | A3G3F        | A3G3F        |              |              |
| 9.02     | 0.5    | A3G3F        | A3G3F        |              |              |

* Enzyme array components are as follows: AB-sialidase, A. ureafaciens neuraminidase; BTφ-gal-ase, bovine testes β-galactosidase; JBβ-hex-ase, jack bean N-acetyl β-hexosaminidase; CLα-fuc-ase, C. lampas α-fucosidase.

The deletion of the C2 domain N-glycosylation site (N263A, Fig. 8b) gave rise to an increase in A3G3 to 74% of the total N-glycan pool. In contrast, the Pterm455 mutant (Fig. 8c), where the whole tailpiece polypeptide was deleted including the N-glycan site at Asn455, gave a glycosylation profile dominated by the biantennary structure, A2G2 (82.9%, peak 22). All glycan assignments were confirmed by the sequencing strategy described above. The data suggest that the C2 glycosylation site contains less processed N-glycans than the tailpiece site.

Recombinant IgA1 and the Carbohydrate-deficient Mutants Interact with Neutrophil FcαR

Wild-type recombinant IgA1 has been shown previously to bind to the myeloid FcαR with similar affinity to that of serum IgA1 (4). In this study, the IgA1 mutant N263A, which lacks the C2 N-glycan, showed a similar capacity to bind the FcαR on neutrophils as wild-type IgA1 (Fig. 9). Both wild-type and mutant N263A antibodies have similar affinities for the receptor, as determined by their comparable percentage rosette formation at different concentrations of coating antibody. Rosetting analysis suggests that the other IgA1 mutant, Pterm455,
also interacts with the receptor with an affinity similar to that of wild-type IgA1.2

**DISCUSSION**

**Occupancy of IgA1 N-Glycosylation Sites**—Although IgA1 is more resistant than IgG or IgM to cleavage by common proteolytic enzymes, it is susceptible to specific proteases secreted by certain pathogenic bacteria (reviewed in Ref. 22). Here IgA1 was cleaved with the C. *ramosus* protease to generate two Fab fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1). The cleavage site, between Pro221 and Val222, allowed the entire fragments and the Fc (Fig. 1).

**Analysis of IgA1 N-Glycans**—Recently developed technology (40) has enabled this first direct analysis of the sialylated glycan pools from recombinant wild-type and N-glycan mutant IgA1 molecules. a, wild-type recombinant IgA1; b, Co2 N-glycan mutant N263A showing structures associated with the tailpiece glycan site; c, recombinant IgA1 lacking the tailpiece showing the structures associated with the Co2 N-glycan site. Peaks were assigned gu values from the dextran hydrolysate shown at the top of each panel. The analysis of the glycans is shown in Table IV.

**TABLE IV**

**N-glycan analysis of recombinant IgA**

| Code | gu value | Oligosaccharide structure | Wild type | N263A | Pterm455 |
|------|----------|---------------------------|-----------|-------|----------|
| 5.5  | A2G0     | <1                        | <1        |<1 |
| 5.90 | A2G0     | <1                        | <1        |<1 |
| 6.2  | Man5     | 4.3                       | 1.5       | 1.9 |
| 6.33 | A2G1(1–6) | 2.0                       | <1        | 6.4 |
| 6.45 | A2G1(1–3) | <1                       | <1        | 1.4 |
| 7.15 | A2G2     | 24.6                      | 14.1      | 82.9 |
| 7.2  | A2G2B    | <1                       | <1        |<1 |
| 7.23 | A2G2B    | <1                       | <1        |<1 |
| 7.57 | A2G2F    | 16.0                      | 7.9       | <1 |
| 7.67 | A2G2F    | <1                       | <1        |<1 |
| 8.32 | A3G3     | 37.1                      | 74.4      | 3.3 |
| 8.66 | A3G3F    | 2.9                       | 3.0       |<1 |
| 10.09| A4G4F    | 5.5                       | 3.0       |<1 |

Increased proportions of oligomannose structures have been noted in human myeloma serum from both polymeric (14%) and monomeric IgA1 (7%; Ref. 15) and have been proposed to act as ligands for the mannose-specific lectin found on type I fimbriae of *Enterobacteriaceae* (56). In pentameric IgM, the predominance of oligomannose glycans attached to the tailpiece (57) is consistent with steric hindrance of the region following polymerization of monomeric IgM in the endoplasmic reticulum (58). Similarly, the presence of J-chain in polymeric IgA, compared with monomeric IgA, may be expected to result in increased

**TABLE III**

**Analysis of the O-glycans associated with IgA1, Fab, and Fc**

The gu values and structures of O-linked glycans released from IgA1, Fab, and Fc separated by NP-HPLC (Fig. 7) are shown. Peaks present at 1% or greater are included. The peak numbers were used to identify the peaks in Fig. 7.

| Peak | gu value | Oligosaccharide structure | Percentage of O-glycan |
|------|----------|---------------------------|------------------------|
| 17   | 1.79     | Galβ1–3GalNAc             | 30.7                   |
| 1.96 |          | 23.1                      | 17.4                   |
| 2.02 |          | 1.51                      | 11.4                   |
| 2.11 |          | 6.9                       | 6.6                    |
| 2.43 |          | <1.0                      | 1.8                    |
| 18   | 2.24     | S(3)Gal                   | 3.1                    |
| 2.4  |          | 16.1                      |                        |
| 2.5  |          | 1.4                       | <1.0                   |
| 2.6  |          | 1.4                       | 7.0                    |
| 19   | 2.9      | Galβ1–3GalNAc             | 37.3                   |
| 3.3  |          | 34.3                      | 26.5                   |
| 3.4  |          | 0.6                       | 3.7                    |
| 3.6  |          | ND                        | 2.1                    |
| 21   | 4.4      | S(3)Galβ1–3GalNAc         | 7.31                   |
|      |          | 1.51                      | 11.8                   |

* ND, not determined.

2 R. J. Pleass, C. M. Anderson, J. I. Dunlop, and J. M. Woof, manuscript in preparation.

3 M. Wormald and Y.-L. Pao, personal communication.
proportions of oligomannose glycans at Asn459 in the tailpiece. The proportion of bisected structures in pooled serum IgA1 (24%) was higher than that in IgG (14%; Ref. 59) although lower than that previously reported for IgA1 isolated from individuals (40%; Ref. 14). This suggests that the proportion of bisected oligosaccharides may differ significantly between individuals.

**Occurrence of Potential O-Glycosylation Sites in the IgA1 Hinge Region**—At particular cycles in the amino acid sequencing, signal depression (Thr225 and Thr235) or absence (Thr238, Ser239, Ser242) was noted for some serine and threonine residues. Other than O-glycosylation, no other co- or post-translational modifications (phosphorylation or acetylation) have been reported on the IgA1 hinge. Therefore, these data indicate that the amino acids at these positions were glycosylated. These data are consistent with jacalin precipitation studies of IgA1 fragments (60), which suggested that jacalin interacts with the Ser239 and/or Ser242 and not Ser238 and Ser240. Variable site occupancy at Thr225 and Thr235, indicated by signal depression rather than absence, suggests that IgA1 exists as an array of glycoforms in which different potential O-glycan sites in the hinge region are occupied. In addition, each site may contain a range of oligosaccharides. This complex range of glycosylated variants may also exist in IgD, where variable site occupancy has been reported (61, 62). The finding that certain myeloma serum IgA1 proteins are glycosylated at all five serine residues (16) may be the result of an up-regulation of N-acetyl-galactosaminyltransferase or a modification in its specificity. Indeed, changes in O-glycosylation are associated with many carcinomas through either altered site occupancy (63, 64) or altered processing (65, 66). This may be significant in diseases such as IgA nephropathy, where altered O-glycosylation has been observed (24, 25, 27). In a preliminary study of serum IgA1 Fc isolated from IgA nephropathy patients, the recovery of O-glycans was significantly reduced, suggesting underoccupancy of O-glycosylation sites in the hinge region (41). The O-glycosylation of both serine and threonine residues found in this study is significant, since this indicates that all IgA1 specific proteases of the serine and metallo types cleave immediately adjacent to carbohydrate side chains, in contrast to the cysteine proteases of *Prevotella* species.

**IgA1 Fc O-Glycans**—The O-glycan pool from IgA1 Fc containing the entire hinge region peptide was analyzed. The sialylated and neutral glycans identified by NP-HPLC were consistent with earlier reports of the analysis of the desialylated glycan pool (14, 16, 26, 54, 55) and confirmed previous findings from this laboratory (14), which indicated that serum IgA1 does not carry the GalNAc neoantigen. The limited repertoire of O-glycan structures attached to serum IgA1, compared with the plasma cells producing IgA1 for mucosal secretion (18, 19), indicates tissue-specific O-glycan processing and may reflect the different environments in which these molecules are required to function.

**Comparison of the N- and O-Glycosylation of IgA1 Fab and Fc**—This study contains the first report of Fab glycosylation in human serum IgA1. The NP-HPLC glycan profile of IgA1 Fab did not account for the heterogeneity in the glycan profile of intact IgA1 (Fig. 5), consistent with Fab glycosylation. The glycan profile of the Fab revealed the glycan structures identified in IgA1 that were absent or reduced in the Fc. 30% of Fab fragments were glycosylated, suggesting that Fab glycosylation is as common in human serum IgA1 as in human serum IgG, where approximately 25% of Fab regions contain sugars (67). Equivalent levels of Fab glycosylation of IgA and IgG may reflect similar variable region utilization. Previous studies of myeloma IgA1 α-chain identified an N-glycosylation acceptor sequon in the variable region (11). However, this sequon was not identified in either the IgA2 subclass (12) or in myeloma IgA1 purified from a different patient (13). The Fab N-glycans were more sialylated than the Fc oligosaccharides. In addition, the Fab region contained the majority of the triantennary oligosaccharides, while most of the neutral glycans were associated with the Fc. In contrast to IgG, where the Fab sugars contain more galactose than those in the Fc (68), there was no significant difference in the extent of galactosylation of IgA1 Fab and Fc, and 95% of structures were fully galactosylated in both fragments. Although other Ig's contain Fab N-glycosylation (69-75), this is the first report of non-myeloma IgA1 N-linked glycosylation and the first report of O-linked Fab glycosylation in human Ig's.

In IgG, the sugars are located in the Cγ2 region on a loop at Asn297-Ser-Thr and are contained in the interstitial space between the two Cγ2 domains. The IgG sugars are involved in noncovalent interactions with the protein surface (76), which further limits their accessibility to the glycosyltransferases. This leads to reduced galactosylation and sialylation (68, 77). Based on the molecular modeling studies (Figs. 10 and 11), analogous sugar-protein interactions appear unlikely in IgA1, and this is consistent with the glycan analysis, which indicates that the IgA1 Fc glycans are fully galactosylated.

**Glycosylation of Recombinant IgA1 Mutants**—Triantennary structures with bisected and fucosylated glycans were predominant in the wild-type anti-NIP recombinant IgA1. In serum IgA1, the major structures were of the biantennary type. These differences may reflect cell-specific glycosylation, highlighting the differences in the glycosylation pathways between human lymphoid cells and the CHO cell culture. Cell culture conditions and the particular host cell are known to influence protein glycosylation (78). However, comparison of the oligosaccharides of recombinant proteins produced by a variety of expression systems has established CHO as one of the most suitable (79). Glycosylation mutants generated by site-directed mutagenesis of recombinant IgA1 were analyzed by comparative NP-HPLC of the released glycans. The glycan profile for the N263A mutant, in which the N-glycosylation site at Asn263 was deleted, showed that the Asn459 N-glycosylation site contained mostly triantennary oligosaccharides (Fig. 8, Table IV). Glycan analysis of the Pterm455 mutant, in which the whole tailpiece was deleted including the Asn459 N-glycosylation site, showed that 83% of the structures were A2G2. This suggested that the Co2 N-glycosylation site contains less processed N-
glycans than the tailpiece N-glycosylation site, and this may reflect the greater accessibility of the tailpiece glycans to the glycosyltransferases. The summation of the two N-glycan profiles for the mutants did not result in a profile comparable with that of the wild-type, suggesting that processing at one Fc N-glycosylation site affects another.

Functional Analysis of Glycosylation Mutants—The CH2 N-glycan in CHO-K1-derived IgA1 does not influence the interaction with the neutrophil FcαR, since mutant N263A mediated rosette formation to the same extent as wild-type IgA1. This is in contrast to previous findings, where the mutation of Asn263 to Glu in IgA expressed in insect cells produced an antibody incapable of mediating rosette formation (80). The differences may arise from the different expression systems used, particularly since insect cells tend to attach unusually large oligosaccharides, which may affect the suitability of the insect cell-derived wild-type antibody for comparative purposes. A detailed carbohydrate analysis of the recombinant antibodies was not presented in this earlier study. Further, although an enzyme-linked immunosorbent assay was used to assess antibody concentration prior to coating RBC, it is possible that problems with mutant assembly, undetectable by enzyme-linked immunosorbent assay, prevented coating levels comparable to that of wild type. We have controlled for this possibility in our study by assessing reactivity with an anti-IgA antibody by fluorescence-activated cell sorting analysis after erythrocyte coating.

The absence of CH2 glycosylation in CHO-K1-expressed IgA1 does not perturb FcαR binding. This is in contrast to the results of studies of the interaction between homologous FcyRs and IgG. IgG molecules lacking CH2 N-glycans have a reduced affinity for FcγRI (31, 32) and FcγRII (81). This may be a consequence of localized perturbation of the CH2 domain structure in aglycosylated IgG (82) in a region proximal to the proposed site of interaction of both FcγRI and FcγRII in the lower hinge region (reviewed in Ref. 83). This difference between the IgA and IgG receptor systems may suggest that the sites recognized by the receptors are not homologous. Indeed, sequence comparisons between IgG1 and IgA1 reveal considerable evolutionary divergence in the lower hinge region, which is evident in the molecular model of the IgA generated here. Recently the Cα2/Cα3 domain interface of IgA has been proposed as a likely binding site for the myeloid FcαR (84).2 Interestingly, the interaction of IgE with the high affinity receptor FcεRI is also independent of the IgE oligosaccharides (85).

A further IgA1 mutant, Pterm455, mediates rosette forma-

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Fig. 10. a, amino acid alignment; b, molecular model of IgA1. a, amino acid alignment for human IgA λ (92) and α (47) chains in the upper sequence, with mouse IgA Fab κ and α chains (29) and human IgG1 Fc (28) in the lower sequence. Molecular modeling of the mouse Fab crystal structure enabled the Fab amino acid alignment to be obtained by matching the conserved intradomain disulfides with those of IgA1 and examination of the structure of the mouse IgA Fab region. Amino acid alignment for IgG1 was as reported (45). Conserved residues are shown in boldface type. ●, a residue absent from the sequence; □, missing residues in the deletion area. b, the molecular model of IgA1 was generated as described under “Experimental Procedures.” The Fc glycan distribution was postulated according to the sequencing data, evaluation of local conformation, and mutagenesis investigations.

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2 Recently the Cα2/Cα3 domain interface of IgA has been proposed as a likely binding site for the myeloid FcαR (84). Interestingly, the interaction of IgE with the high affinity receptor FcεRI is also independent of the IgE oligosaccharides (85). A further IgA1 mutant, Pterm455, mediates rosette forma-
tion comparable with that of wild-type IgA1. This suggests that the tailpiece peptide or oligosaccharide does not play a significant role in FcγR binding.

**Molecular Modeling of IgA1**—A molecular model of an IgA1 glycoform was generated from the crystal structures of related Ig fragments and the glycan sequencing data (Fig. 10b; see “Experimental Procedures”). Displacement of the CH2 domains was required to accommodate the disulfide bond pattern reported in IgA1 (47–49). This leads to a reduced interstitial region and steric crowding around the N-terminal region of the hinge compared with IgG1 (28). The disulfide pattern also restricts the movement of the C-terminal region of the hinge and N-terminal region of the Co2 domains. The orientation and surface location of Cys311 in the CH2 domain does not allow the formation of intra- or inter-α-chain S–S bonds, since these would require disruption of the tertiary structure of the CH2 Ig domain. However, this surface location of Cys311 is consistent with the reported interaction of this residue with secretory component in polymeric IgA (49, 86). The IgA1 hinge region was modeled as an extended structure, based on findings that proline-rich sequences (87) and heavily O-glycosylated mucin peptides (88, 89) adopt extended conformations in solution. As a consequence of the high proline content of the hinge, the hydroxy-side chains extend from the protein backbone at different angles and provide for a continuous coat of glycan along the length of the exposed surface (Fig. 11). The amino acid sequencing data, which indicated that Ser238 and Ser240 are unglycosylated, is consistent with the steric crowding in this region of the hinge observed in the molecular model. As a result of the elongated IgA1 hinge region, each heavy chain diverges from the inter-α-chain disulfide bridge across Cys241, allowing the Fab regions greater conformational freedom relative to the corresponding domains in IgG1.

This model of IgA1 indicated that the Co2 N-linked sites (Asn263) are located toward the C terminus of the domain, with the amide side chains pointing away from the protein backbone. The Co2 N-glycans cannot be confined in the space between the two CH2 domains and are not involved in protein-sugar interactions. This orientation is consistent with the finding that >90% of the N-linked oligosaccharides in the Fc are fully galactosylated and sialylated. In contrast, the Cy2 N-glycans of IgG1 extend from the N terminus of the domain into the interstitial region between the two heavy chains and interact with the protein surface (28, 76). This limits Cy2 N-glycan processing such that 25–35% of glycans are not galactosylated and <10% are sialylated (31, 59, 68, 77). On a molecular level, this suggests that the $K_m$ for the galactosyltransferase is higher for IgG than for IgA and that, in the case of IgG, the enzyme levels are insufficient to ensure that all of the glycans exposed on the CH2 domains are galactosylated. The partial galactosylation of IgG provides a sensitive probe for any changes in the parameters that govern the reaction. In contrast, IgA1 is already fully galactosylated and may accommodate a significant fall in enzyme efficiency before galactosylation is altered.

In patients with rheumatoid arthritis, there is an increase in the proportion of IgG sugar chains lacking terminal galactose residues (90). This is consistent with data from Furukawa et al.

**FIG. 11. Comparison of the molecular models of IgA1 Fc and IgG1 Fc.** Shown is the structure of the Fc regions of IgA1 (upper panel without the tailpiece) and IgG1 (lower panel), including the two heavy chains (blue and gold), cysteine residues (red), and oligosaccharides (yellow). The displacement of the IgA1 CH2 domains resulted in reduced interstitial space. This, together with the altered location of the N-glycosylation site, results in the exposed Co2 N-glycans. In IgG1, these CH2 N-glycans are confined within the interstitial space. The two interchain disulphide bridges in the hinge region of IgG1 limit the flexibility of the region relative to IgA1, where the single C-terminal disulphide bridge allows the hinge polypeptide to diverge from the two CH2 domains. The coating of O-glycans along the accessible hinge region of IgA1 highlights the protective nature of these sugars. IgA1 was modeled as described under “Experimental Procedures,” while IgG1 was modeled using the crystal structure (28).
(91), which demonstrate that the efficiency of the galactosyltransferase reaction is decreased in rheumatoid arthritis B cells. In contrast, studies of IgA1 from rheumatoid arthritis patients (14) indicated that there is no significant reduction in galactosylation of the N-glycans. This suggests that IgG, but not IgA1, is a sensitive marker of altered efficiency of galactosyltransferase in rheumatoid arthritis.

Comparison of the site-specific glycosylation of IgA1 Fc with IgG1 Fc indicates that the protein structure determines the availability of the glycan for processing at each stage of the biosynthetic pathway. Therefore, the three-dimensional structure of the protein can indirectly control the range of glycoforms. Where extensive glycan heterogeneity occurs, this is indicative of unrestricted glycan processing; e.g. CD59 has over 130 glycan structures associated with the single N-glycan sequon (42). Examination of the three-dimensional protein structure around the two IgA1 N-glycan sites indicates greater exposure of the tailpiece glycans relative to those at Asn263. Data from this study also suggest that full occupancy of N-glycans forms. Where extensive glycan heterogeneity occurs, this is exposure of the tailpiece glycans relative to those at Asn 263.

This suggests that the location of the larger N-linked glycan at Asn 249. This conclusion is supported by the glycan sequencing studies of the recombinant IgA1 mutants, where Asn 249 contained more highly processed N-glycans compared with Asn263. Data from this study also suggest that full occupancy of the N-glycan sites is not required to maintain the stability of human serum IgA1 and that neither the tailpiece nor N-glycosylation of the C2 domain plays a major role in IgA1-neutrophil FcRc binding.

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