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Structural analysis of the *N*-glycans from human immunoglobulin A1: comparison of normal human serum immunoglobulin A1 with that isolated from patients with rheumatoid arthritis

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The primary structures of the N-linked oligosaccharides from normal human serum IgA1 were determined by a combination of sequential exoglycosidase digestion, Bio-Gel P-4 chromatography, anion-exchange chromatography and one-dimensional n.m.r. spectroscopy. Three major N-linked disialylated biantennary-complex-type structures were found (55%). The remaining N-linked oligosaccharides consisted of at least nine further structures, some of which (7%) were of the triantennary type and included disialylated triantennary oligosaccharides with outer-arm fucose substitution [Fucα1-3(4)]. Compared with IgG, the N-glycan structures on IgA are more completely processed: the outer arms have a higher proportion of galactose and sialic acid, and only trace levels of incompletely galactosylated oligosaccharides, commonly found on IgG, were detected. Analysis of the sialylated *O*-glycans revealed that 64% were $[NeuAc2\alpha3(6)]_2$Galβ3GalNAc and 9% were [NeuAc2α3(6)]-Galβ4GlcNAcβ6[NeuAc2α3(6)Galβ3]GalNAc, and 27% were monosialylated. The N-linked glycosylation of both serum IgA1 and IgG isolated from a group of six normal individuals was compared with that from ten patients with rheumatoid arthritis (RA). In contrast with the hypogalactosylation found in IgG from diseased sera, there was no evidence of an equivalent decrease in the galactosylation of the IgA1 oligosaccharides. In addition, the N-glycosylation of IgA1 was remarkably consistent within the group of normal individuals. These data suggest that incomplete galactosylation of N-linked glycans and its augmentation in RA does not extend to IgA1 and that the RA-associated galactosyltransferase deficiency may be restricted to cells producing γ-chain.

INTRODUCTION

Relatively little is known about the functions of IgA despite the fact that it is a major isotype in normal human serum (0.5–4.0 mg/ml). Previous studies suggested that the dominant role of serum IgA was the removal of antigenic substances without the generation of an inflammatory response (Van Epps and Williams, 1976; Wilton, 1978). However, more recent work has shown that IgA can activate complement and will efficiently trigger cell-mediated events (Hiemstra et al., 1987; Yeaman and Kerr, 1987; Gorter et al., 1988; Kerr, 1990; Griffiss and Goroff, 1983). IgA receptors have been detected on human neutrophils, monocytes, macrophages, subpopulations of T-cells, B-cells and NK cells (Lawrence et al., 1975; Fanger and Lydyard, 1981; Millet et al., 1988; Chevalier et al., 1989; Fortune et al., 1990; Maliszewski et al., 1990; Mazengera and Kerr, 1990; Monteiro et al., 1990; Stewart and Kerr, 1990). Soluble IgA-binding factors in serum may regulate a class-specific antibody response similarly to IgE-binding factors [Kerr (1990) and references therein].

There are two subclasses of human IgA: IgA1 (80–90% of total serum IgA) and IgA2. The major structural difference between these two subclasses is that IgA1 has an additional 18-amino acid O-glycosylated hinge region (Pro-223–Ser-242) composed exclusively of proline, serine and threonine, which is located between the Cα1 and Cα2 domains. IgA1 contains two N-glycosylation sites per α-chain in the Fc region of the protein, located in the Cα2 (Asn-263) and Cα3 (Asn-459) domains (Putnam et al., 1979), whereas IgA2 contains four sites per α-chain with glycosylation sequons in the Cα1 (Asn-166), Cα2 (Asn-263, -337) and Cα3 (Asn-459) domains (Torano and Putnam, 1978). The IgA2 m2 allotype contains a further glycosylation sequon at Asn-211 in the Cα1 (Tsuzukida et al., 1979).

Previous analyses of the oligosaccharide composition of IgA1 and IgA2 from a group of individuals (Tomana et al., 1976) were consistent with the presence of complex-type structures. Significant variation was found between the samples, suggesting that IgA glycoforms may vary between individuals. Myeloma IgA (Baenziger and Kornfeld, 1974a) contained two N-linked biantennary complex-type structures with sialic acid linked α2–6. Analysis of the O-linked glycans (attached to five substituted serines) from the human myeloma IgA1 by Baenziger and Kornfield (1974b) showed that four of the hinge-region structures were neutral Galβ3GalNAc. In addition, GalNAcα1-*O*-Ser was present at Ser-224, and no sialylated O-linked saccharides were found. More recently we have reported the structures of O-linked glycans from normal serum IgA1 released by β-elimination (Field et al., 1989). In this case it was found that the ratio of incidence of the core structures Galβ3GalNAc and GalNAc was

Abbreviations used: Fuc, fucose; G0–G3, N-linked glycan terminating in zero to three galactose residues; HVE, high-voltage electrophoresis; g.c., gas chromatography; gu, glucose unit; sIgA, secretory IgA; ol, reduced oligosaccharide; OT, oligosaccharide reduced with tritium; RA, rheumatoid arthritis. All monosaccharides discussed in this paper are assumed to be in the D-pyranose configuration, except for fucose which is L.

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maintained at 4:1 but additionally that Galβ3GalNAc was present as both mono- and di-sialylated forms.

Although oligosaccharides of immunoglobulins have been studied extensively, most work has focused on myeloma proteins (Baenziger and Kornfeld, 1974a,b; Chapman and Kornfeld, 1979; Ishihara et al., 1983; Mellis and Baenziger, 1983; Takahashi et al., 1987). It has been reported that the glycosylation of myeloma proteins may differ from their normal serum counterparts (Mizuochi et al., 1982), and therefore the glycans present on the myeloma IgA proteins may not be representative of the normal serum population. In this study we performed a detailed oligosaccharide analysis of the *N*-glycans and the sialylated *O*-glycans released from normal serum IgA1.

In some disease states, most notably adult and juvenile rheumatoid arthritis (RA), the relative proportions of the IgG glycoforms are altered compared with the normal distribution (Rademacher et al., 1988; Rademacher, 1991), and without the appearance of novel glycans. There is an increase in the incidence of *N*-glycans that terminate in *N*-acetylglucosamine and are devoid of non-reducing terminal β-galactose residues (G0-type oligosaccharide chains) (Parekh et al., 1985). In view of this, the terminal substitution (i.e. sialylation and galactosylation) of the *N*-glycans isolated from serum IgA1 from a group of normal individuals was compared with IgA glycans from patients with RA to establish whether the alteration to the glycoform population is restricted to IgG or whether it also affects IgA1.

EXPERIMENTAL

Materials

Jacalin lectin–agarose was obtained from Pierce, Chester, U.K. Antisera were from Sigma, Poole, Dorset, U.K. Enzymes were obtained as follows: bovine testes α-fucosidase (Sigma), bovine testes β-galactosidase and *Streptococcus pneumoniae* β-*N*-acetylhexosaminidase (Boehringer-Mannheim), *Arthrobacter ureafaciens* neuraminidase (Calbiochem, Cambridge Bioscience, Cambridge, U.K.), endoglycosidase H (*Streptomyces plicatus*) (Miles Chemicals); *Achatina fulica* β-mannosidase was a gift from Seikagaku Kogyo Co. (Tokyo, Japan). *Choronia lampas* α-fucosidase and almond emulsion α-fucosidose III (Scudder et al., 1990; Butters et al., 1991) were purified in this laboratory. The sources of other enzymes have been described previously (Ashford et al., 1987; Olafson et al., 1990).

Isolation of human serum IgA1

IgA1 was prepared from serum by the method of Roque-Barreira and Campos-Neto (1985) with some modifications. Briefly, IgA1 was isolated from serum by affinity chromatography on jacalin lectin–agarose. Co-purifying IgM was removed by h.p.l.c. (TSK SWG 3000 column; 100 mM potassium phosphate buffer, pH 7.2; 4 ml/min). IgA1-positive fractions [detected by immunoprecipitation with anti-(human α-chain) serum at 1:25 dilution in barbitone buffer in 1% (w/v) agarose] were pooled and analysed by gel filtration (Zorbax GF 250), reducing SDS/PAGE and immunoelectrophoresis.

Isolation of human serum IgG

IgG was isolated from an aliquot of the individual serum samples used to purify IgA1, using 33%-satd. $(NH_4)_2SO_4$ precipitation and DEAE-cellulose anion-exchange chromatography as described previously (Parekh et al., 1985).

Release of oligosaccharides from IgA1 and IgG

Immunoglobulins were dialysed exhaustively against distilled water (4 °C), freeze-dried and cryogenically dried over activated charcoal at −196 °C (< 0.1 Pa). N- and O-linked oligosaccharides were released with fresh double-vacuum-distilled anhydrous hydrazine and purified at 85 °C, as described previously (Ashford et al., 1987; Parekh et al., 1989b; Olafson et al., 1990).

^{1}H-n.m.r. spectroscopy

Samples were prepared for n.m.r. studies as described previously (Homans et al., 1984). Briefly, neutral reduced oligosaccharides were passed through a tandem column containing 100 μl each of Chelex (Na^+ form), AG50 (X12; H^+ form), AG3 (X4A; OH^- form) and QAE-Sephadex, and charged unreduced or reduced oligosaccharides were passed through a tandem column containing 100 μl each of Chelex (Na^+ form) and AG50 (X12; H^+ form). The samples were filtered through a 0.45 μm-pore-diam. poly(tetrafluoroethylene) filter, evaporated to dryness and then dissolved in ^2H_2O before analysis by one-dimensional n.m.r. spectroscopy (600 MHz, 500 MHz or 300 MHz, using Bruker AM n.m.r. spectrometers).

Radiolabelling of oligosaccharides

Purified oligosaccharides (1 mM glycan) were reduced with 6 mM NaB^3H_4 (specific radioactivity 10 Ci/mmol) at 30 °C in 50 mM NaOH buffered to pH 11.0 with saturated boric acid. After 4 h, an equal volume of 1 M NaB^3H_4 in the same buffer was added and the reaction continued for a further 2 h. The oligosaccharides were subsequently purified from radiochemical contaminants as previously described (Ashford et al., 1987).

Separation of oligosaccharides by charge

Anion-exchange chromatography

Reduced and radiolabelled oligosaccharides were separated by anion-exchange chromatography using a Pharmacia f.p.l.c. system with a Mono Q HR5/5 column. Glycans were applied to the column in 200 μl of distilled water, and neutral species were eluted with water (2.5 column vol.). Charged oligosaccharides were eluted with a linear gradient of ammonium acetate, pH 5.5 (0–100 mM over 15 column vol.; flow rate 1 ml/min). Fractions (1 ml) were monitored by withdrawing portions for liquid-scintillation counting to determine the relative proportions of differently charged species in IgA and to compare the sialic acid content of IgA and IgG. Peak charges were confirmed by analysis of pooled fractions by high-voltage electrophoresis (HVE).

Bio-Gel P-4 chromatography in salt

Unreduced oligosaccharides were applied to a Bio-Gel P-4 (−400 mesh) gel-permeation chromatography column (1.5 cm × 1 m, maintained at 55 °C) and eluted with 50 mM sodium acetate (pH 6.0, containing chlorbutol, 1 mg/ml). Glycans were detected by absorbance at 210 nm. The fractions were desalted on Dowex AG 50W (X12; H^+ form).

Dionex BioLC ion-exchange chromatography

Pooled desalted oligosaccharide fractions from Bio-Gel P-4 chromatography were evaporated to dryness, redissolved in a

minimum of water and injected on to a Dionex BioLC system equipped with a column (4.6 mm × 250 mm) of Dionex Carbo Pac PA1 pellicular anion-exchange material (Dionex U.K. Ltd., Farnborough, Hants., U.K.). Separations were performed by elution with 100 or 200 mM NaOH at a flow rate of 1 ml/min; column temperature was 30 °C. Fractions (250 μl) of eluate were counted for radioactivity on a liquid-scintillation counter.

Separation of neutral oligosaccharides

Glycans were converted into neutral compounds by treatment with 0.1 unit of *Arthrobacter ureafaciens* neuraminidase for 18 h in 60 μl of 0.1 M sodium acetate, pH 5.0, as determined by HVE for 45 min at 80 V/cm in pyridine/acetate/water (3:1:387, by vol.), pH 5.4. Reduced neutral oligosaccharides were fractionated by gel-filtration chromatography through Bio-Gel P-4 (−400 mesh) as previously described (Ashford et al., 1987; Parekh et al., 1989b; Olafson et al., 1990). The eluate was monitored with an h.p.l.c. radioactivity monitor (Berthold model LB503) and in most cases also by withdrawing samples from the fractions for liquid-scintillation counting.

The hydrodynamic volume of the material eluted from the Bio-Gel P-4 columns was determined by comparison with co-injected isomaltose oligomer oligosaccharides (0.5 mg), monitored by refractive index, and is given in glucose units (gu).

Exoglycosidase digestions

Reduced and radiolabelled oligosaccharides were digested with exoglycosidases as described by Ashford et al. (1987), Parekh et al. (1989b) and Olafson et al. (1990) with the following modifications. Jack-bean β-galactosidase digestion was performed in a reaction volume of 30 μl containing 0.3 unit of enzyme. *S. pneumoniae* β-*N*-acetylhexosaminidase digestion was performed at 8 munits/ml for linkage-specific digestions in 30 μl of 100 mM citrate phosphate buffer, pH 6.0, containing 1% BSA, or in a reaction volume of 20 μl at 0.3 unit/ml in 100 mM citrate phosphate buffer, pH 6.0, for arm-specific digestions. Digestion of glycans with bovine testes β-galactosidase was performed in a reaction volume of 25 μl containing 50 munits of enzyme in 100 mM citrate/phosphate buffer, pH 3.5. The specificity of all exoglycosidase reactions was confirmed by co-digestions with standard oligosaccharides as described previously (Parekh et al., 1989b).

Determination of the reduced terminus

The identity of the reducing terminal monosaccharide was determined by using the radioelectrophoretic method (Takasaki et al., 1982). To confirm the identity of the reducing terminal (i.e. GalNAcOT and GlcNAcOT), the radiolabelled reducing terminal structures were also analysed by gas chromatography (radio-g.c.). Monosaccharides prepared by acid hydrolysis were first derivatized to the alditol acetates by incubating them with 10 μl of pyridine and 50 μl of acetic anhydride at 120 °C for 20 min. After evaporation, 100 μl of water and 100 μl of chloroform were added. The aqueous layer was removed, and the chloroform layer evaporated. The peracetylated sample was then redissolved in chloroform, and a 1–2 μl sample injected into a Varian 3600 g.c. system with a SP2380 capillary column (25 m × 0.32 m; Supelco). The sugars were eluted with helium (2.5 ml/min) at 90 °C for 1 min. The temperature was raised at the rate of 30 °C/min to 220 °C, maintained for 13 min, and finally increased to 230 °C at 30 °C/min. Effluent from the g.c. column was mixed with hydrogen (3.5 ml/min), and passed into a catalytic reduction furnace (platinum catalyst/700 °C) within the g.c. oven. The volatile reduction products were then mixed with counting gas [argon/methane (9:1, v/v); 120 ml/min], and radioactivity was detected in a 10 ml counting tube (Raytest, Sheffield, Yorks., U.K.). Retention times of unknown monosaccharide peaks were identified by comparing them with those of radiolabelled standards.

Determination of the incidence of terminal galactose on IgA and IgG

The reduced desialylated glycans derived from IgA1 were fractionated by gel filtration on Bio-Gel P-4 columns. Oligosaccharides which were eluted at hydrodynamic volumes greater than 10 gu were pooled and treated with a mixture of jack-bean α-mannosidase, jack-bean β-*N*-acetylhexosaminidase and bovine testes α-fucosidase as described by Parekh et al. (1988a). This mixed enzyme digestion allows the resolution of complex-type oligosaccharides on Bio-Gel P-4 depending on the number of terminal β-galactose residues they contain.

RESULTS

The overall scheme of analysis is described in Figure 1.

Analysis of IgA1

IgA1 was eluted from the h.p.l.c. gel-permeation column at a position consistent with a molecular mass of 160 kDa, and gave two major bands on reducing SDS/PAGE at 55 and 25 kDa (Figure 2). Immunoelectrophoresis detected only IgA when the purified material was blotted and challenged with anti-(human α-, γ- and μ-chain). C1 esterase can co-isolate with jacalin-lectin-purified IgA (Loomes et al., 1991). C1 esterase is present at approx. 200 μg/ml in normal human serum, about 10% of the level of IgA, and contains six *N*-glycans/molecule, compared with four for IgA (Perkins et al., 1990). Therefore without purification, C1 esterase oligosaccharides would contribute about 15% to the pool of glycans analysed here. C1 esterase (95 kDa) was removed in the gel-filtration step which followed the affinity purification of IgA1 (160 kDa), and it is estimated that the IgA1 glycans analysed here are more than 95% pure. Moreover, the glycans from C1 esterase, as determined in this laboratory (Perkins et al., 1990), are very similar to those found on IgA1; 75% corresponds to glycan A-c (see Figure 9); the remaining C1 esterase glycans are present at negligible levels.

Analysis of IgA glycans

Charge analysis of total *N*- and *O*-glycans

Five IgA samples from normal individuals and five from patients with RA were analysed by Mono Q Sepharose ion-exchange chromatography. Figure 3(a) is a representative chromatogram. The normal mean values were $14.0 \pm 3.6\%$ for neutral, $39.2 \pm 5.5\%$ for A1, $40.5 \pm 5.2\%$ for A2 and $6.3 \pm 1\%$ for A3. The mean values for RA patients were $16.0 \pm 3.8\%$ for neutral, $35.1 \pm 3.0\%$ for A1, $43.4 \pm 6.0\%$ for A2 and $5.5 \pm 2.4\%$ for A3.

The Mono Q profile cannot be used to determine the ratio mono-/di-/tri-sialylated structures. Analysis of these fractions is shown in Figure 3(e). The A1 fraction contained exclusively monosialylated N-linked structures, A2 was a mixture of disialylated N-linked structures and monosialylated O-linked structures, and A3 contained the trisialylated triantennary N-linked and the

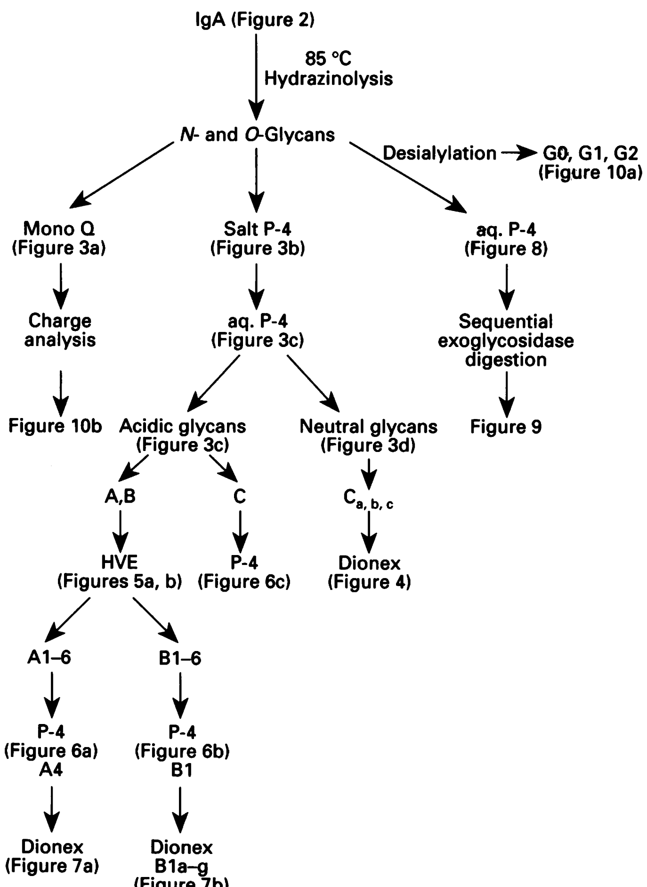

Figure 1 Overall scheme for the analysis of glycans

Hydrazine-released, reduced and radiolabelled IgA *N*- and *O*-glycans were analysed by ion-exchange chromatography using Mono Q Sepharose (Figure 3a). The hydrazine-released sugars before reduction were fractionated, also on the basis of charge, by salt P-4 gel-permeation chromatography (Figure 3b). Naturally neutral structures and charged *N*- and *O*-glycans eluted from the salt P-4 column (Figure 3b) were fractionated on aqueous P-4 (Figures 3c and 3d). Naturally occurring neutral structures were analysed by Dionex (Figure 4); the major acidic *N*- and *O*-glycans eluted in the void were fractionated by HVE (Figures 5a and 5b) and analysed by P-4 (Figures 6a and 6b) and Dionex (Figures 7a and 7b); fraction C void was analysed by P-4 (Figure 6c). O-linked structures eluted from the salt P-4 were analysed by exoglycosidase sequencing and Dionex ion-exchange chromatography, which was also used to confirm the *N*-glycan structures primarily determined from exoglycosidase sequencing of the hydrazine-released, reduced, radiolabelled and desialylated glycans fractionated on aqueous P-4 (Figure 8).

disialylated O-linked structures. This situation is the result of differences in charge contributions between α2-3- and α2-6-linked sialic acids.

Fractionation of total *N*- and *O*-glycans according to charge

The complete set of oligosaccharide structures (N- and O-linked) released by hydrazine from an individual sample of normal serum IgA1 were chromatographed (before reduction) on a Bio-Gel P-4 column eluted with salt (Figure 3b). Three fractions (A, B and C) were reduced with NaB^3H_4 and applied to a Bio-Gel P-4 column eluted with water (Figure 3c). In this latter system charged oligosaccharides were excluded from the gel and eluted at the void volume, and the neutral glycans were eluted according to their hydrodynamic volume. The oligosaccharides present in fractions A (56 % of total) and B (30 % of total) were all eluted

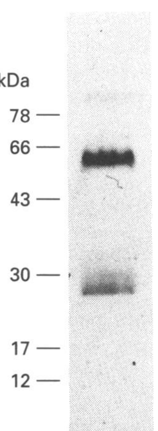

Figure 2 SDS/PAGE reducing gel (8–25 % gradient) of affinity-purified IgA

IgA1 eluted from the h.p.l.c. column gave two major bands on reducing SDS/PAGE at 55 and 25 kDa consistent with the presence of α-chains and light chains. A minor band at approximately 80 kDa has a molecular mass consistent with incompletely reduced heavy plus light chain.

in the void volume. In contrast, in fraction C (14 % of total), 65 % of the oligosaccharides were eluted in the void volume (9 % of total IgA sugars), and a fraction (5 % of total IgA sugars) was eluted between 11 and 15 gu (C_a, C_b, C_c Figure 3d); these fractions contained the naturally occurring neutral *N*-glycans. In summary, 95 % of the oligosaccharide structures recovered from salt P-4 fractionation of this sample of normal IgA were charged and 5 % were neutral.

Exoglycosidase sequencing of natural neutral glycans from fraction C (G0-type oligosaccharides)

Fractions C_a, C_b and C_c shown in Figure 3(d) were analysed by Dionex chromatography (Figures 4a–4c) and the major peaks from fraction C_c, which contained the G0-type sugars, were sequenced. The purified Dionex BioLC ion-exchange fractions of the naturally neutral fraction C_c (Figure 4) were reapplied to the Bio-Gel P-4 column to confirm their hydrodynamic volumes (results not shown.) Peaks C_{c1}, C_{c2} and C_{c4} were all single components eluted at 11.5 gu, whereas C_{c3} contained two components which were eluted at 11.5 and 10.5 gu. Peaks C_{c1} and C_{c2} were resistant to jack-bean α-mannosidase, indicating that they were complex-type oligosaccharides, whereas fraction C_{c4} was completely digested to 5.5 gu (i.e. Manβ4GlcNAcβ4GlcNAcOT), indicating that this fraction contained the oligomannose structure $Man_8GlcNAc_2$. For each mannose residue beyond Man5, the hydrodynamic volume was between 0.85 and 0.9 gu. The oligosaccharides present in fraction C_{c3} were sensitive to α-mannosidase and digested to 9.0 gu, suggesting that the structures were hybrids. The major fraction, C_{c2}, was mixed with the G0 standard GlcNAcβ2Manα6(GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAcOT and found to be co-eluted on Dionex BioLC ion-exchange chromatography.

Further fractionation of acidic glycans (95% of total IgA sugars)

Figures 5a and 5b show HVE profiles for the acidic void fractions of A and B from the Bio-Gel P-4 column eluted with water at 55 °C (Figure 3c). Both fractions A and B were resolved into at least six different acidic fractions. To determine the structures present, each fraction was eluted from the paper,

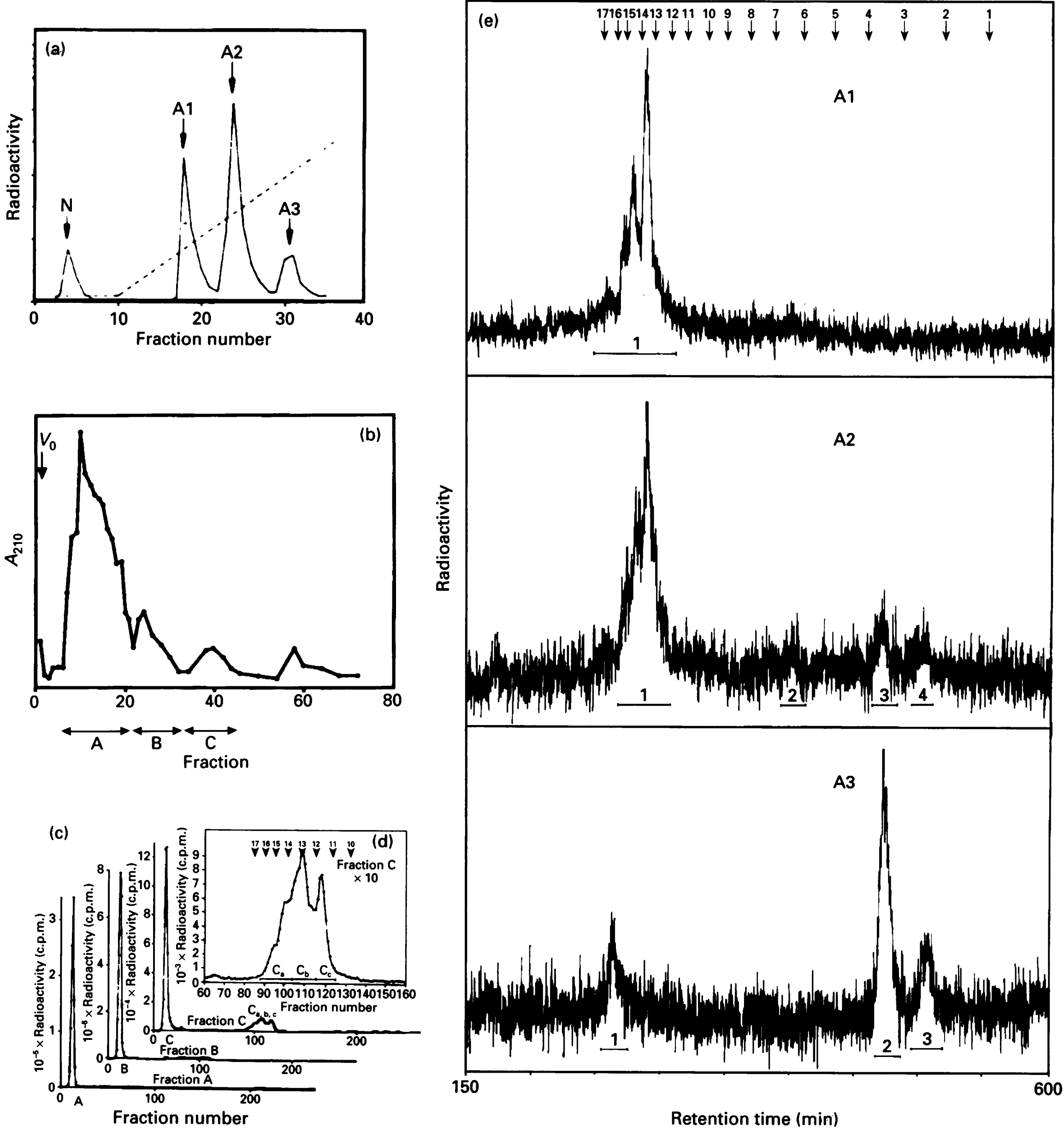

Figure 3 Analysis of IgA glycans

(**a**) Representative profile of normal human IgA1 oligosaccharides analysed by Mono Q chromatography. N, Neutral oligosaccharides (14%); A1 (39%); A2 (41%); A3 (6%). Percentages are the averages obtained from six individual serum samples. (**b**) Elution profile of normal human serum IgA sialylated N- and O-linked oligosaccharides separated by Bio-Gel P-4. The non-reduced oligosaccharide pool was applied to a Bio-Gel P-4 column eluted at 55 °C with sodium acetate containing chlorbutal. Fractions were pooled as indicated: A, 30%; B, 56%; C, 9%. The fractions were monitored by measuring absorbance at 210 nm; sialic acids absorb more strongly at this wavelength, therefore the peak areas do not represent the molar proportions of the glycans. These were determined, after radiolabelling, by HVE. (**c**) Aqueous Bio-Gel P-4 elution profile of fractions A, B and C from salt P-4. The oligosaccharides eluted in fractions A, B and C (**b**) were reduced with NaB^3H_4 and applied to Bio-Gel P-4 gel-filtration columns. The large void peak in each fraction contains the charged oligosaccharides; peaks C_a, C_b and C_c in fraction C contain the naturally neutral glycans. (**d**) An expansion of the naturally neutral glycans present in fraction C. C_a, C_b, C_c were analysed by Dionex chromatography; fraction C_c contains the G0-type oligosaccharides. The numbers at the top indicate the elution position of glucose oligomers co-injected with the sample. (**e**) Biogel P-4 chromatogram of the oligosaccharides. The oligosaccharides eluted at A1, A2 and A3 in (**a**) were incubated with sialidase and applied to a Bio-Gel P-4 column.

treated with sialidase and subjected to Bio-Gel P-4 chromatography (Figure 6). After treatment with neuraminidase, all of the fractions became neutral (results not shown); therefore all the charge on IgA sugars is due to sialic acid.

Analysis of HVE fractions A1–6

Figure 6(a) shows the P-4 elution profiles of the desialylated glycans derived from HVE (Figure 5a) of fraction A (Figure 3c). Fraction A1 (monosialylated) was an overlap of fraction B1 (Figure 5b). Fraction A2 contained large disialylated complex-type oligosaccharides. Fraction A3 contained disialylated biantennary complex glycans. Fraction A4 was predominantly a trisialylated complex-type structure. Fraction A5 (2 %) contained a mixture of tetrasialylated structures. Owing to the small amount of material, fractions A5 and A6 were not analysed further.

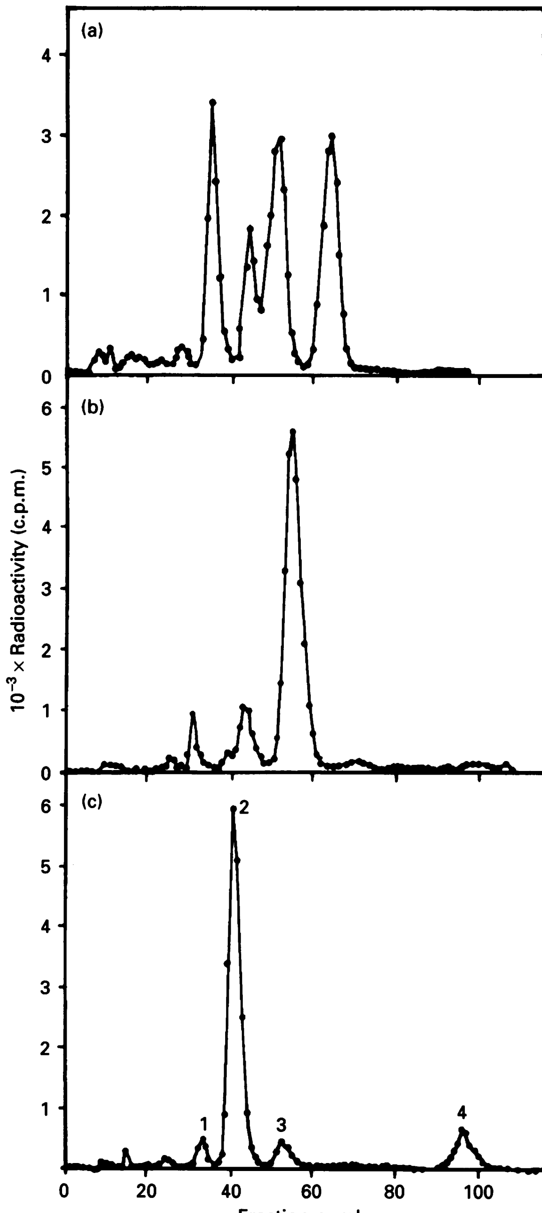

Figure 4 Analysis of naturally neutral oligosaccharides from fractions C_a (a), C_b (b) and C_c (c) (Figures 3b and 3c) by Dionex ion-exchange chromatography

C_{c1} contains complex glycans, C_{c2} contains G0 glycans, C_{c3} contains hybrid glycans and C_{c4} contains oligomannose glycans.

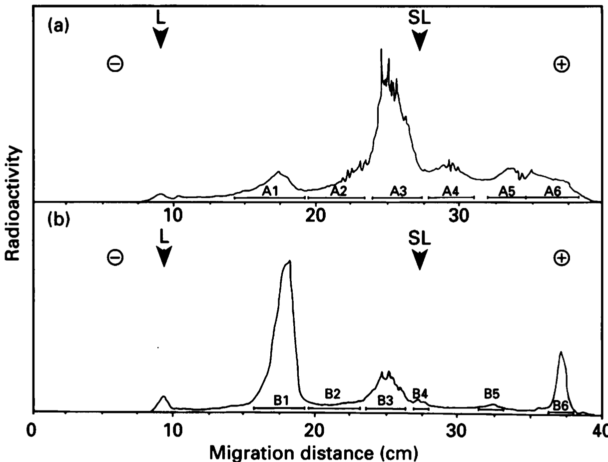

Figure 5 High-voltage electrophoretograms of Bio-Gel P-4 void fractions A and B (Figure 3b)

Oligosaccharides eluted in the void volume of the Bio-Gel P-4 were subjected to HVE (80 V/cm) in pyridine/acetic acid/water (3:1:387, by vol.), pH 5.4. Arrows indicate the positions of [^{3}H]lactitol (L) and 6′(3′)-sialyl-[^{3}H]lactitol (SL). Peak ratios are as follows: (**a**) void of fraction A: A1 (14%), A2 (8%), A3 (67%), A4 (8%), A5 (2%), A6 (1%); (**b**) void of fraction B: B1 (56%), B2 (4%), B3 (13%), B4 (3%), B5 (2%), B6 (14%). The bars indicate the areas of paper that were eluted.

Analysis of HVE fractions B1–6

Figure 6(b) shows the P-4 elution profiles of the desialylated glycans derived from HVE (Figure 5b) of fraction B (Figure 3c). Fraction B1 contained monosialylated biantennary and multi-antennary N-linked structures. Fraction B2 contained three main peaks at 14.5 gu (48%), 9.5 gu (22%) and 6.5 gu (30%). The 14.5 gu structures were an overlap of fraction A3. The 9.5 gu and 6.5 gu structures were monosialylated O-linked structures. Fraction B3 was an overlap fraction of A3. Fraction B4 contained monosialylated 3.5 gu *O*-glycans and was an overlap fraction from fraction C. Fractions B5 and B6 contained disialylated O-linked structures.

Analysis of void fraction C acidic glycans derived from salt P-4 (Figure 3c)

Figure 6(c) shows the P-4 elution positions of the desialylated glycans derived from the void of fraction C (Figure 3c). Structures eluted around 13.5 gu (25%) were an overlap from fraction B (Figure 3b). The 6.5 gu (42%) and 3.5 gu (21%) structures were derived from sialylated *O*-glycans.

Exoglycosidase sequencing analysis of acidic *O*-glycans fractionated by salt P-4/aqueous P-4/HVE/sialidase/aqueous P-4

The results of the enzyme digests are listed below (see also Figure 9, structure O-b).

6.5 gu oligosaccharide present in fractions B5 and B2 (Figure 6b) and B (Figure 6c)

The following data are consistent with the 6.5 gu species having the branched structure Galβ4GlcNAcβ6(Galβ3)GalNAcOT (Figure 9, structure O-b), although the linear structure Galβ4GlcNAcβ6Galβ3GalNAcOT cannot be unambiguously ruled out. The 6.5 gu structure was resistant to jack-bean β-*N*-acetylhexosaminidase, therefore no terminal GlcNAc was present. Digestion with *S. pneumoniae* β-galactosidase gave a 5.5 gu product, indicating the presence of a terminal 4-linked galactose. The 5.5 gu product was resistant to *S. pneumoniae* β-*N*-acetylhexosaminidase and to bovine testes β-galactosidase. Incubation of the 5.5 gu structure with jack-bean β-*N*-acetylhexosaminidase gave a product eluted at 3.5 gu, indicating the loss of a single 3- or 6-linked GlcNAc residue. The 3.5 gu product was sensitive to digestion with bovine testes β-galactosidase giving a product eluted at 2.5 gu. The reducing terminal oligosaccharide was confirmed as *N*-acetylgalactosaminitol by high-voltage borate electrophoresis.

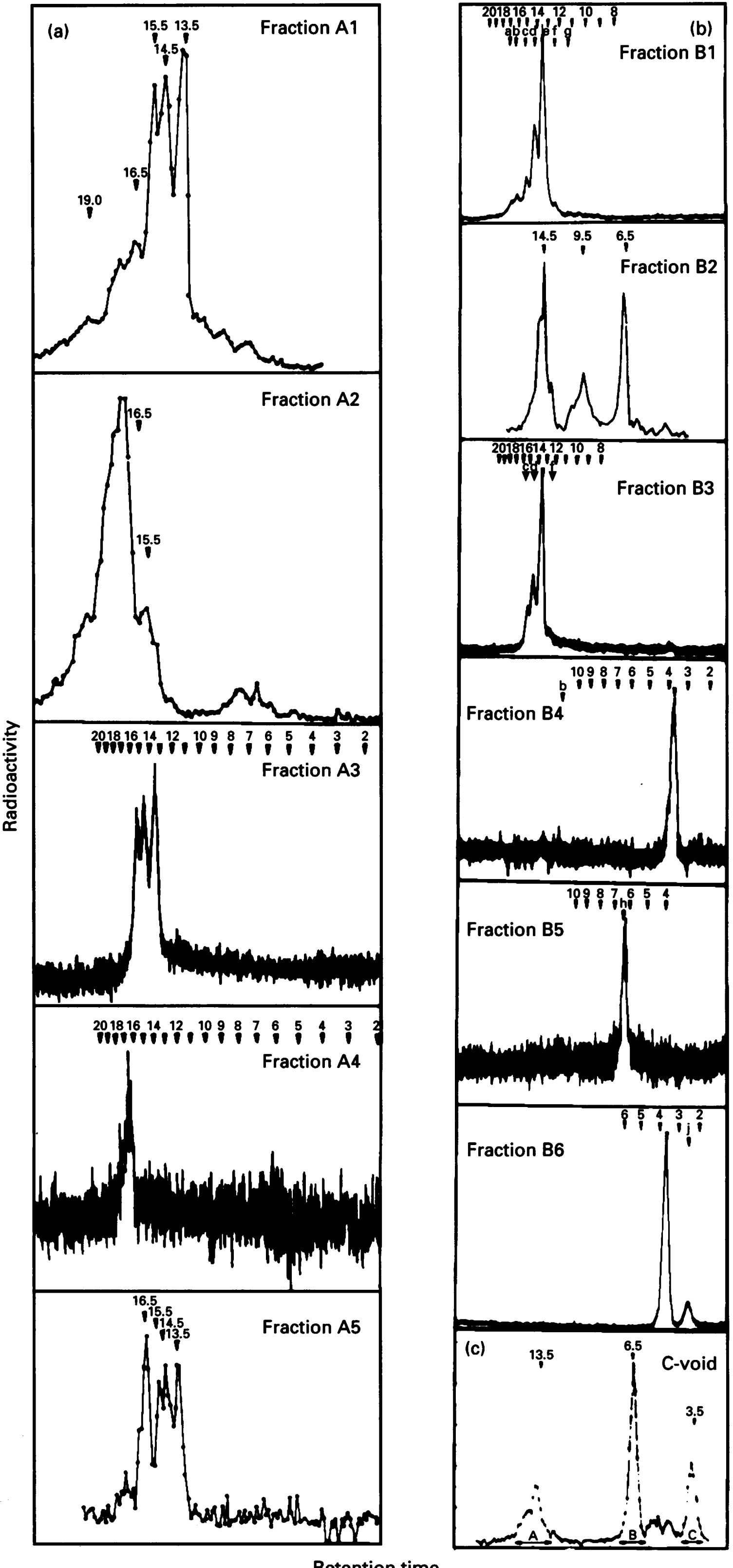

Figure 6 Bio-Gel P-4 chromatogram of the desialylated oligosaccharides eluted in the void of fractions A, B and C (Figure 3c) and fractionated by HVE (Figure 5)

(**a**) Fractions A1–5; (**b**) fractions B1–6. (**c**) Sialidase digest (fraction C-void). A is an overlap of B (Figure 3b); B and C are monosialylated O-links

3.5 gu oligosaccharide present in fractions B4, B6 (Figure 6B) and C (Figure 6c)

The 3.5 gu species was shown to be Galβ3GalNAcOT (Figure 9, structure O-a) by a combination of exoglycosidase digestions and compositional analysis. Fraction B6 was disialylated and B4 was monosialylated. After neuraminidase treatment the neutral cores both ran at 3.5 gu.

The 3.5 gu structure was resistant to *Escherichia coli* β-galactosidase (which cleaves only 4-linked galactose) but susceptible to bovine testes β-galactosidase indicating terminal 3-linked galactose. The 2.5 gu fraction in B6 (Figure 6b) was subjected to descending paper chromatography using pyridine/ethyl acetate/water/acetic acid (5:5:1:3, by vol.) (not shown) and was resolved into three peaks. The major peak (80 %; Apc) and two minor peaks (10 %; Bpc) and (10 %; Cpc) were analysed for the presence of *N*-[^{3}H]acetylgalactosaminitol by radio-g.c. The bulk of the radioactivity was not present in intact monosaccharides (results not shown.) Only the radioactivity in peak Cpc was found to be incorporated into *N*-acetylgalactosaminitol. The radioactivity in peaks Apc and Bpc therefore derived from chemically degraded O-linked oligosaccharides still retaining a sialic acid residue.

Analysis of total *N*-glycans

Dionex ion-exchange chromatographic analyses of *N*-glycans

The glycan structures from the Bio-Gel P-4 separations (Figures 6a and 6b) were further fractionated by Dionex chromatography and compared with standard oligosaccharides. The information was used to confirm the exoglycosidase sequencing analyses of the desialylated *N*-glycans described in the next section. (Most results are not shown.)

Figure 7 shows the Dionex chromatograms of fractions A4 (from Figure 6a) and B1a–g [from B1 (Figure 6b)]. Fraction B1 contained complex-type structures [A-h, A-d, A-b, A-c, A-f (Figure 9) oligomannose and hybrid structures]. Fraction A4 contained trisialylated multiantennary structures. The major fraction was shown to have a hydrodynamic volume of 16.4 gu by P-4 gel filtration.

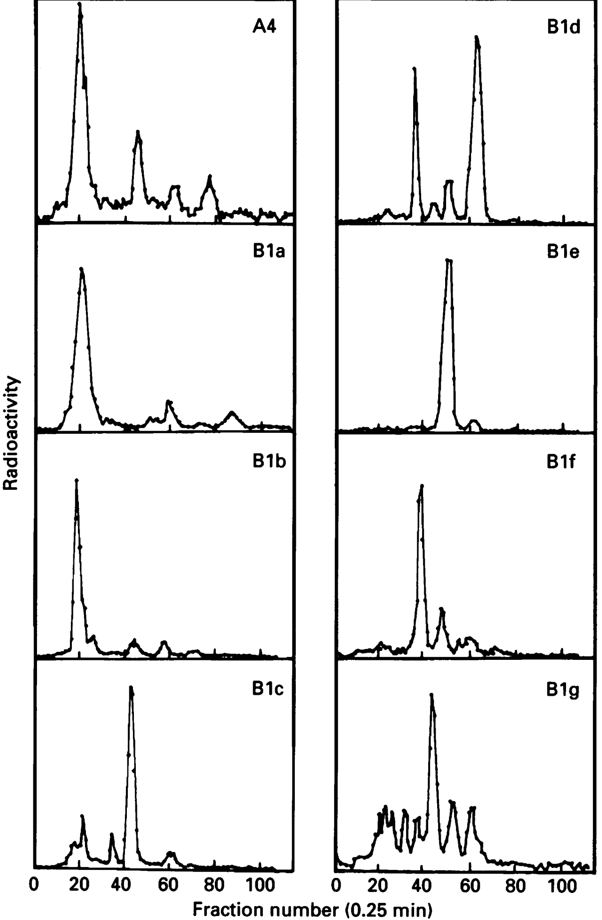

Figure 7 Ion-exchange chromatography of fraction A4 (Figure 4a) and fractions B1a–g

Fraction A4 contained the trisialylated complex structures.

Exoglycosidase sequencing analysis of total *N*-glycans fractionated by aqueous P-4 gel filtration

To determine the molar proportion of the different oligosaccharides present, the total asialo-oligosaccharide pool was treated with sialidase and the neutral oligosaccharides chromatographed on Bio-Gel P-4 columns (Figure 8). The four main fractions V, VI, VII and VIII were then treated with exoglycosidases to give digestive products characteristic of the proposed oligosaccharide structures present in each fraction. The high-resolution Bio-Gel P-4 chromatogram of the N-linked oligosaccharides greater than 11 gu after sialidase treatment (Figure 8) shows three subclasses of glycans. The elution volumes of peaks V–VII are consistent with biantennary complex or hybrid structures, whereas those for peaks I–IV are consistent with multiantennary structures or biantennary/hybrid complex structures containing repeating lactosamine units. Structures which were eluted at less than 13 gu (i.e. fraction VIII) included oligomannose, hybrid and incompletely galactosylated biantennary complex oligosaccharides. Fractions III–VIII from Figure 8 were subjected to detailed sequencing analysis, and in some cases the structures were confirmed by Dionex analysis of co-injected standard samples and by analysis of salt P-4 fractions.

Triantennary structures (Table 1)

Fractions I and II

Insufficient material was present for analysis.

Fraction III

Exoglycosidase sequencing of the 17.2 gu peak gave data consistent with fraction III containing a triantennary complex structure (A-i) (Figure 9). There are three possible isoforms of this structure, in each of which the fucose is associated with a different arm of the trisaccharide. The degree of sialylation was determined from the position at which the glycan migrated in HVE before neuraminidase digestion. The 17.2 gu structure was found only in HVE fraction A2, the position at which the glycan migrated in HVE before neuraminidase digestion.

Fraction IV

Exoglycosidase sequencing of the 16.4 gu peak gave data consistent with fraction IV containing the triantennary complex structure shown (A-h) (Figure 9). The 16.4 gu structure was predominantly found in HVE fraction A4, indicating that it was trisialylated.

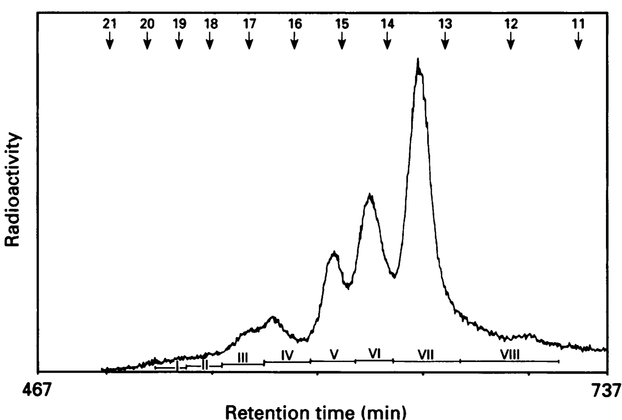

Figure 8 High-resolution Bio-Gel P-4 chromatogram of the desialylated N-linked oligosaccharides released from normal serum IgA

Fractions I–VIII were pooled as shown, and subjected to exoglycosidase sequencing as described in Figure 9. The structures in each pool are also given in Figure 9.

Table 1 Oligosaccharides present in P-4 fractions

Numbers in parentheses are the percentages determined by summation of the structures containing, e.g. +B +F listed in Figure 9. n.d., Not determined; F, fucosylated; B, bisected.

P-4 fraction	Elution volume (gu)	Percentage of total *N*-glycans	Structures present	Percentage of fraction
V	14.7→15.5	14	A-a	84
			A-b	16
VI	13.8→14.7	23	A-b	82
			A-d	12
			A-a	6
VII	12.8→13.8	43	A-c	94
			A-e	4
			A-f	2
V+VI		31	+B +F	45 (43)
			+B −F	7 (7)
			−B +F	49 (50)
			−B −F	–
V+VI+VII +VIII		54	+B +F	22 (22)
			+B −F	19 (18)
			−B +F	17 (24)
			−B −F	42 (46)
VIII	12.8→11.2	6	A-f	23
			A-g	52
			Oligomannose ($Man_8GlcNAc_2$)	11
			Hybrid-type	14
IV	15.5→16.5	7	A-h	100
III	16.5→17.5	4	A-i	40*
I, II	17.5→19.5	3	n.d.	–

* Other structures not determined.

Biantennary structures

Fraction V

Exoglycosidase sequencing of the structures eluted between 15.5 and 14.7 gu gave data consistent with fraction V containing the biantennary complex structures shown in Table 1 [A-a (84 %), A-b (16 %)] (Figure 9).

Fraction B1c (Figure 7) contained the monosialylated oligosaccharides eluted between 14.7 and 15.5 gu from Figure 6b (B1). The major fraction was eluted at 15.2 gu on rechromatography on Bio-Gel P-4 and co-eluted with the standard oligosaccharide Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)(GlcNAcβ4)-Manβ4GlcNAcβ4(Fucα6)GlcNAcOT on Dionex, confirming the structure of the major glycan in fraction V (A-a) (Figure 9).

Fraction VI

Exoglycosidase sequencing of the oligosaccharides eluted between 13.8 and 14.7 gu gave data consistent with fraction VI containing the biantennary complex structures A-b (82 %), A-d (12 %) and A-a (6 %) (Figure 9 and Table 1).

Fraction B1d (Figure 7) contained the monosialylated oligosaccharides eluted between 13.8 and 14.7 gu from fraction B1 (Figure 6b). The structure eluted at 14.5 gu was co-eluted with the standard Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)-Manβ4GlcNAcβ4(Fucα6)GlcNAcOT on Dionex, confirming the structure of A-b. The structure eluted at 14.2 gu (Figure 6b; fraction B1) was co-eluted with the standard Galβ4GlcNAc-β2Manα6(Galβ4GlcNAcβ2Manα3)(GlcNAcβ4)Manβ4Glc-NAcβ4GlcNAcOT or the non-fucosylated form of the structure present in fraction B1c (Figure 7) (Figure 9, structure A-d).

Fraction VII

Exoglycosidase sequencing of the structures eluted between 12.8 and 13.8 gu gave data consistent with fraction VII containing the biantennary complex structures A-f, A-e and A-c (Figure 9 and Table 1).

Fraction B1e (Figure 7) represents the oligosaccharides eluted between 12.8 and 13.8 gu (Figure 6b). The structure eluted at 13.5 gu on Bio-gel P-4 was co-eluted with the standard Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4Glc-NAcβ4GlcNAcOT on Dionex consistent with the data above (structure A-c) (Figure 9).

Fraction VIII

Exoglycosidase sequencing of the oligosaccharides eluted between 11.2 and 12.8 gu gave data consistent with the fractions containing structures A-f and A-g (Figure 9), with oligomannose (11 %) and hybrid-type (14 %) structures respectively (Table 1).

Fraction B1g (Figure 7) also contains oligosaccharides eluted at 11.8 gu (Figure 6b). As these structures were recovered from a monosialylated fraction, they must have been derived from structures containing a terminal sialyl-Gal linkage and therefore probably represent hybrid-type oligosaccharides or truncated monoantennary oligosaccharides. Owing to the small amount of material available, these fractions were not further analysed.

Analysis of core types

Cores V and VI

To confirm the ratios of fucosylated (F) and bisected (B) oligosaccharides in the major fractions, fractions V and VI (Figure 8) were pooled and digested with a mixture of *S. pneumoniae* β-galactosidase and *S. pneumoniae* β-*N*-acetylhexosaminidase. Three products corresponded to the three cores (+B +F) 45 %, (+B −F) 6 %, (−B +F) 49 % and (−B −F) trace. That all structures present contain a standard $Man_3GlcNAc_2$ core was confirmed by digestion of fractions V and VI with bovine testes β-galactosidase and jack-bean β-*N*-acetylhexosaminidase. The products [8.5 gu structure (90 %) and

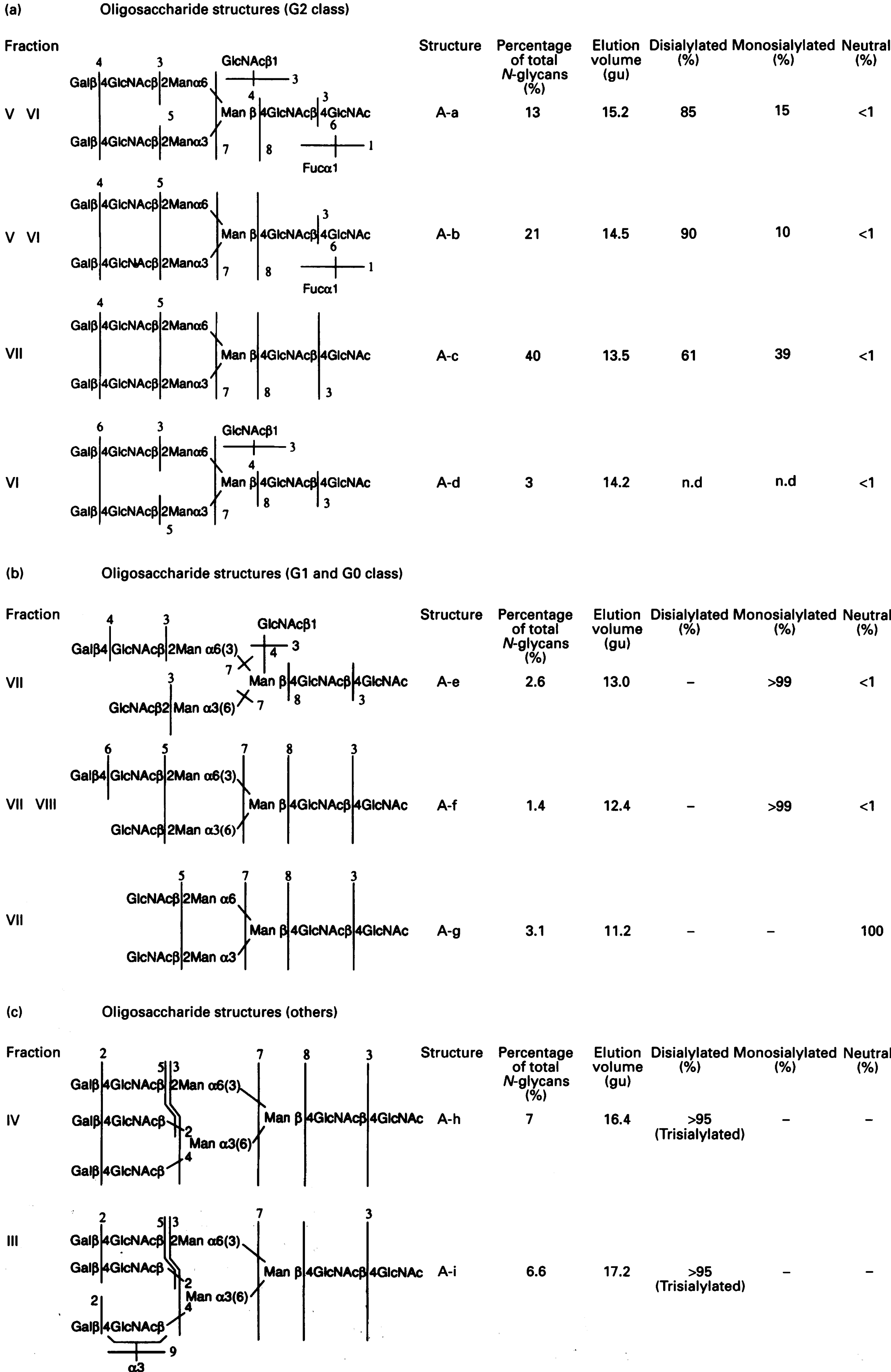

Figure 9 For legend see opposite.

(d) Oligosaccharide structures (O-linked)

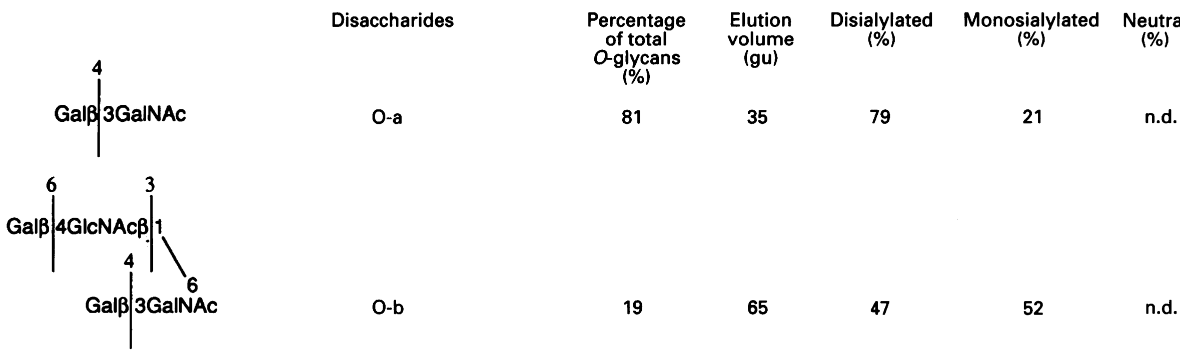

Figure 9 Structures of the neutral and desialylated N-linked oligosaccharides from normal human IgA1 fractionated by P-4 chromatography

Analysis of individual fractions used a combination of sequential exoglycosidase digestion and P-4 gel-permeation chromatography. Changes in the hydrodynamic volume of oligosaccharides were effected by exoglycosidases when used in the following order: oligosaccharide A-a, 3-4-5-3-1-7-8-3; oligosaccharide A-b, 3-4-5-1-7-8-3; oligosaccharide A-c, 3-4-5-7-8-3; oligosaccharide A-d, 3-6-5-3-7-8-3; oligosaccharide A-e, 3-7-4-3-7-8-3; oligosaccharide A-f, 6-5-7-8-3; oligosaccharide A-g, 5-7-8-3; oligosaccharide A-h, 3-2-3-7-8-3, also 2-5-3-7-8-3; oligosaccharide A-i, 3-9-2-5-3-7-8-3, also 3-2-9-2-5-3-7-8-3; oligosaccharide O-a, 4; oligosaccharide O-b, 3-6-3-4. 1, *Choronia lampus* α-fucosidase; 2, jack-bean β-galactosidase; 3, jack-bean β-*N*-acetylhexosaminidase; 4, bovine testes β-galactosidase; 5, *S. pneumoniae* β-*N*-acetylhexosaminidase; 6, *S. pneumoniae* β-galactosidase; 7, jack-bean α-mannosidase; 8, *Achatina fulica* β-mannosidase; 9, almond emulsion α-fucosidase III.

7.5 gu structure (10%)] were digested with jack-bean α-mannosidase followed by snail β-mannosidase followed by jack-bean β-*N*-acetylhexosaminidase. The final products eluted at 3.5 gu (Fucα6GlcNAcOT) and 2.5 gu (GlcNAcOT) were subjected to either chemical or enzymic defucosylation. The 3.5 gu structure gave a single product at 2.5 gu, confirming that the reducing terminal, GlcNAc, was substituted with the fucose residue.

Cores V, VI, VII and VIII

To confirm the ratios of fucosylated (F) and bisected (B) oligosaccharides, fractions V–VIII (Figure 8) were pooled and incubated with a mixture of *S. pneumoniae* β-galactosidase and *S. pneumoniae* β-*N*-acetylhexosaminidase. Four digestion products were found corresponding to the four cores [+B +F (22%), +B −F (19%), −B +F (17%) and −B −F (42%)]. The results are listed in Table 1. Oligosaccharides eluted between 13 and 15 gu were also digested with a mixture of jack-bean β-galactosidase and jack-bean β-*N*-acetylhexosaminidase, yielding equivalent amounts of Manα6(Manα3)Manβ4GlcNAcβ4GlcNAcOT (7.2 gu) and Manα6(Manα3)Manβ4GlcNAcβ4(Fucα6)-GlcNAcOT (8.5 gu).

Core III

Fraction III was digested with a mixture of bovine testes β-galactosidase and jack-bean β-hexosaminidase. The major products were eluted at 8.2 and 7.2 gu, indicating the presence of both −B −F and −B +F cores. A partially digested structure was eluted at 11.5 gu. This structure would be derived from outer-arm fucose-containing oligosaccharides.

Determination of sialic acid linkages and the presence of the bisecting GlcNAc residues by n.m.r. spectroscopy

One-dimensional ^{1}H-n.m.r. was performed on fraction A (Figure 3b). The relative intensities of resonances at 1.7 p.p.m. and 1.80 p.p.m. for the axial sialic acid protons and at 2.67 p.p.m. and 2.76 p.p.m. for the equatorial protons of sialic acid indicated that 90% of the sialic acid present in this fraction was in 2α6 linkage to galactose and 10% was 2α3-linked sialic acid (results not shown). ^{1}H-n.m.r. of the monosialylated *O*-glycans from fraction C (Figure 3b) showed only 2α3 linkages (results not shown). One-dimensional n.m.r. spectroscopy of pooled asialo *N*-glycans showed that essentially all of the N-linked oligosaccharides were of the biantennary complex type (Homans et al., 1984). From the chemical shifts and the *J*1, 2 couplings observed, the anomericity of the residues in the intact glycans could be assigned as follows: galactose and GlcNAc were found as the β anomers [H1 chemical shifts at 4.45 and 4.46 p.p.m. for Gal 6,6′, 4.56 and 4.57 p.p.m. for GlcNAc 5,5′ (non-bisected structures) and 4.47 p.p.m. for the bisecting GlcNAc]. Fucose was detected as the α-anomer (H1 resonance at 4.89 p.p.m.), and mannose was seen as both the α- (H1 5.15 p.p.m. Man 4, 4.92 p.p.m. Man 4′) and β- (H2 at 4.25 p.p.m.) anomers. From the intensity of the three methyl group protons at 1.21 p.p.m. the presence of core fucose was estimated at 30%, and the incidence of bisecting GlcNAc was also estimated at 30% from the ratio of the resonances of the core α-mannose residues in the presence (H1 5.05 p.p.m. Man 4 bi) (H1 4.99 p.p.m. Man 4′ bi) of the bisecting residue.

The n.m.r. spectra were consistent with the assignment of all of the cores as the pentasaccharide Manα6(Manα3)Man-β4GlcNAcβ4GlcNAcol, with fucosylation (30%) and presence of a bisecting GlcNAc (30%). These data are in good agreement with the sequential enzymic sequencing data.

Incidence of terminal galactose of paired IgG and IgA samples

It was found that 95% of the oligosaccharides associated with IgA were of the complex type, and therefore amenable to analysis with mixed exoglycosidases. Of these complex sugars, 86% were biantennary structures such as are present on IgG, and 14% were of the triantennary complex type. The IgG glycans were treated with mixed exoglycosidases, and three peaks were resolved by Bio-Gel P-4 gel filtration, as reported previously (Parekh et al., 1988b). The IgA1 *N*-glycans, subjected to the same treatment, which were resolved into five peaks by Bio-Gel P-4 gel filtration, eluted at 16.5, 13.5, 11.5 (trace), 10.5 and 7.5 gu. With

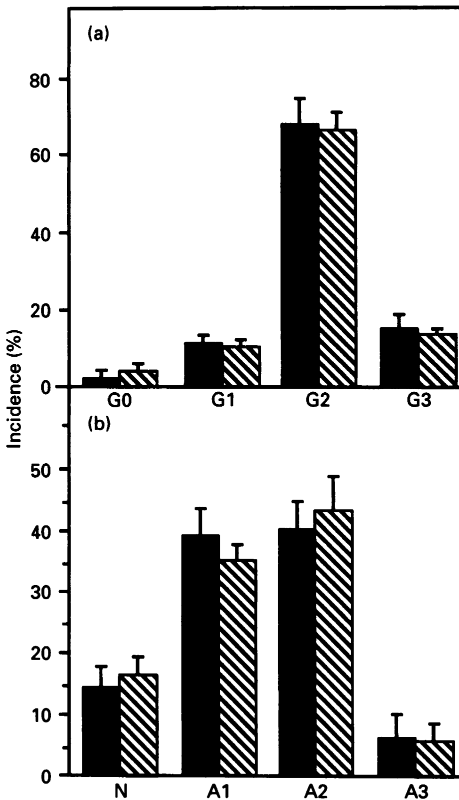

Figure 10 (a) Comparison between the incidence of G0 (no galactose), G1 (monogalactosylated), G2 (digalactosylated) and G3 (trigalactosylated) oligosaccharide structures from normal individuals ($n = 5$) (solid) and patients with RA ($n = 5$) (hatched) and (b) comparison between the incidence of neutral, A1, A2 and A3 oligosaccharide structures from normal individuals (solid) and patients with RA (hatched)

Table 2 Galactosylation of IgA1 and IgG from patients with RA

IgA1 and IgG were isolated from serum and the *N*-glycans released, purified and radiolabelled as described in the Experimental section. A portion of the glycans from each preparation was digested with a mixture of exoglycosidases to allow resolution of the species containing zero to three terminal β-galactose residues, and the products were separated on Bio-Gel P-4. Included in the table are data on the age, disease type and progression for the individuals studied and the G0 values for the IgG and IgA1 glycans.

	Age	Sex	G0 (%)* IgG	G0 (%)* IgA	Disease activity (duration)†
Patients					
P.C.	45	M	31	2.7	Relapse/remission, RF-positive, erosive (14)
M.S.	28	M	43	2.1	Chronic persistent, RF-positive, erosive (2)
C.F.	66	F	35	2.3	Slow, mild, RF-positive, non-erosive (7)
D.B.	60	F	46	2.5	Chronic persistent, RF-positive, erosive (14)
R.S.	56	F	47	2.1	Chronic persistent, RF-positive, erosive (16)
Controls					
G.T.	25	F	16	1.2	Control
J.A.	35	M	17	1.3	Control
N.O.	NA‡	NA‡	21	1.1	Control
A.D.	40	NA‡	23	1.4	Control
M.C.	25	M	25	1.6	Control

* Incidence of structures devoid of terminal galactose, given as percentage of total glycans. Data are given to two significant figures.

† Disease activity description; all patients tested positive for rheumatoid factor (RF), and were diagnosed as definite RA. Disease duration, given in parentheses, is in years.

‡ Data not available.

the exception of the 11.5 gu material, which was a mixture of a hybrid and an oligomannose oligosaccharide, the peaks were a series of diagnostic digestion fragments derived from complex glycans with three to no terminal β-galactose residues respectively.

The average percentage of G0 glycans from IgG and IgA1 from the control group of individuals was 20.4% and 1.3% respectively; the corresponding averages from the patients with RA were 40.4% and 2.3% respectively (Table 2 and Figure 10a). An estimate of the charge distribution of the glycans of IgA from normal and RA patients was determined using Mono Q Sepharose; no differences were found (the results are summarized in Figure 10b).

DISCUSSION

Glycosylation of normal human serum IgA compared with IgG

The data in this report demonstrate that more than 95% of the N-linked carbohydrate moieties of human serum IgA1 were of the complex-type (Figure 9). Biantennary oligosaccharides accounted for 86% of the N-linked glycans on IgA1 and 14% of the oligosaccharides were multiantennary or extended. A triantennary glycan accounted for about 7% of the total N-linked carbohydrate. Less than 2% of structures recovered from IgA1 were asialo/agalacto. As most glycans are fully substituted with galactose, the heterogeneity in IgA1 glycosylation was considerably less than in IgG. This is interesting because IgA1 has two conserved sequons in each heavy chain; site-specific glycan processing might be expected to result in increased heterogeneity of IgA compared with IgG, which has only one. All four of the usual types of core substitution (+fucose, ±bisecting GlcNAc) were detected (see Table 1): 41% of biantennary structures were core fucosylated, and 40% contained bisecting GlcNAc. Compared with IgG (78%, Fc fragment), the incidence of core fucose was low. By contrast, the prevalence of the bisecting GlcNAc residue was higher for IgA1 than for IgG (13%, Fc fragment). The incidence of different structures was remarkably constant between individuals (Table 3 and Figure 10).

Comparison with other immunoglobulins

IgM, IgD, IgE

No evidence was found for significant levels of oligomannose glycans (i.e. > 5%), which have been reported for human IgM, IgD and IgE (Chapman and Kornfeld, 1979; Ishihara et al., 1983; Rearick et al., 1983).

Myeloma IgA

These data are in general agreement with an earlier study where *N*-glycans from an IgA1 myeloma protein were analysed (Baenziger and Kornfeld, 1974a), although the glycosylation of IgA1 from normal human serum was more heterogeneous than that reported for the myeloma protein. The two structures found on the myeloma protein correspond to oligosaccharides A-c (monosialylated) and A-d (disialylated) (Figure 9). In the present

Table 3 Conservation of IgA1 glycosylation pattern between individuals

Analysis of the IgA1 glycan structure by determination of galactose and sialic acid levels was performed as described in the text. Data are shown as percentage incidence of total, together with the S.D. and mean for each parameter. Structures are described in the text. IgA1 glycans were isolated from sera obtained from individuals (initials given) without RA. ND, Not determined.

	Individual						
Structure	M.C.	J.A.	A.D.	G.T.	R.G.	A.M.	Mean ± S.D.
N	10.7	11.4	10.5	17.1	15.6	18.8	14.0 ± 3.6
A1	44.1	40.5	34.3	40.8	44.8	30.9	39.2 ± 5.5
A2	39.6	41.7	47.0	36.0	33.5	44.9	40.5 ± 5.2
A3	5.6	6.4	8.2	6.1	6.1	5.6	6.3 ± 1.0
G0	1.6	1.3	1.4	1.2	ND	3.3	1.8 ± 0.8
G1	8.9	9.2	12.5	13.3	ND	11.8	11.1 ± 2.0
G2	71.3	73.2	66.7	55.7	ND	68.2	67.0 ± 6.8
G3	12.7	11.2	13.0	21.4	ND	11.4	13.9 ± 4.7

study, disialylated A-b (19%) and disialylated A-c (24%) were the major species and only 3% of structure A-d was detected.

Secretory IgA

N-Glycans of both secretory IgA (sIgA) and secretory component from human milk are biantennary complex-type oligosaccharides (Mizoguchi et al., 1982; Pierce-Cretel et al., 1982, 1984). In contrast with serum IgA, the acidic oligosaccharides of sIgA are not bisected (Pierce-Cretel et al., 1982). Three extensions of the basic biantennary structure were found on sIgA: a fucose in α1-3 linkage to GlcNAc-5′, a galactose residue in β1-3 linkage to the terminal Gal on the Gal 6′ and a fucose in α1-6 linkage to GlcNAc-5′. Both neutral biantennary *N*-acetyl-lactosamine-type and extended biantennary *N*-acetyl-lactosamine-type structures extended by additional fucosyl *N*-acetyl-lactosamine-type structures are present on sIgA (Pierce-Cretel et al., 1982), some of which may be derived from the secretory component (Mizoguchi et al., 1982). The presence of fucosyl-*N*-acetyl-lactosamine structures on serum IgA1, as reported here, suggests that either serum contains an appreciable quantity of sIgA or serum IgA1 has some oligosaccharide structural characteristics in common with sIgA. However, the presence of a trisialylated triantennary structure on serum IgA1 and the recovery of oligosaccharides containing the fucosyl-*N*-acetyl-lactosamine group in a sialic acid-containing fraction, rather than in the neutral fraction as for sIgA, suggests a unique glycosylation pattern for serum IgA1. Although we have not been able to establish whether the sialyl α(2-3) Lewis X group is present, its occurrence on IgA1 would potentially give IgA a unique effector function amongst the immunoglobulins.

Serum IgA O-linked structures

We have previously reported that approx. 64% of *O*-glycans released by β-elimination from normal serum IgA were sialylated (Field et al., 1989) and 19% were the monosaccharide GalNAc. However, the predominant structure present (54%) was NeuNAc2α3Galβ3GalNAc, and 10% of the disialylated species was also recovered. In the present study, the sugars were released by hydrazine at 85 °C, and only sialylated O-linked structures were recovered; 64% were NeuAc2α3Galβ3(NeuAc2α6)GalNAc and 9% were the disialylated form of Galβ4GlcNAc β6(Galβ3)GalNAc. In our experience the O-linked structures recovered from IgA depend on the methods used to release and purify the oligosaccharides. The pattern of O-linked glycosylation reported here is more similar to that found on the hinge region of SIgA obtained from human milk (Pierce-Cretel et al., 1981) than to the IgA1 myeloma in which Baenziger and Kornfeld (1974b) reported the presence of only the neutral Galβ3GalNAc and GalNAc oligosaccharides.

IgA and RA

Increased levels of incompletely galactosylated IgG are observed in a restricted group of diseases (Parekh et al., 1989a), including juvenile (Parekh et al., 1988b) and adult (Parekh et al., 1985) RA. *N*-Glycans from five normal and five RA IgA1 serum samples were analysed for similar RA-associated alterations in glycosylation. The incidence of glycans terminating in 0–3 β-galactose residues (G0, G1, G2, G3) was remarkably constant, despite a wide variation in the galactosylation of IgG isolated from the same serum (Table 2). The incidence of galactosylation is much greater for IgA1 glycans than for IgG glycans. For the entire study group, IgA1 G0 varied between 1.1 and 2.7%, whereas the IgG G0 varied between 16 and 47%. We note that on a percentage basis the increase in G0 for IgA between normal (1.3%) and RA (2.3%) is essentially the same as for the increase in G0 found for IgG between normal (20%) and RA (40%). Although it cannot be ruled out that there may be a small percentage of IgA clones that secrete G0 IgA and that the number of these clones has doubled in patients with RA, it seems unlikely that a 1% increase in G0 IgA in patients with RA would be of physiological significance.

The lower levels of IgG galactosylation associated with RA may result from decreased levels of galactosyltransferase (Axford et al., 1992). Given the high levels of fully galactosylated glycans associated with IgA1, this effect would be expected to be much more pronounced. That it is not supports the conclusion that the alteration in galactosyltransferase levels is restricted to those cells making IgG, or some other mechanism is involved.

The conformation of the IgG Fc polypeptide may play an important role in regulating the processing of *N*-glycans (Schachter, 1986; Lee et al., 1990). Crystallographic studies have shown that the two CH2 domains in IgG do not form extensive lateral associations. The resulting interstitial region accommodates the complex oligosaccharides which are attached to Asn-297 on each heavy chain such that one of the α-(1,3) antennae interacts with the trimannosyl core of the opposing oligosaccharide. The α(1-6) antennae interact with hydrophobic and polar residues on the domain surface. It has been proposed that this restricts the addition of both galactose and sialic acid to the Fc oligosaccharides and is supported by the observation that glycans associated with the Fab fragment of human IgG are more sialylated and galactosylated than the oligosaccharides associated with the Fc fragment (Rademacher et al., 1986). In the case of IgA1, there are two sequons positioned in the Fc, at Cα2 (Asn-263) and Cα3 (Asn-459), neither of which is at a position comparable with the site on IgG (Young et al., 1990). The glycosylation of IgA1 therefore resembles more closely IgG Fab glycosylation (bisect 67%) than IgG Fc glycosylation (bisect 13%).

Alternatively, the biosynthetic apparatus may generate a similar range of glycoforms for both immunoglobulins, but because the IgA1 oligosaccharides are fully exposed, glycoforms bearing terminal galactose or GlcNAc may be removed from the circulation at an accelerated rate compared with either the sialylated IgA1 glycoforms or IgG. Interestingly, serum IgA is catabolized five times faster than IgG (Kerr, 1990). If the IgG

glycans are partially inaccessible to macromolecular ligands by being sterically shielded, then IgG carrying incompletely substituted glycans may evade regulatory mechanisms that *in vivo* are responsible for either preventing secretion or for removing such glycoproteins from the circulation (Wilson et al., 1993). In the case of IgA1, the positions of the sequons suggest that the glycans are not shielded by the polypeptide (Young et al., 1990), and the high incidence of both sialic acid and galactose on these glycans is strong evidence that there is no restriction to the access of the relevant glycan-processing enzymes (Shao et al., 1987). This suggests that the oligosaccharides are potential ligands, and therefore more complete construction of the IgA1 oligosaccharides may not only be possible but necessary to ensure efficient secretion, a reasonable serum half-life and to prevent autoimmune stimulation which may result from the exposure of unusual oligosaccharide determinants.

It will be interesting to determine whether IgA1 glycosylation is altered in other disease states, such as those in which IgG glycosylation has been shown to alter (e.g. tuberculosis or Crohn's disease) or where there is no evidence for an IgG-related disorder (e.g. IgA nephropathy).

Note added in proof (Received 8 February 1994)

Since submission of this manuscript Boren et al. (1993) have reported that the H-2 antigen (terminal Fucα1-2Gal) is present on serum IgA. This conclusion was drawn from the observation that serum IgA binds *Ulex europaeus* type-1 lectin on a Western blot. We do not report the presence of either Lewis^y (Le^y) (contains Fucα1-2Gal and Fucα1-3GlcNAc) or H-2. Our results do not exclude the possibility that these glycans are present, but suggest that they are at very low levels. Le^y would elute in fractions I and II (Figure 8), which contained very low amounts of oligosaccharides. H-2 would elute in fraction III and was not observed, suggesting that it exists at levels lower than the limits of detection of this technique.

Also in this paper we report that serum IgA contains low amounts of Lewis^x (Le^x) (Fucα1-3GlcNAc). This is compatible with the observation by Boren et al. (1993) that IgA recognizes *Aleuria aurantia* lectin as in addition to binding core fucose this lectin also binds weakly to outer-arm fucose residues (Furukawa and Kobata, 1993).

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