Site-specific glycosylation of human immunoglobulin G is altered in four rheumatoid arthritis patients

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Alterations in the glycosylation of human IgG have been shown to occur in rheumatoid arthritis (RA). However, the precise nature and location of these changes have not been fully established. Therefore we carried out a detailed analysis of the oligosaccharides chemically released from intact human serum IgG and fragments of the molecule. Serum samples were from three healthy ('normal') individuals, and from four patients with RA. Site-specific glycosylation of the glycoprotein was shown to occur, which extended to sites even within the Fab fragment. There were differences in galactosylation, sialylation and the presence of a bisecting *N*-acetylglucosamine. Disease-related alterations were also shown to be site-specific. In particular, an increase in the proportion of agalactosylated oligosaccharides occurred on the Fc fragment in RA ($P = 0.057$), but, in contrast to previous reports, there was an increase on the light chain in the proportion of fully galactosylated, bisected and core fucosylated oligosaccharides (from 13% of total in normal to between 18 and 35% in RA, $P = 0.057$)). There was also an Fab-specific increase in oligosaccharides bearing a bisecting *N*-acetylglucosamine and a core fucose ($P = 0.057$). The site-specific glycosylation changes described in this paper reveal the complexity of the regulatory mechanism, perhaps reflecting the many levels at which regulation can occur.

INTRODUCTION

Human IgG is a glycoprotein with 2.8 ± 0.4 mol of oligosaccharide per mol of IgG [1]. Most of the carbohydrate is present on the Fc fragment (2.0 mol/mol of Fc) at a conserved glycosylation site (Asn-297) whereas the remaining sugar occurs at variable positions within Fab fragments. The distribution of oligosaccharides between the Fd and light (L) chain portions of the Fab fragment has not been determined. Extensive microheterogeneity in the oligosaccharides present on IgG is observed; more than 30 structures of the complex, biantennary type have been identified [2]. The heterogeneity arises from: variations in outer-arm sialic acid and galactose content; the presence or absence of a bisecting GlcNAc, or of a core-linked fucose. The occurrence of these different oligosaccharides is not the result of performing the analysis on polyclonal IgG, because heterogeneity is also found on human myeloma proteins [3].

The incidence of the various oligosaccharide structures is age-related [4] and alters during pregnancy and disease. In pregnancy, there is a marked decrease in the percentage of oligosaccharides lacking outer-arm galactose residues [G(0)] [5,6] whereas in rheumatoid arthritis (RA), juvenile arthritis, Crohn's disease, systemic lupus erythematosus with Sjogren's syndrome, tuberculosis, erythema nodosum leprosum and Lyme disease-associated arthritis, the percentage of G(0) structures increases [2,5,7–12]. In RA, changes in the percentage of G(0) have been shown to correlate with disease activity; there is a decrease during remission and an increase at onset [6,9]. An increase in the percentage of G(0) in serum IgG has been shown to be directly associated with pathogenicity in a mouse model of RA [13], although its role in the pathophysiology of RA remains unclear. A compositional analysis demonstrated an increase in the amount of GlcNAc in RA [5] but this increase was not detected in earlier sequencing studies [2,8]. A small decrease in sialylation of oligosaccharides has been reported for rheumatoid serum IgG [2], but this has not been observed by others [5,8]. Previous studies suggested that the decrease in outer-arm galactosylation associated with RA is restricted to the Fc fragment [14,15], although unspecified disease-related alterations on the Fab fragment were also reported [15]. These changes may be restricted to the heavy (H) chain portion of the molecule [15].

The aim of this paper is to perform a site-specific glycosylation analysis of both normal and RA serum IgG in order to locate on the molecule the glycosylation changes that occur in RA. We have determined the incidence of sialylated oligosaccharides, and of the 12 most abundant structures after desialylation, from normal and RA serum IgG and the Fab, Fc, H-chain and L-chain fragments. Our results indicate that the sugars present at different sites on human IgG are dissimilar in sialylation, galactosylation and bisecting GlcNAc; that in RA, in contrast with previous reports, changes in galactosylation occur at all sites, although not to the same degree; and that in RA there is an Fab fragment-specific increase in oligosaccharides with a bisecting GlcNAc and core fucose.

MATERIALS AND METHODS

Materials

Protein G–Sepharose was obtained from Pharmacia Ltd. (Milton Keynes, Bucks., U.K.). *Aleuria aurantia* lectin (AAL) (obtained from Boehringer Mannheim, Lewes, Sussex, U.K.) was coupled to Affigel-10 (purchased from BioRad Lab Ltd., Watford, Herts., U.K.) according to the manufacturer's instructions. RCA 120-

Abbreviations used: AAL, *Aleuria aurantia* lectin; B, bisecting GlcNAc; Con A, concanavalin A; F, core fucose; G(0), G(1), G(2), outer-arm galactose residues; GalTase, β1-4 galactosyltransferase; g.u., glucose units; H, heavy; L, light; RA, rheumatoid arthritis; RCA-120, *Ricinus communis* agglutinin 1.

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Agarose was from Vector Laboratories (Peterborough, Cambs., U.K.). Con A–Sepharose, papain, and alkaline phosphatase-conjugated antibodies cross-adsorbed with human serum were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Affinity-purified Fab and Fc fragment specific antibodies (goat) were obtained from ICN Flow (High Wycombe, Bucks., U.K.), and affinity-purified H- and L-chain specific antibodies (rabbit) were from Dakopatts Ltd. (High Wycombe, Bucks, U.K.). Neuraminidase (*Arthrobacter ureafaciens*) was from Oxford Glycosystems (Abingdon, Oxon., U.K.) and the radiolabelled alditols used as standards were either from Oxford Glycosystems or were prepared enzymically.

Purification of IgG

Serum samples were diluted 1:1 with 0.1 M sodium phosphate, pH 7.2, and applied at a flow rate of 0.8 ml/min to a Protein G–Sepharose column (10 cm × 0.5 cm) equilibrated with the phosphate buffer. The column was washed until the A_{280} of the eluate was zero and then eluted with 0.1 M glycine/HCl, pH 2.7. The IgG samples were immediately neutralized with 1 M Tris to pH 7.

Isolation of Fab and Fc fragments

Papain was activated by pre-incubation at 37 °C for 30 min with 10 mM L-cysteine in 0.2 M Tris/HCl, pH 7.5, containing 4 mM EDTA. The papain was desalted into 100 mM Tris/HCl, pH 7.5, with 2 mM EDTA by using a PD-10 column (Pharmacia) before use. IgG samples (40 mg) were incubated with 2 % (w/w) activated papain for 1 h at 37 °C in 100 mM Tris/HCl, pH 7.5, containing 2 mM EDTA. Under these conditions cleavage was specific to the hinge region of IgG, giving Fab and Fc fragments. However, only about 67 % of normal IgG was digested, whereas about 90 % of RA IgG was cleaved. Papain was inactivated by addition of iodoacetic acid to a concentration of 10 mM. The mixtures were applied to a Protein G–Sepharose column (10 cm × 0.5 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 1 M guanidine hydrochloride at a flow rate of 0.8 ml/min. The column was washed until the A_{280} of the eluate was zero and then eluted with 0.1 M glycine/HCl, pH 2.7. Unbound and bound pools were concentrated by ultrafiltration (Amicon YM10 membrane) and fragments purified by gel filtration with a TSK SW3000G column (60 cm × 21 mm) equilibrated with 0.25 M sodium acetate, pH 6.0, at a flow rate of 4 ml/min. Fab fragments were passed again through the Protein G–Sepharose and TSK columns.

Isolation of heavy and light chains

Freeze-dried IgG samples (40 mg) were dissolved in 2 ml of 0.4 M Tris/HCl, pH 8.6, containing 6 M guanidine hydrochloride and 2 mM EDTA. The samples were flushed with argon and incubated at 50 °C for 1 h. Dithiothreitol (72 mg) was added, the samples were flushed again with argon and incubated at 50 °C for 3 h. After cooling to 25 °C, 200 mg of iodoacetic acid in 2 ml of 0.4 M Tris/HCl, pH 7.5, containing 6 M guanidine hydrochloride was added. The pH was maintained at 8.6 for 5 min at 25 °C by the addition of NaOH, and then the samples were incubated for a further 15 min at 50 °C. Samples were applied to a TSK G3000SW column (60 cm × 21 mm) equilibrated in 0.4 M sodium phosphate buffer, pH 7.0, containing 5 M guanidine hydrochloride at a flow rate of 2 ml/min. L-chains were reapplied to the TSK column.

SDS/PAGE

Samples were analysed with a Phast System (Pharmacia) on 10–15 % (w/v) polyacrylamide gels.

Western blotting

Polypeptides were transferred from polyacrylamide gels to nitrocellulose by electroblotting with a Phast Transfer system. The nitrocellulose was incubated with 3 % (w/v) BSA in PBS for 2 h. Specific antisera were added and the incubation was continued for a further 1 h. After being washed three times with PBS the nitrocellulose was incubated with the appropriate secondary antibody conjugated with alkaline phosphatase for 1 h. The nitrocellulose was washed three times with PBS, and once with alkaline phosphatase buffer (0.1 M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH to 10.4 with NaOH) before adding 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium in alkaline phosphatase buffer to reveal the bound antibodies.

Storage

Samples were stored at −20 °C for up to one year.

Release and analysis of N-linked oligosaccharides

Hydrazinolysis, isolation of oligosaccharides and their subsequent reduction with tritiated sodium borohydride, high-voltage paper electrophoresis, desialylation and gel filtration with Bio-Gel P4 were all performed as previously described [16,17].

Fractionation of radiolabelled alditols with concanavalin A (Con A) was based on previously described methods [18,19] with the following modifications. Con A–Sepharose columns (2.5 cm × 0.5 cm) were washed sequentially with 5 ml of 0.1 M HCl and 5 ml of 10 mM sodium acetate buffer, pH 5.0, containing 5 mM $MnCl_2$ and 5 mM $CaCl_2$, and finally equilibrated with 10 mM sodium acetate, pH 5.0, at a flow rate of 0.1 ml/min. Samples were applied to the columns, which were then washed with 5 ml of 10 mM sodium acetate, pH 5.0, containing 100 mM NaCl and then eluted with 10 ml of 100 mM HCl. The unbound and bound pools were desalted by passage through tandem columns of Dowex AG50 and AG3 before drying under vacuum. Fractionation of radiolabelled alditols by using AAL and RCA 120 was based on previously described methods [20,21]. The pools were desalted as before. HPLC separation of radiolabelled alditols was performed on a Micropak AX5 column (Varian, 25 cm × 0.46 cm) with an acetonitrile/water mobile phase [22]. The flow rate was 1 ml/min and the gradient was 30–45 % water over 90 min with a further 15 min at 45 % water before re-equilibration at 30 % water. All of these analytical methods were validated with appropriate oligosaccharide standards and were highly reproducible.

Statistical analysis

Two-tailed Mann–Whitney *U*-tests were performed with software provided by SPSS U.K. Ltd. (Chertsey, Surrey, U.K.)

RESULTS

Purity of IgG and fragments

For this study IgG and its fragments were isolated from seven serum samples; four were from patients with RA (RA1–RA4),

Table 1 Clinical details

(a) Patients*

Sample	Sex	Age (years)	Disease activity†	Rheumatoid factor status	Serum concentration of IgG (mg/ml)
RA1	Female	58	Moderate	Positive	9.3
RA2	Male	67	Moderate	Negative	5.2
RA3	Female	52	Severe	Positive	12
RA4	Male	60	Moderate	Positive	8.0

(b) Controls

Sample	Sex	Age (years)	Serum concentration of IgG (mg/ml)
N1	Male	52	8.0
N2	Female	58	7.2
N3	Male	68	4.9

* All the patients fulfilled the American College of Rheumatology's revised criteria for the classification of RA [43].
† The disease activity in these patients was assessed according to a previously published index [44].

and three from healthy (' normal') adults (N1–N3). The normal and RA samples were age-matched. All the RA patients showed features typical of the disease, and their clinical details are given in Table 1. The purity of the samples was assessed by SDS/PAGE and Western blotting (Figures 1 and 2). For the IgG samples, in addition to the expected polypeptides corresponding in molecular mass to H- and L-chains, polypeptides of high molecular mass are present on the overloaded Coomassie Blue-stained gels. These polypeptides react with γ-chain specific antibodies indicating that they are incompletely reduced IgG molecules. The Coomassie Blue-stained gels of the fragments of IgG all have present as the predominant species polypeptides of the expected molecular masses. Minor components are visible in each sample of a fragment but in all cases these react with specific antibodies to that fragment (results not shown). To confirm the purity of the fragments, Western blots with specific antibodies to the major possible contaminant were performed. For the Fc fragment samples contamination is less than 5%; in all the other samples contamination is less than 2%.

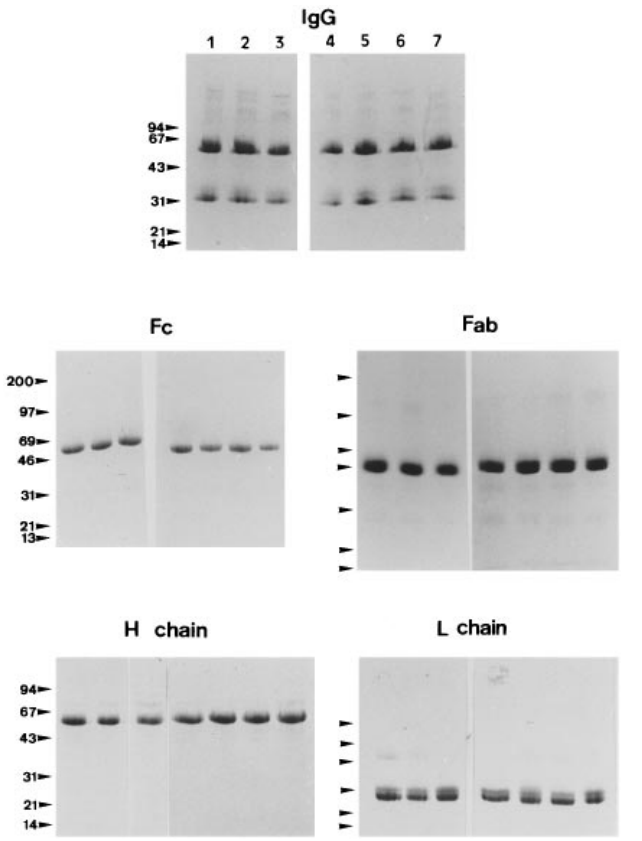

Figure 1 SDS/PAGE analysis of IgG and fragments

Samples (1 μg) were analysed on 10–15% polyacrylamide gels stained with Coomassie Blue. The IgG samples were reduced; the others were not. The positions and masses (in kDa) of standards are indicated. Lanes 1–7 (left to right in all cases): N1, N2, N3, RA1, RA2, RA3, RA4.

Oligosaccharide analysis

The scheme presented in Figure 3 was followed to analyse the glycosylation of IgG and its fragments. After sugar release and radiolabelling, a small amount (5%) of each sample was used to determine the percentage incidence of neutral, monosialylated and disialylated oligosaccharides by high-voltage electrophoresis. The electrophoretograms of samples N1 and RA1 are given in Figure 4, and the results of this analysis for all the samples are presented in Table 2. After digestion of the remaining sugars with neuraminidase, they were applied to Bio-Gel P4 gel-filtration columns to determine the hydrodynamic volumes of the sugars present (results not shown). The chromatograms of some of the H-chain samples had a peak at 11.2 glucose units (g.u.), which was a major component unlike the corresponding IgG sample chromatogram, which had only a trace amount of the 11.2 g.u. peak. Because H-chains are heavily glycosylated and contribute the majority of the sugar present on IgG, the large amount of the 11.2 g.u. peak on the chromatogram of the H-chain compared with IgG was unexpected. This peak was isolated and assigned by sequential exoglycosidase digestion as GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)X, where X is a sugar with a hydrodynamic volume of 1.0 or 1.5 g.u. (results not shown). Further analysis by gas chromatography demonstrated that the retention times of these sugars differ from

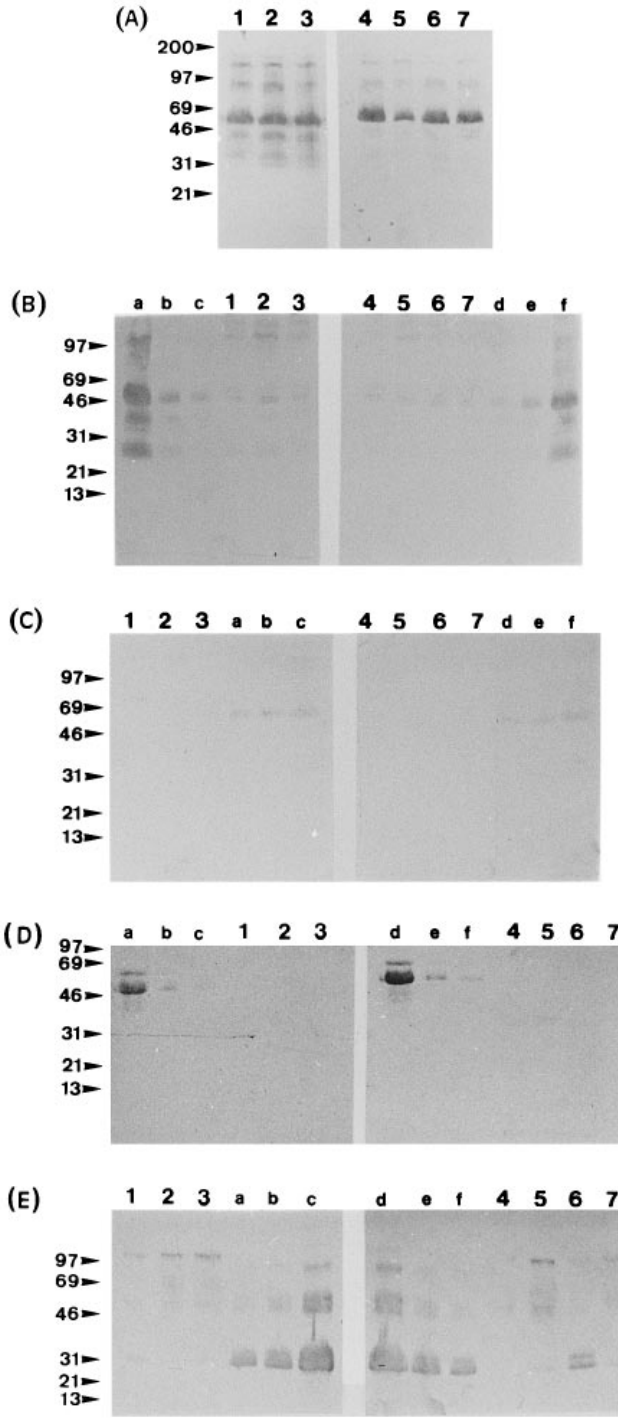

Figure 2 Western blot analysis of IgG and fragments

Samples (lanes 1–3, N1–3; lanes 4–7, RA 1–4) of 1 μg in all cases were analysed after electrophoresis on 10–15% polyacrylamide gels under reducing (**A**) or non-reducing (**B**–**E**) conditions. The positions and masses (in kDa) of standards are indicated. Positive controls are shown in lanes a–f for each primary antibody used. (**A**) IgG incubated with anti-γ. (**B**) Fc fragments incubated with anti-Fab. Lanes a and f, 1 μg of Fab fragments; lanes b and e, 0.05 μg; lanes c and d, 0.02 μg. (**C**) Fab fragments incubated with anti-Fc. Lanes a and d, 0.02 μg of Fc fragments; lanes b and e, 0.05 μg; lanes c and f, 0.2 μg. (**D**) L-chains incubated with anti-γ. Lanes a and d, 1 μg of H-chains; lanes b and e, 0.05 μg; lanes c and f, 0.02 μg. (**E**) H-chains incubated with anti-κ and anti-λ. Lanes a and f, 0.02 μg of L-chains; lanes b and e, 0.05 μg; lanes c and d, 1 μg.

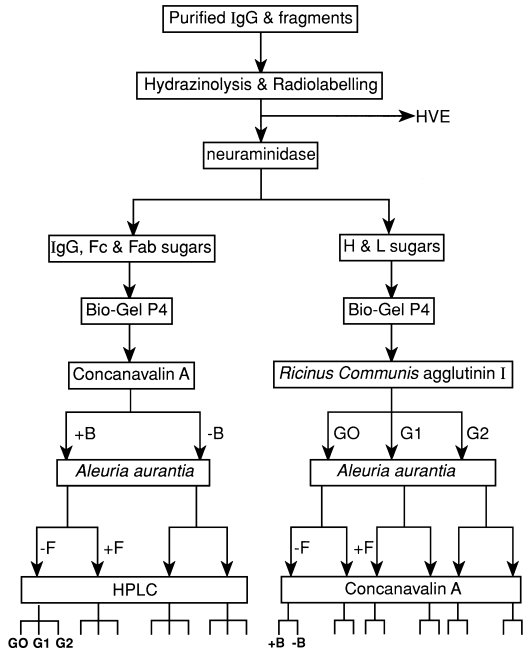

Figure 3 Scheme followed to analyse glycosylation of IgG and fragments

+/— B, sugars with and without a bisecting GlcNAc; +/— F, sugars with and without a core fucose; G0, G1, G2, sugars with zero, one or two outer-arm galactose residues.

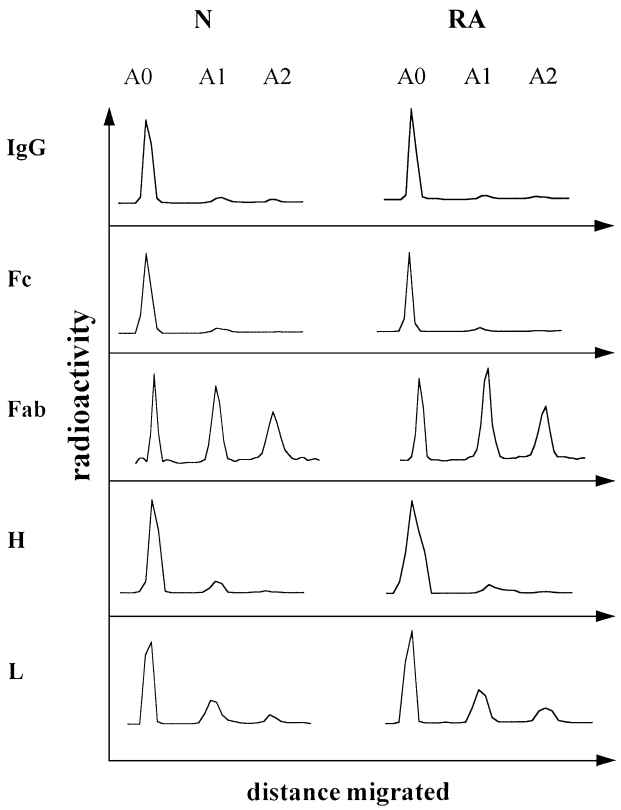

Figure 4 High-voltage electrophoresis of sugars released from IgG and its fragments

A0, neutral sugars; A1, monosialylated; A2, disialylated.

Table 2 Percentage incidence of sialylated oligosaccharides on IgG and its fragments

Oligosaccharides were determined as neutral (A0), monosialylated (A1) or disialylated (A2) by high-voltage electrophoresis.

	IgG			Fc			Fab			H			L		
Sample	A0	A1	A2	A0	A1	A2	A0	A1	A2	A0	A1	A2	A0	A1	A2
N1	81	13	6	88	12	0	27	40	33	85	13	2	60	28	12
N2	77	19	4	87	13	0	34	37	29	84	13	3	78	15	7
N3	69	24	7	84	16	0	25	38	37	83	14	3	63	22	15
RA1	83	12	5	92	8	0	28	46	27	82	14	4	53	30	17
RA2	83	10	7	87	13	0	30	37	33	71	20	9	49	35	16
RA3	84	9	7	96	4	0	37	40	23	83	13	4	63	23	13
RA4	84	10	5	94	6	0	25	42	34	88	9	3	48	26	27

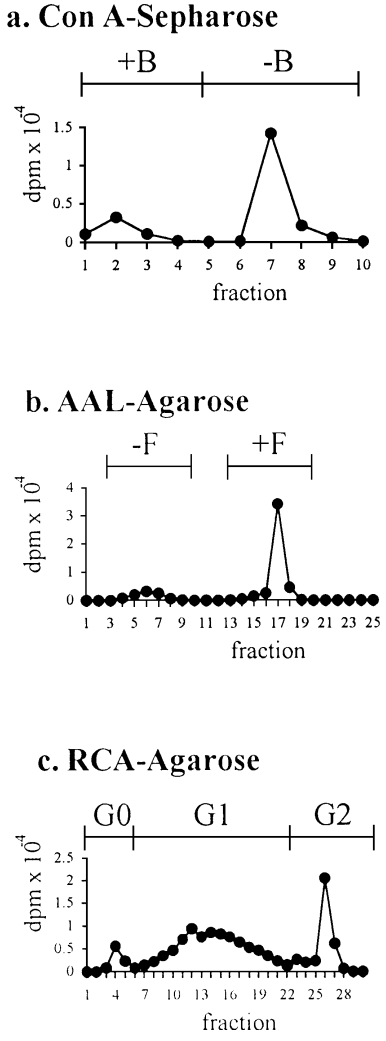

Figure 5 Lectin affinity chromatography

Pooled fractions containing sugars with or without (+/−) a bisecting GlcNAc (B), a core fucose (F), and zero, one or two outer-arm galactose residues (G) are presented in (**a**), (**b**) and (**c**) respectively.

N-acetylglucosaminitol (results not shown). It appears that during sugar release for some of the H- and L-chain samples an abnormal reducing terminus was formed. No other artefacts caused by the hydrazinolysis procedure were detected on the chromatograms; for example, the common problem of loss of monosaccharides from the reducing terminus (known as peeling) did not occur because peaks at less than 11.2 g.u. were not present.

Because of this problem two schemes were used to determine the percentage incidence of the 12 most abundant asialo-oligosaccharides present on IgG and its fragments. For intact IgG and the Fc and Fab fragments, the asialo-oligosaccharides were subjected to lectin affinity chromatography: first on Con A to fractionate on the basis of the presence or absence of a bisecting GlcNAc, followed by AAL to differentiate on the basis of core fucosylation. Sugars were separated further into those carrying none, one or two outer-arm galactose residues by normal-phase HPLC with a Micropak AX5 column. For the H- and L-chain sugars a different scheme was used because the altered reducing terminus of some of the H- and L-chain sugars precluded the use of the HPLC separation. The new scheme comprised a series of lectins, beginning with RCA 120 lectin, which fractionated according to the number of outer-arm galactose residues, followed by AAL and Con A. Neither of these schemes could differentiate the arm specificity for those sugars bearing a single galactose. All the techniques were validated for their ability to bind appropriate sugars, particularly with regard to those structures with the altered reducing terminus (results not shown). The resolution of mixtures of standards by each lectin column is given in Figure 5, and the reproducibility and recovery data for each column are presented in Table 3. The results of the lectin affinity chromatography of sugars released from IgG, Fab and Fc fragments are presented in Table 4. Chromatograms of the normal-phase separation of sugars with each of the four possible core structures for samples N1 and RA1 are given in Figure 6. The results of this analysis for all the IgG, Fab and Fc fragments are presented in Table 5. The overall percentage incidence of sugars present on all the IgG, Fab and Fc fragment samples is given in Table 6, which also presents the lectin affinity chromatography data for all the H- and L-chain samples.

Site-specific glycosylation of normal IgG

The sialylation of the various fragments of IgG is dissimilar: Fc fragment and H-chain sugars are predominantly neutral, Fab fragment sugars are mainly sialylated, whereas L-chain sugars have an intermediate level of sialylation (Table 2). The difference in sialylation between Fab fragments and L-chains indicates that Fd fragment sugars must be largely sialylated. This is supported by the presence of disialylated sugars on H-chains but not Fc fragments.

Table 3 Reproducibility of and recovery from lectin affinity chromatography

	Percentage of radiolabel recovered									
	Con A–Sepharose*			AAL–agarose†			RCA–agarose‡			
Experiment	+B	−B	Overall	−F	+F	Overall	G0	G1	G2	Overall
1	22	78	100	19	81	95	9	38	53	94
2	23	77	92	19	81	98	9	37	54	102
3	24	76	96	–	–	–	10	39	51	90

* Sugars with and without (+/−) a bisecting GlcNAc (B).
† Sugars with and without (+/−) a core fucose (F).
‡ Sugars with zero, one or two outer-arm galactose residues (G).

Table 4 Percentage incidence of core structures on IgG and its fragments

	+B+F*	+B−F	−B+F	−B−F
IgG				
N1	11	2	80	7
N2	12	2	77	9
N3	12	4	77	7
RA1	16	3	76	5
RA2	13	4	76	7
RA3	13	2	80	5
RA4	13	2	76	9
Fc				
N1	13	1	80	6
N2	13	2	79	6
N3	12	2	75	11
RA1	9	1	87	3
RA2	13	2	77	8
RA3	15	1	81	3
RA4	11	1	79	9
Fab				
N1	52	15	26	7
N2	45	14	31	10
N3	52	13	26	9
RA1	65	8	22	5
RA2	60	14	19	7
RA3	58	8	28	6
RA4	59	12	23	6

* Sugars with and without (+/−) a bisecting GlcNAc (B) and core fucose (F).

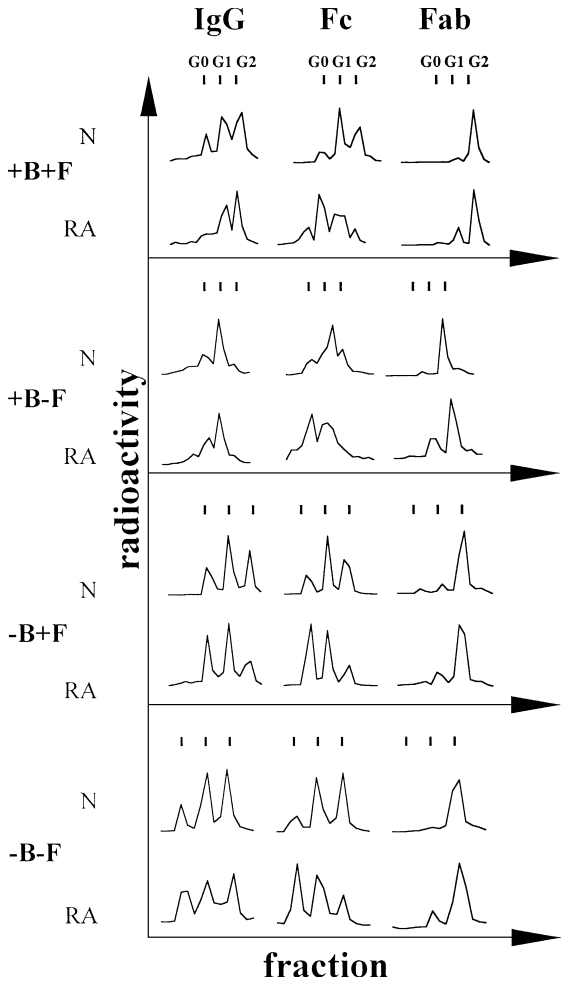

Figure 6 HPLC separation of sugars

For each core structure the retention times of standards with zero, one or two outer-arm galactose residues (G) are indicated. +, With; −, without; B, bisecting GlcNAc; F, core fucose.

The percentage incidence of the 12 most abundant asialo-oligosaccharides present on IgG and its fragments is presented in Table 6. The glycosylation of intact IgG, the Fc fragments and H-chains is similar, with the predominant core structure being −B+F (about 80%). This result is in accord with the observed similarity in sialylation for these samples, and is consistent with the finding that most of the sugar present on IgG is located at the conserved glycosylation site at Asn-297. The glycosylation of Fab fragments and of L-chains are dissimilar to those of IgG and the other fragments, and to each other. The oligosaccharides present on Fab fragments are mainly digalactosyl (about 75%), with the core structure +B+F being predominant (about 50%). These results are consistent with the sialylation data for Fab fragments because digalactosyl sugars are usually sialylated. The glycosylation of L-chains is more similar to IgG, Fc fragments and H-chains than to Fab fragments because the predominant core structure is −B+F (about 60%) and the relative amounts of G(0), G(1) and G(2) (about 25%, 45% and 30% respectively) are comparable for IgG, Fc fragments, H- and L-chains. For the

Table 5 Percentage incidence of outer-arm galactosylation of IgG and its fragments

	+B+F*			+B−F			−B+F			−B−F		
	G0†	G1	G2	G0	G1	G2	G0	G1	G2	G0	G1	G2
IgG												
N1	21	40	39	9	26	65	24	45	31	16	42	42
N2	17	41	42	7	26	67	26	41	33	18	42	40
N3	27	39	34	9	35	56	31	41	28	22	45	33
RA1	15	46	39	9	31	60	34	43	23	28	34	38
RA2	23	39	38	18	40	42	34	43	24	38	40	22
RA3	32	36	32	15	33	52	53	33	14	36	34	30
RA4	28	40	32	18	32	50	38	40	22	32	43	25
Fc												
N1	12	50	38	20	55	25	19	48	33	10	49	41
N2	28	52	20	20	51	29	22	43	35	15	44	41
N3	35	46	19	26	47	27	25	38	37	21	41	38
RA1	48	40	12	43	46	11	46	39	15	38	40	22
RA2	33	42	25	27	36	37	38	41	21	16	51	33
RA3	61	33	6	48	41	11	59	30	11	51	33	16
RA4	55	37	8	44	45	11	44	42	14	43	40	17
Fab												
N1	1	10	89	1	9	90	7	12	81	2	8	90
N2	2	15	83	1	16	83	11	17	72	1	4	95
N3	3	16	81	1	17	82	7	22	71	2	8	90
RA1	4	23	73	4	30	66	7	16	77	1	18	81
RA2	3	16	81	1	17	82	8	18	74	1	11	88
RA3	5	22	73	4	33	63	8	19	73	4	20	76
RA4	1	21	78	1	24	75	5	21	74	1	3	96

* Sugars with and without (+/−) a bisecting GlcNAc (B) and core fucose (F).
† Sugars with zero, one or two outer-arm galactose residues (G).

L-chains about 30% of the oligosaccharides have the core structure +B+F, which is less than in Fab fragments but more than in IgG, Fc fragments and H-chains (about 10%). The differences between the Fab fragments and the L-chains reveal that the sugars of Fd fragments must be predominantly digalactosyl and bisected.

Site-specific changes in glycosylation of RA IgG

The sialylation of the RA IgG samples and their fragments is similar to the normal ones (Table 2). However, there are changes in glycosylation between RA and normal IgG and their fragments (Table 6). For RA IgG, Fc fragments and H-chains there is an increase ($P = 0.057$) in the percentage incidence of agalactosyl sugars with the core structure −B+F. The RA Fab fragments have an increase in the percentage incidence of monogalactosyl sugars ($P = 0.057$) with the core structure +B+F. The RA L-chains have an increase in the percentage incidence of digalactosyl sugars with the core structure +B+F ($P = 0.057$) with a corresponding decrease in the incidence of monogalactosyl sugars with the core structure −B+F.

DISCUSSION

The approach taken in this study to investigate site-specific glycosylation of human IgG was to analyse oligosaccharides released from fragments generated by chemical and enzymic treatments. The conditions employed to reduce and alkylate the disulphide bonds of IgG resulted in their essentially complete disruption, allowing purification of the released H- and L-chains. However, this was not the case for the papain digestion because under the mild conditions used the cleavage did not go to completion; moreover the extent of digestion differed for the normal and RA samples. This is an important observation and could indicate an altered conformation of the IgG molecule around the hinge region in the disease state. However, this observation was also of concern because papain shows IgG subclass specificity [23], and subclass-specific glycosylation of IgG has been demonstrated [24]. For this reason a sugar analysis of the uncleaved IgG was performed and was found to be the same as untreated IgG (results not shown). In addition $F(ab')_2$ fragments isolated from IgG digested almost to completion with pepsin [25], for one normal and three RA samples, were found to have identical glycosylation profiles with the corresponding Fab fragments (results not shown). Thus this approach to the analysis of site-specific glycosylation of IgG is justified, especially for polyclonal IgG, which has tremendous polypeptide heterogeneity making isolation of glycopeptides extremely difficult, if not impossible. The glycosylation of Fd fragments could only be inferred because the purity of Fd fragments and L-chains isolated from $F(ab')_2$ fragments by a method based on a published procedure [26] was inadequate for sugar analysis (about 70% polypeptide purity was achieved; results not shown).

An earlier study describes the glycosylation of Fab fragments of human myeloma IgG [27]. The structures found on this fragment are similar to those of Fab fragments isolated from polyclonal IgG determined in this study, and confirm previous reports that sugars present on Fab fragments are more highly bisected, galactosylated and sialylated than Fc fragment oligosaccharides [18,28]. Analysis of the glycosylation of three Bence-

Table 6 Percentage incidence of oligosaccharides present on IgG and its fragments

	+B+F*			+B−F			−B+F			−B−F		
	G0†	G1	G2	G0	G1	G2	G0	G1	G2	G0	G1	G2
IgG												
N1	2‡	4	4	0	1	1	19	36	25	1	3	3
N2	2	5	5	0	1	1	20	32	25	2	4	4
N3	3	5	4	0	1	2	24	32	22	2	3	2
RA1	2	7	6	0	1	2	26	33	17	1	2	2
RA2	3	5	5	1	2	2	26	33	18	3	3	2
RA3	4	5	4	0	1	1	42	26	11	2	2	2
RA4	4	5	4	0	1	1	29	30	17	3	4	2
Fc												
N1	2	7	5	0	1	0	15	38	26	1	3	2
N2	4	7	3	0	1	1,	15	34	28	1	3	2
N3	4	6	2	1	1	1	19	29	28	2	5	4
RA1	4	4	1	0	1	0	40	34	13	1	1	1
RA2	4	5	3	1	1	1	29	32	16	1	4	3
RA3	9	5	1	0	0	0	48	24	9	2	1	0
RA4	6	4	1	0	0	0	35	33	11	4	4	2
Fab												
N1	1	5	46	0	1	14	2	3	21	0	1	6
N2	1	7	37	0	2	12	3	5	22	0	0	10
N3	2	8	42	0	2	11	2	6	18	0	1	8
RA1	3	15	47	0	2	5	2	4	17	0	1	4
RA2	2	10	49	0	2	11	2	3	14	0	1	6
RA3	3	13	42	0	3	5	2	5	20	0	1	5
RA4	1	12	46	0	3	9	1	5	17	0	0	6
H-chains												
N1	2	4	7	1	0	1	16	39	26	1	2	1
N2	3	4	5	1	0	1	23	40	20	2	2	1
N3	3	4	5	3	1	1	27	36	16	3	2	1
RA1	3	12	3	1	1	0	37	23	16	3	1	0
RA2	2	5	5	3	1	1	35	33	10	5	1	1
RA3	4	5	1	7	1	0	47	22	9	4	1	1
RA4	3	3	5	2	1	1	39	30	10	4	2	1
L-chains												
N1	4	11	12	4	2	2	11	33	13	3	3	2
N2	4	6	13	1	2	2	22	29	17	1	1	1
N3	5	17	13	1	2	2	17	30	9	1	2	1
RA1	6	15	35	1	1	3	11	13	13	1	1	2
RA2	2	8	23	1	1	2	11	22	27	1	1	2
RA3	8	11	18	2	2	2	26	16	12	1	1	1
RA4	6	9	29	2	1	0	13	21	14	1	1	3

* Sugars with and without (+/−) a bisecting GlcNAc (B) and core fucose (F).
† Sugars with zero, one or two outer-arm galactose residues (G).
‡ Values are presented to the nearest integer.

Jones proteins revealed that each contained a single, but different, asialo-oligosaccharide structure [29]. These glycoproteins are extremely unusual in not displaying oligosaccharide micro-heterogeneity, and do not reflect the glycosylation of L-chains isolated from polyclonal IgG described in this study.

The results presented in this report indicate that site-specific glycosylation of IgG occurs, which is tightly regulated under normal physiological conditions because the pattern of glycosylation at different sites within the molecule was conserved in all three healthy individuals. The large differences in glycosylation between Fc and Fab fragments, and between H- and L-chains, together with the similarity in glycosylation of intact IgG, Fc fragments and H-chains confirm that most of the sugar present on IgG is found within the Fc fragment (2 mol/mol of Fc, at Asn-297). The lack of knowledge of the amount of sugar present on each fragment, and of the error in the measurement of the percentage incidence of each structure present, prevented verification of the study by comparing the glycosylation of intact IgG with the sum of the sugars present on the fragments. Nevertheless the patterns of glycosylation are consistent for the fragments of the different samples, allowing these conclusions to be drawn. A recent analysis of the amounts of sugar present on Fab fragments from a normal and an RA individual gave values of 0.1 and 0.3 mol oligosaccharide per mol of Fab fragment respectively (results not shown). Thus the ratio of the amount of sugar on Fc compared with Fab fragments differs between these individuals (normal 20:1, RA 6.7:1). At present the regulatory mechanism underlying site-specific glycosylation of IgG is not understood; although the conformation of the polypeptide, differences in accessibility of the nascent sugars to glycosyl

transferases, and the activity and specificity of glycosyltransferases in B cells may be important [24,30–33].

The regulation of glycosylation of IgG is perturbed in pregnancy and in several disease states. In this study, by summing the percentage incidences of a-, mono- and digalactosyl structures present on the fragments of IgG, we have localized the previously described increase in the percentage of G(0) of RA IgG to the conserved glycosylation site at Asn-297, because RA Fc fragments and H-chains, but not Fab fragments or L-chains, have this increase in G(0). Unexpectedly, for RA L-chains an increase in the relative amount of digalactosyl sugars compared with normal was observed, whereas for RA Fab fragments an increase in monogalactosyl sugars was detected. These results indicate that RA Fd fragments have a greater relative amount of monogalactosyl sugars than normal. Differences in sialylation between the fragments of normal and RA IgG were not detected in this study. The previously reported increased amount of GlcNAc in RA [5] is consistent with the Fab fragment and L-chain-specific increase in the percentage incidence of sugars with a bisecting GlcNAc observed for the RA samples.

The glycosylation of circulating rheumatoid serum IgG might not reflect the glycosylation of nascent IgG because oligosaccharide-dependent clearance mechanisms for plasma proteins have been described [34]. Sugars with an exposed GlcNAc could be cleared rapidly from the circulation. Fab fragment sugars are thought to be more exposed than Fc sugars and hence IgG molecules with agalactosyl Fab sugars could be more rapidly cleared. Whether such mechanisms are involved in regulating the serum half-life of IgG is unclear, bearing in mind that only a small proportion of IgG molecules contain Fab sugars. In addition, clearance of IgG with agalactosyl oligosaccharides on the Fab cannot explain the greater incidence of digalactosyl oligosaccharides present on rheumatoid L-chains, nor does it explain the increased incidence of bisecting GlcNAc on rheumatoid Fab fragments.

At present the cause of the altered glycosylation of IgG in RA is not clear, but it does seem to have a biosynthetic origin [35]. Although the decreased levels of β1-4 galactosyltransferase (GalTase) activity in peripheral blood B and T lymphocytes isolated from RA patients [33,36] correlates with the decreased galactosylation of serum IgG, this does not explain the site-specific changes in galactosylation described in this study, nor does it explain the normal levels of galactose detected on rheumatoid serum IgA_1 [37]. Analysis of oligosaccharides released from IgG and its fragments gives no information about the combination of the sugars present on a molecule. Thus it is possible that an RA IgG molecule which has an increase in galactosylation of its L-chains is synthesized by a different population of B cells than an IgG molecule which has a decrease in galactosylation of its Fc fragment. Because B cells have differing levels of glycosyltransferase activities [24] this provides a straightforward explanation for site-specific galactosylation. Evidence to support changes in the population of B cells in RA is provided by the finding that there is an increase in the number of B lymphocytes which are $CD5^+$ [38,39] and also by the discovery that IgG subclass distribution is altered [40]. An alternative explanation is that multiple GalTases of differing protein (and hence IgG fragment) specificity are present in B cells. An IgG-specific GalTase of reduced activity in RA has been reported [30,33], but there is no other evidence to confirm this interesting finding. Destruction of galactose caused by reactive oxygen species has also been proposed to contribute to lower levels of galactose in RA [41], but it is difficult to envisage how this mechanism could lead to the site-specific changes in galactosylation reported in this study.

At present little is understood about the mechanisms that regulate the glycosylation of human IgG. This detailed structural study describes the site-specific changes in glycosylation of IgG that occur in a disease state, giving insight into the complexity of the regulatory mechanism. Recent advances in methods to culture B cells without transformation [42] should provide the means to investigate the levels and properties of the glycosyltransferases present in different B cells, and how they are altered in disease.

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