Characterization of Changes in IgG Associated Oligosaccharide Profiles in Rheumatoid Arthritis, Psoriatic Arthritis, and Ankylosing Spondylitis Using Fluorophore Linked Carbohydrate Electrophoresis

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ABSTRACT. ***Objective.*** To investigate fluorophore linked carbohydrate electrophoresis (FCE) as a method of analyzing serum immunoglobulin G (IgG) oligosaccharides in healthy individuals and those with rheumatic disease and compare with lectin binding assays of carbohydrate composition.

Methods. IgG was isolated from patients with rheumatoid arthritis (RA) (n = 21), ankylosing spondylitis (AS) (n = 20), psoriatic arthritis (PsA) (n = 20), and healthy adults (n = 36). IgG oligosaccharides were released enzymatically, fluorescently labelled using 8 aminonaphthalene-136 trisulfonic acid; and identification of the oligosaccharide bands was by stepwise enzymatic degradation. Comparison of FCE was made with lectin binding analysis in which the lectins *Ricinus communis* (RCA1) and *Bandeiraea simplicifolia* (BSII) were used to detect galactose (Gal) and N-acetylglucosamine (GlcNAc), respectively.

Results. Each disease could be differentiated from healthy adults on the basis of Band 1 asialo-digalacto core fucosylated oligosaccharide (gf2) intensity ($p = 0.001$), but not from each other. Reduced levels of different sugars were associated with specific diseases: reduced gf2 with RA ($p < 0.001$), PsA ($p < 0.001$) and AS ($p < 0.02$), reduced Band 5 disialo-digalacto core fucosylated (a2f) oligosaccharide with AS ($p < 0.001$), reduced Band 6 disialo-digalacto (a2) oligosaccharide with AS ($p < 0.001$) and PsA ($p = 0.021$). All diseases were associated with a significant increase in Band 4 asialo-agalacto core fucosylated oligosaccharide (g0f) ($p < 0.001$). In RA, FCE band intensities correlated with sugar quantity when identified using lectin binding analysis ($p \leq 0.003$). In contrast, there was no correlation between the same bands in healthy individuals.

Conclusion. FCE is an accurate method of analyzing IgG associated oligosaccharides and reveals unique band patterns or sugar prints associated with healthy adults and patients with RA, PsA, and AS, and comparison with lectin binding analysis suggests undetected RA glycoprotein structural differences. FCE has potential in the early diagnosis and differentiation of rheumatic diseases. (J Rheumatol 2001;28:1531–6)

Key Indexing Terms:
RHEUMATOID ARTHRITIS   PSORIATIC ARTHRITIS   ANKYLOSING SPONDYLITIS
IMMUNOGLOBULIN G   OLIGOSACCHARIDE

Immunoglobulin G (IgG) consists of 2 heavy and 2 light polypeptide chains linked by disulfide bridges. In the Fc the 2cgII domains each contain a conserved glycosylation site, asparagine 297, to which complex biantennary oligosaccharides are covalently linked[1].

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Various methods have been employed to study the oligosaccharides associated with IgG. Parekh, *et al*[2] used the laborious method of hydrazinolysis followed by exoglycosidase digestion and Bio-Gel P-4 column chromatography, to characterize fully IgG associated oligosaccharides. Sumar, *et al*[3] developed a rapid immunodot-blot technique to detect galactose and N-acetylglucosamine using specific lectin binding to *Ricinus communis* (RCA1) and *Bandeiraea simplicifolia* (BSII). Tsuchiya, *et al*[4] used another lectin, *Psathyrella velutina*, to detect N-acetylglucosamine. Youinou, *et al*[5] devised an ELISA based on the binding of RCAl to detect galactosylated IgG. Rook, *et al*[6] used a monoclonal antibody raised against Group A streptococci N-acetylglucosamine (GlcNAc) to detect agalactosyl IgG.

Polyacrylamide gel electrophoresis of 8-aminonaphthalene-1,3,6-trisulfonic acid labeled oligosaccharides was first described by Jackson in 1990[7]. This rapid technique can be combined with enzymic release of N-linked oligosaccharides using peptide N-glycosidase F to analyze the oligosaccharide components of glycoproteins[8]. This technique is much more specific than both the lectin and antibody tests as described.

IgG glycosylation has been studied on a number of different diseases. Several authors[1-3] have found a higher content of agalactosylated oligosaccharides associated with serum IgG in patients with rheumatoid arthritis (RA) compared to healthy adults[9].

A study of the predictive value of IgG oligosaccharide abnormalities in early synovitis[10] included one patient with a normal level of agalactosylated IgG who was later shown to have psoriatic arthritis.

Patients with juvenile chronic arthritis[2,11], Crohn's disease[9,12], primary Sjögren's syndrome[5], systemic lupus erythematosus[12], and mycobacterial infections[9] have all been shown to have an increase in agalactosylated IgG.

In a study utilizing high performance liquid chromatography (HPLC) technology[13], 16 different IgG oligosaccharide structures were analyzed from a spectrum of rheumatic diseases. It was observed that disease associated changes were not only confined to the presence of agalactosylated sugars, but that sugar prints consisting of permutations of different sugar structures were present and were specifically associated with each rheumatic disease. This raised the possibility that IgG sugar printing could be useful not only in differentiating rheumatic diseases, but also in distinguishing disease associated pathogenic processes.

Pathophysiological studies have also revealed that IgG glycosylation changes are not merely epiphenomena. When GlcNAc is the terminal sugar on IgG, it is accessible for binding with mannose binding protein, and this in turn may result in activation of complement[14]. Additionally, most rheumatoid factors selectively bind IgG that is hypogalactosylated[15] and this may explain why immune complexes are abundant in RA.

We investigated whether fluorophore linked carbohydrate electrophoresis (FCE) could be used to analyze oligosaccharides from serum IgG of healthy adults and patients with RA, psoriatic arthritis (PsA), and ankylosing spondylitis (AS), and to compare the findings with lectin binding assays.

MATERIALS AND METHODS

Patients. Serum samples were obtained from patients with RA (n = 21, mean age 59 yrs, range 43–76 yrs; 6 men, 15 women who fulfilled the American Rheumatism Association 1987 criteria[16]), patients with active AS (n = 20, mean age 45 yrs, range 18–58 yrs; 8 men, 2 women who fulfilled the New York criteria[17]), patients with PsA (n = 20, mean age 46 yrs, range 21–73, 15 men, 5 women; defined as seronegative oligo and poly inflammatory arthritis associated with psoriasis), and healthy blood donors (n = 36, mean age 43 yrs, range 24–64; 16 men, 20 women). For the comparison with lectin binding data, serum samples from 21 healthy subjects and 15 patients with RA were used. Serum samples were stored at –20˚C until required.

Age matching of samples was not utilized in this study — although age was suspected of being a confounding variable in patients with osteoarthritis, this has not been confirmed in subsequent larger studies utilizing lectin analysis[1] and HPLC[13].

Purification of IgG. IgG was purified from sera using a column of coarse Sephadex G-25 (Pharmacia, Uppsala, Sweden) and DE52 diethylaminoethyl cellulose according to the method of Sumar and Bond[3,18].

Protein estimation. Protein estimations were performed using the Pierce BCA protein assay (Pierce Europe, Oud-Beijerlands, Netherlands).

Assessment of purity of IgG. Purified IgG samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; 5 mg samples were mixed with 20 ml of sample buffer [1.0 ml 0.5 M Tris-HCl, pH 6.8; 0.8 ml glycerol; 1.6 ml 10% (w/v) SDS; 0.4 ml 2 beta-mercaptoethanol; 0.2 ml 0.05% (w/v) bromophenol blue; 4.0 ml distilled water] and boiled 4 min.

The reduced samples were applied to 12% gels and electrophoresis was performed at 200 volts for 40 min using the Bio-rad minigel apparatus (Bio-rad Laboratories, Hertfordshire, England). The gels were stained with Coomassie blue. Protein bands at 51 kDa and 22 kDa were identified and assumed to represent heavy and light chains of IgG, respectively. The relative proportions of IgG and contaminants were assessed densitometrically using a Hewlett-Packard desk scanner attached to a Macintosh IIsi computer and analyzed using Collage software (Techgen).

Oligosaccharide sequencing. Sequencing of the oligosaccharide bands from one representative oligosaccharide profile from the IgG of a healthy individual was performed using the FCE N-linked oligosaccharide sequencing kit (Glyko Inc., San Francisco, CA, USA). Briefly, the isolated oligosaccharides were eluted from a preparative gel and dried. Stepwise degradation of aliquots of each oligosaccharide was performed using neuraminidase (which removes α-2-3,6,8 linked N-acetylneuraminic acid), ß-galactosidase (which removes ß-1-3,4 linked galactose), ß-N-acetylhexosaminidase (which removes ß-1-2,3,4,6 linked GlcNAc), α-mannosidase (which removes α-1-2,3,6 linked mannose), and α-fucosidase (which removes α-1-6 linked fucose).

The degraded products were then loaded onto a polyacrylamide gel and electrophoresis was performed at 5–8˚C, 20 mA per gel, for 1 h. The gels were then removed from their cassettes and photographed as above. Changes in the relative mobility of the oligosaccharide relative to a standard oligosaccharide ladder were used to analyze the structures represented by each oligosaccharide band (Glyko Inc.).

FCE N-linked oligosaccharide profiling. Fluorophore linked carbohydrate electrophoresis was performed using the FCE N-linked oligosaccharide profiling kit (Glyko Inc. and Millipore, London, UK). The procedure was modified as follows. Before analysis, purified IgG samples were dialyzed against distilled water for 16 h at 4˚C. 500 mg samples of purified IgG (undenatured) were dried in a centrifugal vacuum evaporator (CVE), dissolved in 45 ml oligo profiling enzyme buffer, and incubated with 5 mU of peptide *N*-glycosidase F at 37˚C for 16 h to release N-linked oligosaccharides. The protein was precipitated by adding 3 volumes of cold ethanol, storing on ice for 10 min, and centrifuging to pellet the protein. The supernatant was removed stored on ice for a further 10 min. Centrifugation was repeated and the resulting supernatant dried in a CVE.

Fluorescent labeling of the oligosaccharides was performed using 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and sodium cyanoborohydride. The resulting labeled oligosaccharides were dried using a CVE, resuspended in 5 ml distilled water, and stored at –70˚C until electrophoresis.

Electrophoresis was performed at 5–8˚C, 15 mA per gel, for 1 h, 40 min to allow optimum separation of the oligosaccharides. The gels were then removed from their cassettes and photographed on a UV light box using a Polaroid MP-4 Land camera, Wratten 2A (Kodak) and 58 (Tri-green, Lee Filters, Andover, England) filters, an aperture of f4.5, exposure time 50 s, Type 55 Polaroid film.

Densitometric analysis of the bands representing the oligosaccharides was performed using the equipment as above. The pixel score of each individual band was expressed as a percentage of the total score for all the oligosaccharide bands in each lane (relative intensity, RI). The reproducibility of the assay was assessed by repeating the procedure from purification of IgG to measurement of RI for one healthy adult 6 times and one patient with RA 4 times. The coefficients of variation were between 3% and 16%.

Detection of IgG glycoforms using lectin binding analysis. Lectin binding analysis was performed as described[18]. IgG samples were purified using ion exchange chromatography as above and blotted onto nitrocellulose. The blots were boiled in phosphate buffered saline. After blocking with 1% bovine serum albumin, the blots were probed with one of 2 biotinylated lectins: *Ricinus communis* agglutinin (RCAI) was used to detect galactose and *Bandeiraea simplicifolia* (BSII; Vector Laboratories, Peterborough, England) was used to detect N-acetylglucosamine. The blots were developed with the substrate 4-chloro-1-naphthol and scanned using the equipment described above.

Statistical analysis. By inspection the data appeared normally distributed and the variances were similar. Lilliefors test for normality confirmed that $0.01 > p < 0.05$ for only 2 of 24 distributions. Tests for statistical differences between means were therefore performed using ANOVA and Tukey's honestly significant difference test. Confidence intervals for each band for each disease type were calculated. Comparisons between FCE and lectin binding data were made using Pearson's correlation coefficient.

RESULTS

Assessment of purity of the IgG samples. Purity of the IgG samples varied from 71 to 78%. The same contaminant proteins in similar quantities were seen in IgG samples from the healthy adults and all disease groups.

Oligosaccharide profiling. Six bands were seen in the oligosaccharide profiles, which were the same for all subjects (Figure 1). There was no relationship between the RI of Bands 1 to 6 from the healthy individuals and either age or sex of the subjects.

Significant differences ($p = 0.001$) were seen in the RI of Bands 1 (g2f), 2 (g1f), 4 (g0f), 5 (a2f), and 6 (a2) between the disease groups (Figure 2). No significant difference in RI of Band 3 (a2f) was found between the groups.

The RI of the 6 bands from the disease groups were compared to those from the healthy subjects (Figure 2). The RI of the least electrophoretically mobile band, Band 1 (g2f), was significantly reduced in patients with RA ($p < 0.001$) and PsA ($p < 0.001$) and less markedly so in those with AS ($p < 0.02$). Patients with RA also revealed a significant reduction in Band 2 RI (g1f) ($p < 0.01$). All the disease groups showed a significant reciprocal increase in the RI of Band 4 RI (g0f) ($p < 0.001$). Band 5 RI (a2f) was reduced in AS patients ($p<0.001$) and band 6 RI (a2) was reduced in both AS ($p < 0.001$) and PsA ($p = 0.021$).

Oligosaccharide sequencing. The oligosaccharide associated with each band is as follows — Band 1: asialo-digalacto core fucosylated oligosaccharide (g2f), Band 2: asialo-monogalacto core fucosylated oligosaccharide (g1f), Band 3: disialo-digalacto core fucosylated oligosaccharide

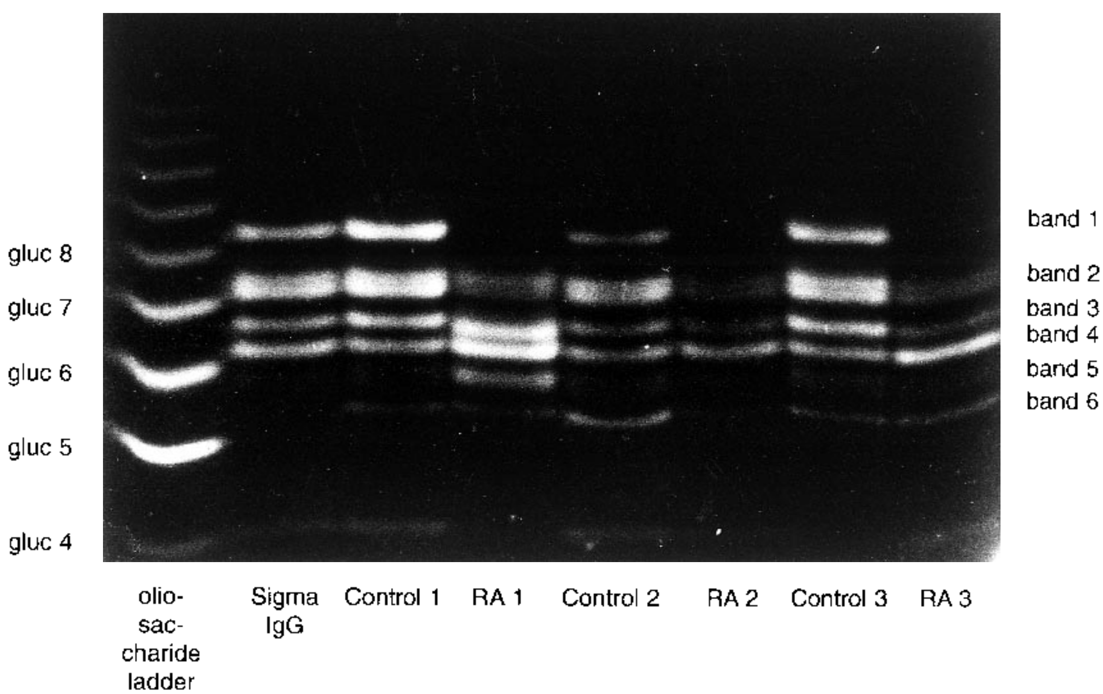

Figure 1. N-linked oligosaccharide profiling gel showing the 6 bands released from IgG. In the first lane is a standard oligosaccharide ladder (gluc8–gluc4 representing 8–4 glucose units). In the second, a commercial preparation of IgG (Sigma). Carbohydrates released from 3 IgG samples from healthy adults (controls 1–3) and 3 IgG samples from patients with RA (RA1–3) are run in the next 6 lanes. Six bands of differing intensities are produced by these samples (bands 1–6).

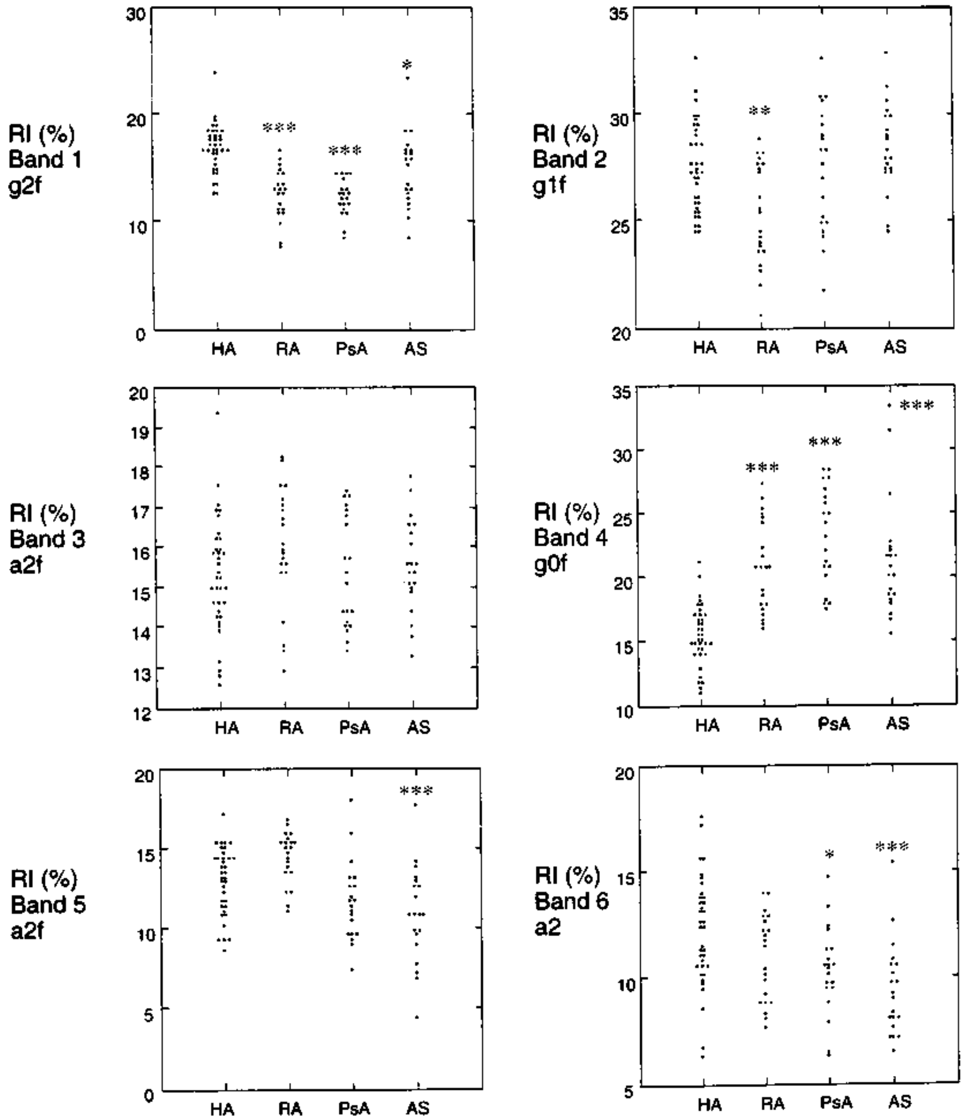

Figure 2. Data from N-linked oligosaccharide profiling gels. Relative intensities (%) of bands 1–6 (on vertical axis). Disease groups on horizontal axis. HA: healthy adults, n = 36, mean (95% CI), band 1 RI (%) 16.8 (16.1–17.6), band 2 RI (%) 27.4 (26.7–28.1), band 3 RI (%) 15.3 (14.8–15.7), band 4 RI (%) 15.4 (14.7–16.2), band 5 RI (%) 12.9 (12.2–13.6), band 6 RI (%) 12.1 (11.3–13.0). RA: n = 21, mean (95% CI), band 1 RI (%) 12.5 (11.5–13.6), band 2 RI (%) 25.3 (24.2–26.4), band 3 RI (%) 15.9 (15.3–16.6), band 4 RI (%) 20.7 (19.2–22.3), band 5 RI (%) 14.4 (13.7–15.1), band 6 RI (%) 10.9 (9.9–11.8). PsA: n = 20, mean (95% CI), band 1 RI (%) 12.2 (11.4–13.0), band 2 RI (%) 27.4 (26.0–28.8), band 3 RI (%) 15.3 (14.7–16.0), band 4 RI (%) 23.2 (21.4–25.0), band 5 RI (%) 11.6 (10.4–12.8), band 6 RI (%) 10.3 (9.3–11.2). AS: n = 20, mean (95% CI), band 1 RI (%) 14.8 (13.2–16.4), band 2 RI (%) 28.4 (27.4–29.3), band 3 RI (%) 15.5 (15.0–16.0), band 4 RI (%) 21.3 (19.1–23.4), band 5 RI (%) 10.8 (9.4–12.2), band 6 RI (%) 9.3 (8.3–10.4). *p < 0.02, **p < 0.01, ***p < 0.001.

sialic acid is probably α2,3 linked (a2f), Band 4: asialo-agalacto core fucosylated oligosaccharide (g0f), Band 5: disialo-digalacto core fucosylated oligosaccharide (sialic acid probably α2,6 linked) (a2f), Band 6: disialo-digalacto (a2) oligosaccharide sialic acid probably α2,6 linked.

Comparison of FCE and lectin binding analysis. The RI of Band 1 (g2f) from the patients with RA showed a strong positive correlation with RCAI binding gal ($p < 0.001$), and a negative correlation with BSII binding GlcNAc ($p = 0.002$) (Figure 3). The RI of Band 4 (g0f) from patients with RA showed a strong negative correlation with RCAI binding gal ($p < 0.001$) and a positive correlation with BSII binding GlcNAc ($p = 0.003$).

In contrast, there was no correlation between the RI of the same bands with either RCAI or BSII binding in the healthy individuals.

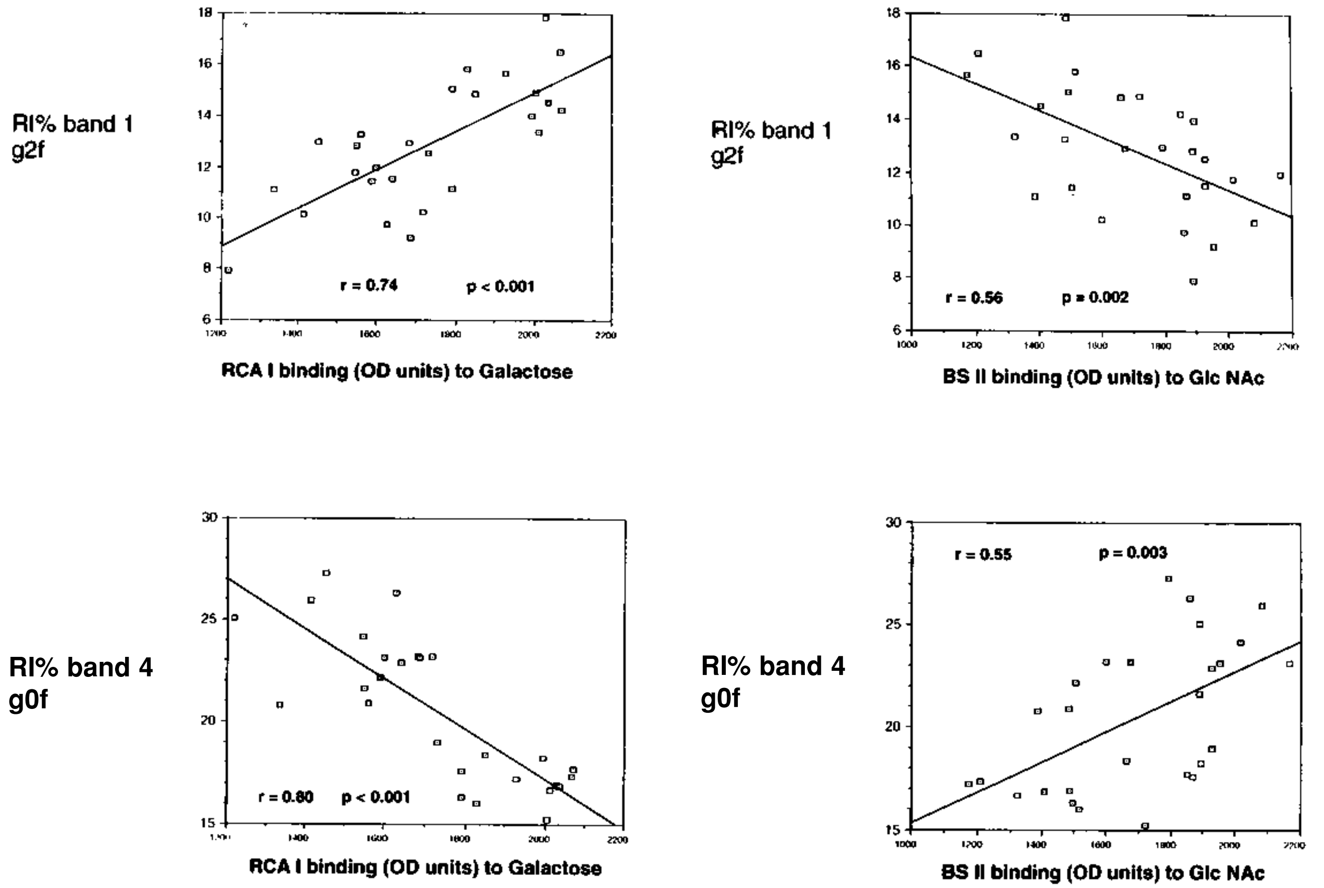

Figure 3. Comparison of lectin binding and FCE data in patients with RA showing the correlations between RCA1 and BSII binding (absorbance units on the horizontal axis) and the relative intensities (%) of bands 1 and 4 (vertical axis).

DISCUSSION

Using fluorophore linked carbohydrate electrophoresis we were able to elucidate 6 oligosaccharide bands from the serum IgG taken from healthy individuals and those with rheumatic diseases. The same bands were found in all samples tested but there were significant differences in their relative intensities. The identity of the oligosaccharide contained within each band was determined by enzymatic digestion of the oligosaccharide released from IgG of the healthy individual. The difference in disialodigalactose core fucosylated oligosaccharide electrophoretic mobility (bands 3 and 5) was probably due to different sialic acid linkage (α2,3 and α2,6). This enabled us to distinguish a range of oligosaccharides from the asialoagalacto to the disialodigalacto variety. When the oligosaccharides were analyzed for each rheumatic disease group, it was found that their profiles were significantly different from each other and in comparison to the healthy individuals.

In all 3 disease groups there was a significant reduction in asialylated digalactosyl structures (band 1) in comparison to healthy individuals, and there was a reciprocal increase in agalactosyl structures (band 4) in all the disease groups investigated. RA was unique in having significant reduction in monogalactosylated structures (band 2), AS was associated with a significant reduction in some disialylated structures (bands 5 and 6), and PsA was associated with a significant reduction in disialo structures without fucose (band 6).

This is the first reported electrophoretic analysis of IgG oligosaccharides associated with rheumatic disease. Even within the confines of the limited number of oligosaccharide structures elucidated, it would appear that this technique has the potential ability to differentiate one rheumatic disease from another.

This raises the possibility not only that this technique could be useful in the early diagnosis of these diseases, where perhaps they may appear clinically similar to each other and to self-limiting viral arthritides, but that it might also indicate that different oligosaccharide related pathogenic mechanisms associated with inflammation occur in these diseases. It is now important to investigate patients with early synovitis and quiescent disease to analyze these concepts more fully, where longitudinal studies will be important in the assessment of potential disease and therapy effect on sugar profiles.

The possibility that the 3-dimensional appearance of the

IgG glycoprotein may be different in healthy individuals and patients with RA, even if they contain similar oligosaccharide compositions, is suggested when the relative intensities of the asialylated digalactosyl (band 1) and agalactosyl (band 4) are correlated with the extent of lectin binding to gal and GlcNAc. In individuals with RA we investigated, very good correlation was found indicating that an increase in the appearance of agalactosyl structures can be recognized by both techniques. However, no such correlations were apparent in healthy individuals. There are a number of possible reasons for this. It may be that other sugar variations are occurring such that, perhaps, there are differences in the extent of core fucosylation, bisecting GlcNAc or Fab glycosylation. This seems unlikely, as the electrophoretic mobility of the 6 bands was similar in all individuals investigated, and conservation of sugar structure has also been confirmed with HPLC technology. Alternatively, perhaps there are subtle amino acid changes that result in the sugars adopting a different spacial configuration, which results in more effective binding of the 2 lectins in individuals with RA. This is unlikely, as such RA associated differences have not previously been detected.

We report the successful use of fluorophore linked carbohydrate electrophoresis in the analysis of IgG associated oligosaccharides in healthy individuals and patients with rheumatic diseases. Unique oligosaccharide profiles or sugar prints have been shown to be associated with RA, PsA, and AS, and these were not solely associated with agalactosylation.

In addition, it would appear that the RA IgG glycoprotein has a different appearance from that derived from healthy individuals. These data raise the possibility that FCE may be utilized in the early diagnosis and differentiation of rheumatic diseases and may also be an additional tool to investigate the pathogenic mechanisms leading to these disorders.

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