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. Various methods have been employed to study the oligosaccharides associated with IgG. Parekh, et al. 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. developed a rapid immunodot-blot technique to detect galactose (Gal) and N-acetylglucosamine (GlcNAc), respectively.

Polyacrylamide gel electrophoresis of 8-aminonaphthalene-1,3,6-trisulfonic acid labeled oligosaccharides was first described by Jackson in 1990. This rapid technique can be combined with enzymic release of N-linked oligosacch...
rides using peptide N-glycosidase F to analyze the oligosaccharide components of glycoproteins. 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 have found a higher content of agalactosylated oligosaccharides associated with serum IgG in patients with rheumatoid arthritis (RA) compared to healthy adults.

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

Patients with juvenile chronic arthritis, Crohn’s disease, primary Sjögren’s syndrome, systemic lupus erythematosus, and mycobacterial infections have all been shown to have an increase in agalactosylated IgG.

In a study utilizing high performance liquid chromatography (HPLC) technology, 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. Additionally, most rheumatoid factors selectively bind IgG that is hypogalactosylated, 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), patients with active AS (n = 20, mean age 45 yrs, range 18–58 yrs; 8 men, 2 women who fulfilled the New York criteria), 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 and HPLC.

**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. Purified IgG was desalted by passage through a Sephadex G-10 column. The purity of the IgG was assessed by Coomassie blue staining. The purity of the IgG was assessed by Coomassie blue staining.

**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 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. 300 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 mM 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-aminonaphthaleine-1,3,6-trisulfonic acid (ANTS) and sodium cyanoborohydrate. 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, Watten 2A (Kodak) and 58 (Tri-green, Lee Filters, Andover, England) filters, an aperture of f/4.5, 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 indi-
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
sialic acid is probably $\alpha_2,3$ linked (a2f), Band 4: asialo-agalacto core fucosylated oligosaccharide (g0f), Band 5: disialo-digalacto core fucosylated oligosaccharide (sialic acid probably $\alpha_2,6$ linked) (a2f), Band 6: disialo-digalacto (a2) oligosaccharide sialic acid probably $\alpha_2,6$ 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 = 0.003) (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.
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 (\(\alpha 2,3\) and \(\alpha 2,6\)). 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 spatial 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.

ACKNOWLEDGMENT
We thank Dr. O. FitzGerald and Dr. D. Mulherin of University College Dublin for sera from patients with psoriatic arthritis, and Dr. A. Ebringer of the Division of Biomolecular Sciences, King’s College, University of London, for sera from patients with ankylosing spondylitis.

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The Journal of Rheumatology 2001; 28:7