Reduction in IgG galactose in juvenile and adult onset rheumatoid arthritis measured by a lectin binding method and its relation to rheumatoid factor

N Sumar, D A Isenberg, K B Bodman, A Soltys, A Young, A M Leak, J Round, F C Hay, I M Roitt

Abstract
Glycosylation changes in patients with juvenile chronic and adult onset rheumatoid arthritis have been studied using a novel binding method. Both these major types of arthritis showed decreased galactosylation of serum IgG, which confirms earlier studies using a different, more complex chemical method.

No significant correlation between serum IgG, IgM, and IgA rheumatoid factors and age corrected G(0) (percentage of oligosaccharide chains lacking galactose) was found. The possibility that the less glycosylated IgG is preferentially confined to circulating IgM/IgG immune complexes cannot be excluded, however.

Analysis of the IgG glycoforms of serum IgG has shown that a major difference exists between normal subjects and those with adult onset rheumatoid arthritis. Thirty-two patients invariably have an increased incidence in the number of oligosaccharides, attached to the Cy2 domain of IgG, which lack terminal galactose and terminate in N-acetylgalactosamine (G(0)). Such aglycosylated immunoglobulins might lose the ability to bind monocyte and macrophage receptors and to induce cellular cytotoxicity. In addition, complexes formed by aglycosylated immunoglobulins with antigen fail to be eliminated rapidly from the circulation. Lack of these terminal galactose residues in the Cy2 domain of IgG Fc might also contribute to autotorienticity in this region. Given the many clinical and serological differences between patients with adult rheumatoid arthritis and those with juvenile chronic arthritis (reviewed in ref 5), the finding of an identical glycosylation abnormality in the younger onset cases was surprising. It is, however, notable that although rheumatoid arthritis in childhood is usually reported to be IgM rheumatoid factor negative, IgG rheumatoid factor has been reported and 'hidden' rheumatoid factors in this group have been described.

It was felt important to repeat the measurement of agalactosylated (G(0)) IgG concentrations in patients with juvenile chronic arthritis, and to compare the results with rheumatoid factor concentrations of several isotopes in both the juvenile and adult onset forms of rheumatoid arthritis. We have shown previously that it is essential to relate G(0) levels to age. A recently introduced lectin binding method to measure G(0) was used with an age corrected curve. We now show that using this method raised levels of G(0) are commonly found in patients with juvenile chronic arthritis, but that there is no statistically significant correlation between IgG, IgM, and IgA rheumatoid factors and age corrected G(0) levels in patients with adult or childhood onset.

Patients, materials, and methods
PATIENTS
Serum samples from 17 children with active juvenile chronic arthritis were studied. Their ages ranged from 2 to 16 years (mean (SD) 9.2 (4.4)). Seven had had a polyarticular onset of their disease and five each had pauciarticular and systemic onsets. Erythrocyte sedimentation rate, C reactive protein, and total immunoglobulin concentrations (determined by standard laboratory techniques) were available for all these patients. One patient was regarded as having severely active disease clinically and the remainder had mildly or moderately active disease. Thirteen patients with adult onset rheumatoid arthritis were studied. Each patient had four or more of the American Rheumatism Association's revised criteria for the diagnosis. Their ages ranged from 27 to 63 years (mean (SD) 49.5 (11.8)). Ten patients with systemic lupus erythematosus were studied as disease controls. These patients had four or more of the American Rheumatism Association's revised criteria for the classification of the disease. Their ages ranged from 11.75 to 71 years (mean (SD) 42.3 (16.7)). Erythrocyte sedimentation rates from the dates of the venesection of patients with adult onset rheumatoid arthritis and of those with systemic lupus erythematosus were available. As healthy controls 12 adults (age range 23-77 years, mean (SD) 45 (19)) and 13 children (age range 9-12 years, mean (SD) 10.1 (1.1)) were studied.

LECTIN BINDING METHODS FOR DETERMINING G(0) LEVELS
Full details of this method are described elsewhere. Briefly, purified IgG samples (1-2 μg) and IgG standards of known percentage G(0) values were dot blotted onto nitrocellulose using a Biorad dot blotter. Two identical blots were prepared. The blots were boiled in 1% sodium dodecyl sulfate in saline or in 100 μl of carbonate buffered saline at 90°C for 15 minutes followed by blocking in PBS-Tween-20-bovine serum albumin (PBS-Tween-BSA). The following steps were carried out at room temperature and the blots shaken on a horizontal shaker. The blocked blots were treated with biotinylated ricin agglutinin, RCA1, (1/250 dilution) and biotinylated Bandeiraea lectin, BSAII, (1/100 dilution) for 15 minutes and washed. Biotinylated lectin was then washed away and the slides incubated for 30 minutes with avidin-biotin-peroxidase complex (ABC) diluted 1/200 in PBST. The slides were washed and then incubated for 30 minutes with 0.05% H2O2, 0.1% diaminobenzidine, 0.02% nickel ammonium sulphate in Tris-HCl buffer and then washed. The slides were counterstained with hematoxylin and dried.
dilution) for two and a half hours. After washing the blots with PBS-Tween-BSA (5×10 minute washes), streptavidin-biotin-horseradish peroxidase conjugate was added (1/1000 dilution) and the blots incubated for two hours. The blots were then washed with PBS-Tween developed with chloronaphthol, and scanned using a Biorad video densitometer. The ratios of BSII binding/RCAl binding for the standards were plotted against percentage G(o) values and the test samples read off against this curve.

RHEUMATOID FACTOR MEASUREMENT
Half of a microtitre plate (Nunc Immunon 2) was coated with 10 μg/ml horse IgG (Miles Laboratories) in bicarbonate/carbonate buffer pH 9·6 (100 μl per well). The other half of the plate was filled with buffer alone. The plate was incubated overnight at 4°C. The wells were washed four times with PBS and blocked with 2% BSA in PBS (200 μl/well) for two hours at room temperature. The wells were washed four times with PBS, and samples and controls were added in duplicate to the wells both on the coated and uncoated sides of the plate (100 μl/well). Samples and controls were diluted 1/500 in PBS/BSA with 0-05% Tween-20. Plates were incubated for two hours at room temperature. The plates were washed four times in PBS-Tween. Goat antihuman IgG, IgA, or IgM F(ab)2 peroxidase conjugate (Sigma) diluted 1/1000 in PBS-Tween was added to the wells (100 μl/well). Plates were incubated for two hours at room temperature. The plates were washed four times in PBS-Tween. 2,2'-Azinobis(3-ethylbenzthiazoline) sulphonate acid (ABTS) substrate solution (100 μl) was added to each well (50 mg ABTS in 100 ml 0-018 M citrate/phosphate buffer pH 4 with 50 μl H2O2). After 10 minutes colour development was stopped by the addition of 50 μl sodium fluoride (96 mg/50 ml) to each well, and the wells were read at 405 nm. Background values were subtracted from values from the coated side of the plate and the results expressed as arbitrary units.

STATISTICS
Student's t test was used to test for significance of differences in means, and Pearson's correlation coefficient was used to examine associations between variables.

Results
In view of our previous demonstration10 that G(o) levels in healthy subjects fluctuate with age, a 'normal age curve' was constructed using the lectin binding method.11 All the G(o) levels were compared with this curve.

The figure shows a scattergram of the G(o) and rheumatoid factor levels in different groups. Raised IgM rheumatoid factor levels were largely confined to the patients with adult onset rheumatoid arthritis (p=0·003). Thus only two of the 17 patients with juvenile chronic arthritis and one of the 10 lupus patients had high IgM rheumatoid factor values. In contrast, there was little difference in IgG or IgA rheumatoid factor levels between any of the

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Scattergrams of the (A) IgM, (B) IgA, and (C) IgG rheumatoid factor results in each of the patient groups tested. The results represent the optical density values by enzyme linked immunosorbent assay (ELISA) minus the background binding as described in the Methods section. A scattergram of the G(o) results is shown in (D). The results in (D) are shown as the number of standard deviations above or below the age corrected mean. The numbers shown below some of the columns (above the abscissa) represent subjects whose background ELISA values were a little greater than the antigen coated side of the plate. JCA=juvenile chronic arthritis, JCAc=juvenile chronic arthritis control, ARA=adult rheumatoid arthritis, ARAc=adult rheumatoid arthritis controls, SLE=systemic lupus erythematosus.
Reduction in IgG galactose in rheumatoid arthritis

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*p<0.05; **p<0.01; ***p<0.001.
†ESR=erythrocyte sedimentation rate; CRP=C reactive protein;
G(o)=percentage of oligosaccharide chains lacking galactose.

Discussion

The initial studies of changes in the glycosylation of IgG in patients with rheumatoid arthritis were performed using a precise, but complex chemical method. This method requires the use of hydrazine to release oligosaccharides associated with IgG. Subsequently, these oligosaccharides are digested with exoglycosidases of defined specificity. Although this method produces direct structural information, it is extremely time consuming. To obtain equivalent results by a faster method the differential binding properties of lectins for particular sugars have been used.13

With this new technique we have now confirmed our original, unexpected results showing raised G(o) in all three major types of juvenile chronic arthritis. The method has been used to confirm that adults with rheumatoid arthritis commonly have high levels, but in contrast, values for our control patients with systemic lupus erythematosus were invariably normal when a correction for age was made.

Given the major difference between adult and juvenile chronic arthritis—that is, the usual absence of rheumatoid factor in the latter—we have now extended our studies to look at the relation between G(o) levels and IgG, IgM, and IgA rheumatoid factor. This was important since the defects in agalactosylated IgG suggest potential explanations for the existence of rheumatoid factors—which remains uncertain since their first description over 50 years ago. For example IgG molecules lacking terminal galactose and sialic acid moieties may expose determinants on the Fc which are usually concealed. Alternatively, the truncated oligosaccharides may alone, or in tandem with adjacent peptides, form an antigenic epitope.

As we clearly show, however, there is no statistical correlation between any of the three isotypes of rheumatoid factors measured and the levels of G(o) in any of the groups of patients and controls tested. Does this therefore render impossible a link between the glycosylation abnormalities we noted and rheumatoid factor? A clue that the answer to the question may still be 'no' is offered by our observations of a highly significant correlation between G(o) and background binding of serum to the ELISA plate. If this background binding of serum is due to immune complexes sticking to the plastic surface then G(o) may be related to complex formation. This is now being investigated and it may be relevant to note that IgG seemed to behave as an acute phase reactant in showing a significant association with erythrocyte sedimentation rate and C reactive protein, but was also highly correlated with G(o). This observation thus confirms previous studies, which have reported a mean IgG concentration in control children of 8-14 to 9-8 g/l;15 in children with active juvenile chronic arthritis it is generally 11-3* to 13-8 g/l.16 It is interesting to speculate whether most of the increased IgG found in patients with juvenile chronic arthritis is in the G(o) form.

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