Rheumatic Disease Differentiation Using Immunoglobulin G Sugar Printing by High Density Electrophoresis

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ABSTRACT. ***Objective.*** To determine whether immunoglobulin G (IgG) sugar printing using high density electrophoresis can be a diagnostic and prognostic test to rapidly differentiate early rheumatoid arthritis (ERA), rheumatoid arthritis (RA), and other rheumatic diseases from each other.

Methods. One hundred fifty-three patients with ERA/RA, psoriatic arthritis (PsA), early psoriatic arthritis (EPsA) ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), juvenile idiopathic arthritis (JIA), early undifferentiated seronegative arthritis (UA), and osteoarthritis (OA) were investigated. Samples of their serum IgG were purified, and sugars were released enzymatically and fluorophore-labelled, then subjected to high density electrophoresis, and relative quantities of each sugar were determined by optical density.

Results. Sugar prints of 9 sugars were compiled for each of the 9 disease groups. Specific disease-associated sugar changes were determined by comparison with OA. For example, agalactosylated structures were increased in ERA/RA and EPsA/PsA ($p = 0.0001–0.004$) and digalactosylated structures were decreased in PsA, AS, and JIA ($p = 0.0001–0.04$). When the disease groups were compared, each disease was characterized by a unique sugar print comprising 7 of the 9 sugars ($p = 0.001–0.005$); only g0fb and a1f were not associated. ERA/RA differed in the quantities of monogalactosyl and sialylated sugars ($p = 0.006–0.007$). The presence of agalactosyl sugars enabled correct prediction of RA in 71.2% of individuals, with a specificity of 84.2% and sensitivity of 50.0%. The area under the sensitivity versus specificity curve was 0.7812.

Conclusion. IgG sugar printing was found to be effective in differentiation of rheumatic diseases and can differentiate ERA and RA from each other and from other rheumatic diseases; and hence may constitute a relatively rapid diagnostic and prognostic test for patients presenting with arthritis. (J Rheumatol 2003;30:2540–6)

Key Indexing Terms:
RHEUMATIC DISEASE IMMUNOGLOBULIN G SUGARS
ELECTROPHORESIS EARLY ARTHRITIS

Immunoglobulin G (IgG) consists of 4 polypeptide chains linked together by disulfide bridges. The molecule consists of Fab and Fc regions, which are responsible for antigen binding and effector functions, respectively. In the Fc, a conserved glycosylation site at asparagine 297 is found in the cg2 domain. At this site complex biantennary sugars (Figure 1) are covalently linked and an average of 2.5 N-linked sugars are attached to each IgG molecule[1].

There are at least 16 different neutral sugars associated with IgG, and these can be grouped into 3 sets depending on whether they contain 0, 1, or 2 galactose residues on their outer arms (g0, g1, g2, respectively)[2-4]. The galactose molecules may be situated on either the α-1,3 or the α-1,6 arm of the sugar molecule, and further heterogeneity can be achieved by the presence or absence of core fucose and bisecting N-acetylglucosamine, and in addition the g1 and g2-type structures may be sialylated.

The rheumatic diseases are heterogeneous, and overlapping disease syndromes may be found[5]. At present, there is no single diagnostic test capable of differentiating one disease from another. In some human diseases there are well documented changes in the relative proportions of the sugar structures on serum IgG. For example, in rheumatoid arthritis (RA) there is an increase in the number of sugar structures that lack the terminal galactose residue[1]. Extensive investigations have been carried out to determine whether IgG sugar changes are merely a reflection of inflammation, and it has been conclusively shown that these changes are indeed disease-specific[6].

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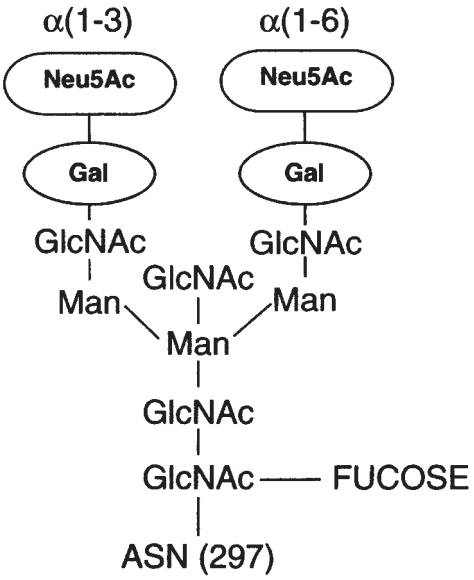

Figure 1. Complex N-linked biantennary oligosaccharide attached to the H-chain Asn 297 in the Fc moiety of IgG. GlcNAc: N-acetylglucosamine; Man: mannose; Gal: galactose; Neu5Ac: α2-6 sialic acid.

Further, these sugar changes seem to be linked with RA pathological mechanisms. Increased levels of IgG hypogalactosylation in early synovitis have been associated with the development of RA[7], and the concentrations of IgG galactose in RA pregnancy fluctuate in parallel with clinical improvement during pregnancy and clinical relapse after delivery[8]. Glycosylation-related pathology is not unique to IgG, as, for example, disease-related sugar changes have also been reported to involve α-1 acid glycosylation[9-11].

At the molecular level, RA circulating immune complexes are preferentially hypogalactosylated[12] and in relation to this, rheumatoid factors have been found to selectively bind hypogalactosylated IgG[13]. These data may explain why immune complexes are abundant in RA.

IgG sugar changes may have further proinflammatory consequences, as absence of galactose means that terminal N-acetyl glucosamine becomes available for binding with mannose binding protein, and this in turn may result in activation of complement with its consequent proinflammatory effects[14,15].

To focus further on the etiology of these sugar changes, our work concerning the glycoimmunology of RA and other rheumatic diseases has revealed a significant defect in the ß1,4-galactosyltransferase enzymes that result in hypogalactosylation of IgG[16,17]. Disruption in glycosylation homeostasis is thought to involve the generation of unique galactosyltransferase isoenzymes that are functionally different from those present in healthy individuals[18]. These changes have been found to be integrally associated with the pathogenic mechanisms associated with inflammation in RA[19].

Using normal-phase high performance liquid chromatography (HPLC), we compared[20] the sugar pools released from serum IgG from patients with a spectrum of rheumatic diseases. This technique allowed us to resolve the sugar pools into 16 peaks, and the relative proportions of the peaks in each disease group were significantly different from those in healthy controls. A characteristic serum IgG sugar profile or sugar print for each disease was found.

To clarify this, we have developed a sugar electrophoresis method that can screen multiple samples relatively rapidly and economically[21,22]. This method has now been optimized to give good resolution of neutral glycans associated with IgG.

We investigated whether IgG sugar printing can differentiate early RA (ERA), RA, and other rheumatic diseases from each other. We wished to determine whether IgG sugar printing using high density electrophoresis might constitute a relatively rapid diagnostic and prognostic test for patients presenting with arthritis.

MATERIALS AND METHODS

Patients (Table 1). Serum samples were obtained from 2 clinics. Patients with established rheumatic disease were selected from the rheumatology clinic at St. George's Hospital, London, including patients with RA, ankylosing spondylitis (AS), psoriatic arthritis (PsA), systemic lupus erythematosus (SLE), juvenile idiopathic arthritis (JIA), and osteoarthritis (OA). The latter was used as a noninflammatory disease control.

Patients with early synovitis were selected from the early arthritis clinic at St Vincent's University Hospital, Dublin. Early synovitis was defined as the presence of arthritic symptoms of less than 6 months' duration. These patients were diagnosed as follows: early RA (ERA), early PsA (EPsA), and undifferentiated seronegative arthritis (UA). Final diagnoses were made after extensive evaluation at followup.

IgG sugar analysis. IgG was purified from serum by a single operation using a column of coarse Sephadex G-25 and DE52[23]. A novel high density electrophoresis-based imaging system was devised[21] that essentially involves release of sugar from IgG using the endoglycosidase peptide-N-glycosidase F, fluorophore-labelling the sugars with 2-amino benzamide acid, resolving a known total quantity of the sugar on a polyacrylamide based gel, and analyzing the sugar bands by optical density with a GAS7000 (UVI-Pro, UVItec, Cambridge, UK) gel scanning system (Figure 2) to determine relative quantities of each sugar. The process of IgG purification, glycan release, and sugar analysis was repeated 11 times on different days with 2 operators using the same sample of serum. The coefficient of variance was calculated for 6 of the resolved bands. The effect of dilution on fluorescence band volume was determined by diluting a stock glycan solution and loading 4 µl in each serially diluted sample. The coefficient of variance of the bands utilized in this analysis was 0.04. Figure 2 illustrates the process. Analysis of lanes was carried out on the complete gel with a homopolymer glucose ladder in one lane and a disease control (OA) in another. The following 9 sugars were identified — asialodigalactose core fucose (g2f), asialomonogalactose (g1), asialoagalactose core fucose bisecting N-acetylglucosamine (g0fb), asialoagalactose core fucose (g0f), which was the most intense band of all the agalactosylated glycan bands, asialoagalactose (g0), monosialodigalactose core fucose (a1f), monosialodigalactose (a1), diasialodigalactose core fucose (a2f), and disialodigalactose (a2).

Statistics. Mean concentrations of each sugar were compared between the 6 disease groups and the patient controls. For 3 sugars, a1, a2f, and a2, a natural log transformation was used to achieve an approximate normal

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Table 1. The sex and average age of patients in each disease group.

Diagnosis	Male	Female	Sample Size	Average Age, yrs	Age Range, yrs
RA	4	16	25	52	22–76
ERA	12	21	33	48	17–75
PsA	1	4	5	54	23–65
EPsA	6	3	9	43	22–73
AS	11	2	13	42	24–72
JIA	2	10	9	12	4–16
SLE	1	19	20	40	23–69
OA	2	18	19	48	11–75
UA	11	9	20	38	15–64

RA: rheumatoid arthritis, ERA: early rheumatoid arthritis, PsA: psoriatic arthritis, EPsA: early psoriatic arthritis, AS: ankylosing spondylitis, JIA: juvenile idiopathic arthritis, SLE: systemic lupus erythematosis, UA: undifferentiated seronegative arthritis.

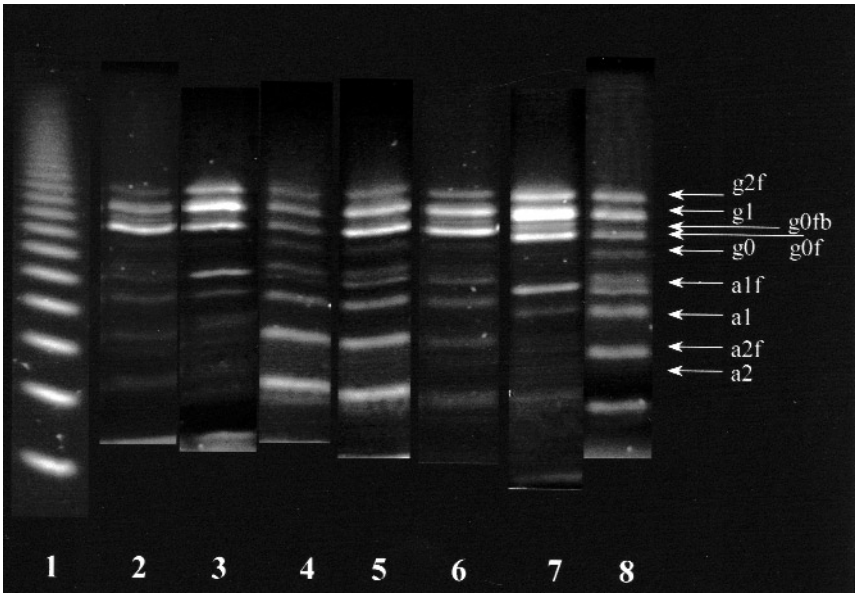

Figure 2. Resolution of IgG N-glycans from 6 rheumatic diseases. Two aminobenzoic acid-labelled IgG N-glycans resolved on 30% polyacrylamide gels with Tris-glycine running buffer. Lane 1: homopolymer glucose ladder; lane 2: RA; lane 3: psoriatic arthritis; lane 4: ankylosing spondylitis, lane 5: JIA, lane 6: inflammatory polyarthritis, lane 7: SLE, lane 8: OA disease control. Identity of glycan bands is shown on the right.

distribution because the distributions were skewed. For each sugar, hospitals and diagnoses were compared using analysis of covariance. Comparison of individual sugar quantities between disease groups was carried out using the Student's t test, and comparison was made to the OA disease control to further analyze each disease/sugar association. RA diagnosis was predicted from sugars using logistic and multinomial regression. The predictors that had the largest p value were removed until all remaining predicting variables had p values less than the conventional 0.05. The final models were used to estimate the probabilities that each subject had RA. If the estimated probability exceeded 0.50, the subject was classified as RA. This prediction was compared to the actual diagnosis using sensitivity, specificity, and proportion predicted correctly. ERA/RA and EPsA/PsA data were combined for analyses concerning RA predictive diagnosis. All analyses were done using Stata 5.0 (Stata Corp., College Station, TX, USA). The receiver operating characteristic (ROC) curve is essentially a plot of sensitivity vs 1 – specificity and is a measure of how good the test is, and is an estimate of the probability that an RA case will have a higher score than a non-RA case. If it is 1.0, then the test is ideal.

RESULTS

Specific disease-associated sugar changes (Table 2). The quantity of each sugar was determined for each of the 9 disease groups and a sugar print compiled (Figure 3).

Disease-associated sugar changes were determined by comparison with OA. There were many significant differences, and no disease group was similar.

Agalactosyl structures. The g0f structure was increased in ERA. The g0 structure was decreased in ERA/RA, EPsA/PsA, and SLE.

Monogalactosylated structures. Significant increases and decreases in quantity were seen. There was a significant increase in g1 in RA and EPsA. A significant decrease was seen with g1 in JIA.

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Table 2. Disease-specific sugar associations. The rows show the relationship between a specific sugar and disease. The columns show the changes in each sugar for a particular disease. The numbers in each box refer to the following (top to bottom): mean sugar quantity, standard deviation, ratio to OA, and p value. The boxes with a bold outline indicate a significant increase in glycan sugar relative to OA disease control. Boxes with broken inner lines and bold outline indicate a significant decrease in glycan intensity relative to OA disease control.

Sugar	RA (25)	ERA (33)	PsA (5)	EPsA (9)	AS (13)	JIA (9)	UA (20)	SLE (20)	OA (19)
g2f	13.2 5.2 0.9 0.2	12.6 5.9 0.8 0.09	10.8 3.6 0.7 **0.04**	12.7 5.5 0.4 0.2	14.0 2.7 0.09 **0.0001**	11.0 2.8 0.7 **0.01**	15.5 4.0 1.0 0.9	17.7 5.1 1.2 0.1	15.3 4.3
g1	31.9 3.4 1.2 **0.001**	28.1 4.9 1.1 0.2	28.0 4.5 1.1 0.4	30.2 4.1 1.2 **0.04**	25.2 5.7 1.0 0.6	21.8 3.8 0.8 **0.03**	29.5 7.4 1.1 0.1	29.1 5.6 1.1 0.08	26.1 4.9
g0fb	3.5 2.4 1.3 0.3	3.4 2.2 1.3 0.2	4.0 3.7 1.5 0.3	2.7 2.5 1.0 0.9	3.0 3.0 1.1 0.8	2.4 2.2 0.9 0.7	2.9 2.7 1.0 0.8	2.6 2.5 1.0 0.8	2.7 2.1
g0f	27.6 7.4 1.6 **0.0001**	26.4 10.6 1.5 **0.002**	31.0 8.9 1.7 **0.0006**	27.8 10.8 1.6 **0.004**	18.0 5.1 1.0 0.8	17.3 5.5 1.0 0.8	21.4 7.0 1.2 0.1	18.1 6.9 1.0 0.9	17.8 5.9
g0	1.8 0.86 0.4 **0.0001**	2.1 2.0 0.5 **0.0001**	2.4 2.7 0.5 **0.02**	1.8 3.2 0.3 **0.0001**	4.3 2.9 1.0 0.7	5.3 1.8 1.2 0.2	2.4 2.0 0.5 **0.0005**	3.1 1.7 0.7 **0.008**	4.5 1.5
a1f	8.6 2.4 0.82 **0.05**	9.0 3.5 0.9 0.1	6.9 2.6 0.6 0.06	10.0 3.4 1.0 0.4	10 2.3 1.0 0.7	8.9 1.8 0.8 0.2	9.3 2.8 0.9 0.3	11.0 3.2 1.0 0.7	10.5 4.0
a1	5.0 1.6 0.59 **0.0001**	5.7 2.3 0.7 **0.0001**	5 2.5 0.6 **0.005**	5.1 2.0 0.7 **0.001**	7.3 3.6 0.9 0.1	9.9 2.9 1.1 0.3	6.1 2.8 1.1 **0.003**	6.4 1.8 0.7 **0.007**	8.6 3.0
a2f	3.4 2.26 0.46 **0.0001**	5.2 4.3 0.7 **0.01**	4.7 2.3 0.1 0.06	3.4 2.2 0.4 **0.003**	9.3 6.3 1.2 0.6	12.9 5.3 1.7 **0.008**	5.4 4.1 0.7 **0.05**	6.2 3.8 0.8 0.2	7.7 4.8
a2	1.5 0.83 0.4 **0.0001**	2.7 1.9 0.7 **0.01**	2.2 .8 0.6 **0.04**	2.6 1.4 0.7 **0.05**	5.9 2.4 1.6 **0.008**	5.6 1.7 1.5 **0.02**	3.5 3.5 0.9 0.1	3.0 1.3 **0.8** 0.9	3.7 1.8

Digalactosylated structures. A significant decrease in the quantity of g2f was seen in PsA, AS, and JIA.

Sialylated structures. The monosialylated structure a1 was significantly decreased in ERA/RA, EPsA/PsA, UA, and SLE. The disialylated structure was significantly decreased in ERA/RA and EPsA/PsA, but increased in AS and JIA. With the addition of core fucose, a2f was decreased in ERA/RA, EPsA, and UA, and increased in JIA.

Quantitative analysis of sugar prints. To examine the profile of the sugar print in more detail (Figure 3), all 9 disease groups were compared with each other. Seven of the 9 sugars were present in statistically significant amounts when each disease group was analyzed: g2f, $p = 0.01$; g1, $p < 0.01$; g0fb, $p < 0.05$; g0f, $p < 0.01$; g0, $p < 0.01$; a1f,

2546$p < 0.05$; a1, $p < 0.0001$; a2f, $p < 0.001$; a2, $p < 0.001$. Only g0fb and a1f were not present in significant amounts. Thus, each disease group could be characterized by a unique sugar print.

Comparison of early and established arthritis. Analysis of the 9 sugars was carried out to determine the significant sugar profile differences between ERA/RA and EPsA/PsA. The quantities of g1 ($p = 0.007$), a2f ($p = 0.006$), and a2 ($p = 0.0070$) sugars were significantly different comparing ERA/RA. No differences were found in the PsA groups.

Comparison of UA with other disease groups. Analysis of the 9 sugars showed that there was no significant sugar profile difference between UA and the 8 other rheumatic disease groups.

RA disease prediction using the sugar print (Figure 4). The 7 sugars identified showing a significant relationship to diagnosis (omitting sugars g1, a2f, and a2, which were shown to differentiate ERA/RA) were used to predict a diagnosis of RA. Using g0f and g0 alone, RA was diagnosed with a specificity of 84.2% and sensitivity of 50.0%, such that the diagnosis was correct for 71.2% of individuals. The area under the ROC curve for this prediction was 0.7812.

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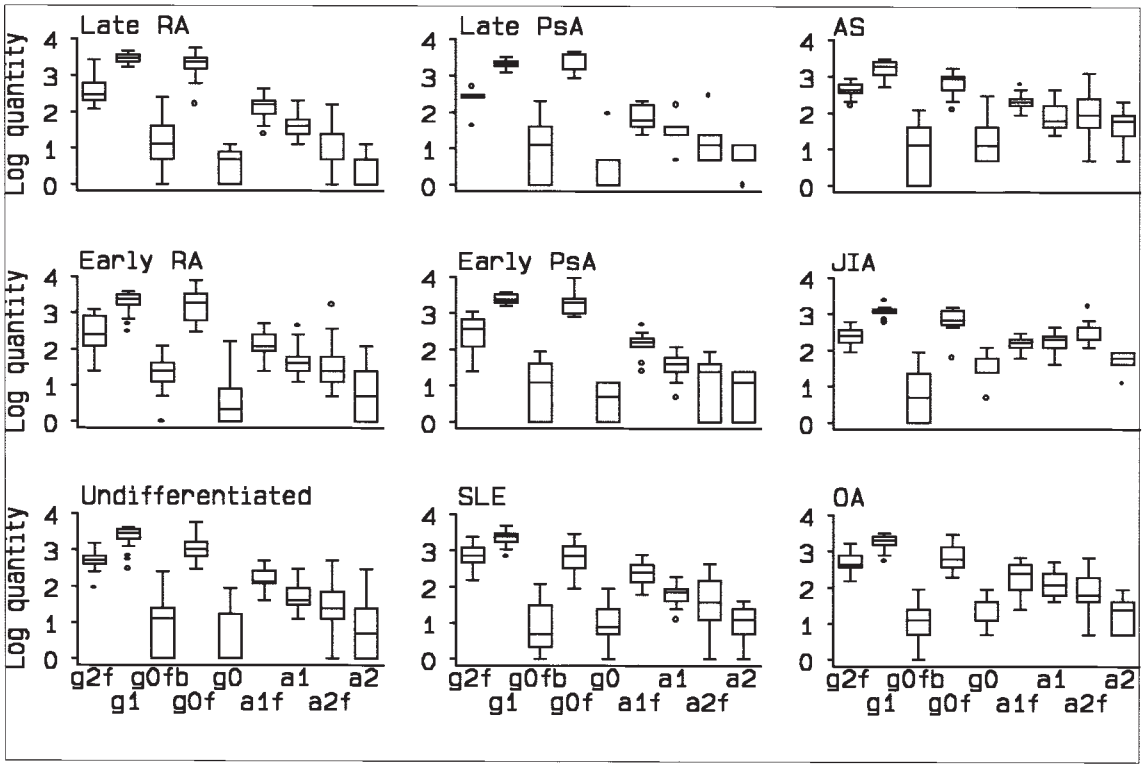

Figure 3. Rheumatic disease sugar print. Graphs show box and whisker plots for each disease. Each plot shows the maximum, 3rd quartile, median, first quartile, and minimum. Because some sugars produce much higher quantities than others, a logarithmic scale has been used.

DISCUSSION

These findings represent a continuation of our previous studies[20,22] indicating that the IgG sugar profiles from a spectrum of rheumatic disorders can be used to differentiate these diseases from each other, a process we call sugar printing. In particular, our primary aim and objective was fulfilled: we observed that sugar printing can differentiate ERA, RA, and other rheumatic diseases from each other and might therefore constitute a relatively rapid diagnostic and prognostic test for patients presenting with arthritis.

We present data from an increased number of patients with a broader spectrum of disease, and in particular, we have attempted to differentiate ERA/RA and PsA from each other, as well as from other diseases. We have again demonstrated the utility of sugar printing as a means of differentiating rheumatic disease. In the case of RA, we found that ERA/RA are distinguished from other diseases by their agalactosyl sugar profiles, but differ from each other by their monogalactosyl and sialylated sugar profiles. UA, in contrast, had no specific distinguishing features, as one would expect from what is probably a heterogeneous group of pathologies. It would appear from the data that PsA behaves similarly to RA, but to confirm this, an increased number of psoriatic patients now need to be analyzed.

Comparing our previous and the present data, it is apparent that the association between g0f, the most abundant band identified with RA, and PsA, is especially strong, as again, it is shown that the agalactosyl association is not a reflection of inflammation per se, as the 5 other rheumatic disorders investigated were in active phase at the time of sampling. The strength of the association with RA has been further confirmed by our ability to use g0f and g0 to predict RA from the pool of patients investigated in this study. Although the sensitivity of the exercise was only 50%, the specificity was 84.2%, and roughly 75% of RA patients were correctly identified as having the disease. However, the study was not designed for the purpose of evaluating a diagnostic tool, and indeed, now we know the sugars upon which to focus, these figures may improve in parallel with our experimental technique. In addition, it is appreciated that a different cohort of RA patients must be examined before this optimistic observation can be taken further.

More studies are required to ascertain the precise sugar disease associations exactly. For example, we report that g0, the least abundant sugar identified, is negatively associated with RA, PsA, UA, and SLE. Previously, we had not made this observation[22]. In addition, we also previously noted a significant compensatory reduction in digalactose sugars in RA patients, and although a reduction was noted in RA and PsA in this study, its magnitude was not so great. Previous data also hinted at a digalactosyl IgG association with lupus[20], but we have been unable to repeat this finding in

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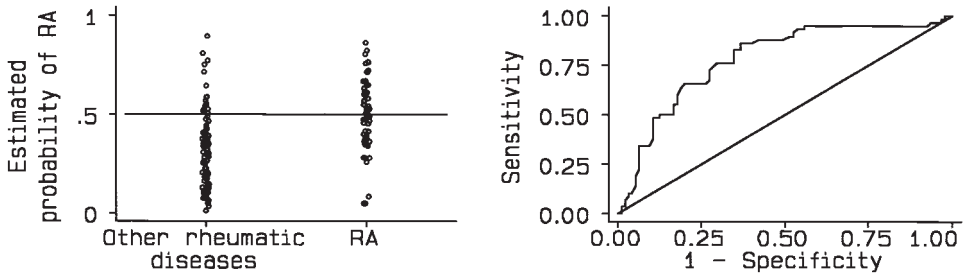

Figure 4. RA disease prediction using the sugar print. Scatter diagram and ROC curve (sensitivity vs specificity) show the estimated probability of RA by regression on best of sugars g0f and g0, which did not differentiate ERA and RA.

this study, with a larger cohort of lupus patients. We now need to determine the exact composition of the electrophoretic bands, although a previous experiment indicated[21] that each band does not seem to be specifically contaminated with mixtures of sugars. Additionally, we should focus upon specific diseases such as lupus and determine whether clinical subdivisions of, for example, patients who have arthritis reveal an association with a recognized sugar print.

Our studies also demonstrated more disease associations with sialylated structures. In particular, there are associations with both RA and PsA, and associations with AS, JIA, and lupus have now been identified. Again, the relationship between these structures and potential pathological mechanisms deserves further elucidation. It is undoubted that every aspect of an autoimmune response[17] is associated to a greater or lesser extent with either glycosylation or protein glycosylation interactions. Areas undergoing active research include the stimulation of T and B lymphocytes by remnant glycosylated epitopes from denatured type II collagen[24]; cell trafficking of these lymphocytes that is dependent upon glycosylation-dependent cell adhesion processes[25]; the involvement of galectins, which have a carbohydrate recognition domain and are involved in specific inflammatory and immunological functions[26]; and the consequences of human interleukin expressions (e.g., interleukins 2, 3, 6, and 7), which have lectin activities and hence are specifically associated with binding to oligosaccharide-containing receptor complexes[27]. In addition, mannose binding protein and complement interactions may result in activation of the autoimmune inflammation cascade, and this requires elucidation in relation to arthritis pathogenesis[28,29].

We hypothesize that the spectrum of autoimmune rheumatic diseases and their overlap is in part a consequence of variable expression of distinct sugar-associated inflammatory mechanisms. Early RA is distinct from RA as different but overlapping inflammatory mechanisms are activated, and although similar mechanisms may be present in patients with PsA (as indicated by their sugar print), other mechanisms may also be switched on that are more specifically associated with psoriatic pathology. By tracking glycosylation changes in a patient's sugar prints, it is hypothesized that a picture of the predominant inflammatory mechanism may be obtained. In this way, we may obtain insight into disease progression, effects of treatment, and apparent switching of one disease to another.

IgG sugar printing has been shown to be effective in differentiation of rheumatic diseases, and we have demonstrated that ERA can be differentiated from both other early arthritic pathologies and their more chronic counterparts. Sugar printing therefore opens the possibility of improving the diagnosis of patients with arthritic disease and may enable the provision of earlier and more appropriate therapy.

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