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Sialyl Lewis^x expression on IgG in rheumatoid arthritis and other arthritic conditions: a preliminary study

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Both infiltrating leukocytes and soluble immunoglobulin form aggregates in synovial fluid during the inflammatory process in rheumatoid arthritis (RA). Some of these changes are probably mediated by the adhesion molecule, E-selectin, which increases its expression with disease activity. As glycosylation changes in IgG in RA are well established, the current study was undertaken to measure the expression of the carbohydrate antigen sialyl Lewis x (sLe^x), on IgG in RA. sLe^x is a major ligand for E-selectin. Using a recently developed ELISA, sLe^x expression was determined in IgG isolated from 8 healthy individuals, 20 RA sufferers (10 early and 10 with more long-standing disease) and 20 patients with other rheumatic conditions (osteoarthritis, ankylosing spondylitis, systemic lupus erythematosus). S Le^x expression on IgG was elevated above the reference range in all but one of the RA patients and this change was highly significant ($P < 0.0006$). Expression of this antigen on IgG was also significantly different from normal in the other arthritic groups ($P < 0.02$), but the changes were much less than that observed for RA. In early RA, sLe^x was inversely correlated with parameters used to measure disease activity. This was not observed with the established RA, where there was weak positive association. These preliminary results indicate that a change in sLe^x expression on IgG is an early finding in the development of RA, which may be important in the development of the disease or for predicting its outcome.

Introduction

In rheumatoid arthritis (RA), there is increased $CD4^+$ expression on lymphocytes in the synovium, increased leukocyte trafficking to the inflamed joint and increased immune complex formation (rheumatoid factors) [1,2]. These processes probably involve some or all of the recently characterized adhesion molecules. E-selectin is one such adhesion molecule [1]. It is found on the cell surface of endothelial cells and it plays an important role in attracting infiltrating leukocyte to a site of inflammation [3]. It has been shown that the expression of E-selectin is elevated on synovial endothelial cells in RA [4–6] and that the level of soluble E-selectin in synovial fluid is correlated with synovial fluid leukocyte counts [7]. Increased expression of E-selectin on the synovial cells is also correlated with increased disease activity [6].

A major ligand for E-selectin is the tetrasaccharide antigen, sialyl Lewis^x (N-acetyl neuraminic acid α2-3Galactose β1-4 (Fucose α1-3)N-acetylglucosamine) [3]. This grouping is found on the membranes of leukocytes and various other cell types [8], and on O- and N-linked oligosaccharide chains of some soluble glycoconjugate macromolecules [9–11]. Increased sLe^x expression occurs in many tissues as part of the pathophysiological process and treatment with soluble sLe^x can inhibit inflammation-induced tissue damage [12].

A well-documented carbohydrate change in RA is the undergalactosylation of N-glycan chains on the Fc region of serum IgG [13]. There is some evidence that this change could lead to the increased formation of rheumatoid factors [14] and/or the fixation of complement [15], two processes that could be important in the pathogenesis of RA. However, definitive evidence for a cause and effect relationship between this undergalactosylation and RA is still forthcoming.

Because of this change in the glycosylation of IgG in RA and the association between E-selectin and RA, it is proposed that there may also be changes in the expression of

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the carbohydrate antigen, sLe^x, on IgG in this disease. In a previous study, the presence of sLe^x on IgG was detected in every specimen from 81 healthy individuals, but no information was given on the extent of this expression [16]. In this study, we have used a recently developed method that quantitates sLe^x [10], in order to compare its expression on IgG isolated from healthy individuals, RA sufferers and patients with other rheumatic conditions.

Materials and methods

Specimens

Blood samples were obtained by venepuncture from 4 women and 4 men (median age 51, range 22–64 years) with no known disease (HY); 9 women and 1 man with seropositive RA of no longer than 9 months duration, "early" (median age 41 years, range 27–75 years); 7 women and 3 men with seropositive RA of generally longer duration, 'later' (median age 50 years, range 40–79 years); 4 women and 1 man with osteoarthritis (OA) (median age 55 years, range 50–76 years); 1 women and 4 men with ankylosing spondylitis (AS) (median age 35 years, range 23–69 years) and 9 women and 1 man with systemic lupus erythematosus (SLE) (median age 35 years, range 21–71 years). Sera were separated by low speed centrifugation ($600 \times g$) and stored at -20 °C until required for analysis. The HY individuals were attending a blood donor session and none were taking oral contraceptives or any other form of medication. The patients with 'early' RA were attending a rheumatology clinic in Dublin (RAD), whereas all the other patients, including the other group of RA patients (RAL), were attending a rheumatology clinic at St George's Hospital in London. All disease groups were diagnosed by the criteria recommended by the ACR [17]. Table 1 gives clinical details for all the patients with RA. The median disease duration for the RAD and RAL were 6 and 36 months respectively. In the OA, AS and SLE groups, therapy was given to 1/5 (*Voltarol*), 2/5 (*NSAID, Naproxen*) and 6/10 (3 *Prednisolone,* 1 *Azathioprine,* 1 *Hydroxychoriquine,* 1 *Cyclosporin*) patients respectively.

Preparation of IgG

Immunoglobulin G was isolated from sera by an affinity chromatography method that was similar to that described for the isolation of haptoglobin [19]. Briefly, 50µl aliquot of serum was mixed with 100µl Protein G Sepharose beads (Pharmacia Ltd.), incubated for 2h at room temperature,

Table 1. A summary of the clinical details and the sLe^x expression on serum IgG for patients with rheumatoid arthritis

Pat No	*Sex*	*Age (y)*	*Medication*	*ESR (mm)*	*Ritchie Score*	*SJ/EMS (no/min)*	*VAS (mm)*	*Duration (months)*	*SLEX (A^{450})*
D1	F	40	No	40	13	20	1	1.5	0.81
D2	F	25	No	6	8	8	1	4	1.2
D3	F	30	No	55	12	25	8	6	0.94
D4	F	71	No	41	0	0	1	6	1.07
D5	M	27	Sulphasalazine	21	12	12	4	9	0.91
D6	F	42	No	37	7	21	4	8	0.98
D7	F	64	No	29	8	20	5	4	0.99
D8	F	75	No	10	5	3	2	2	1.12
D9	F	59	No	32	7	N/A	4	3	1.01
D10	F	40	No	96	8	18	5	6	0.99
L1	M	50	Gold	150	7	60	8	84	1.08
L2	F	79	Methotrexate	37	0	8	1	15	0.98
L3	F	42	Methotrexate	11	5	30	5	84	0.9
L4	F	40	Methotrexate	2	5	960	6	36	1.53
L5	F	67	Gold	78	16	960	9	36	1.31
L6	F	49	Methotrexate	45	3	30	4	96	1.2
L7	F	43	No	42	N/A	0	N/A	2	1
L8	F	69	Methotrexate	19	N/A	960	1	4	0.76
L9	M	54	Methotrexate	7	N/A	N/A	N/A	12	1.12
L10	M	50	Sulphasalazine	22	3	30	2	156	0.89

ESR, Ritchie score, SJ, EMS, VAS are abbreviations for erythrocyte sedimentation rate, Ritchie articular index (18), Swollen joint number, Early morning stiffness (min), and Visual analogue scale (scale 0–10) for pain. For the 'Dublin' patients, only SJ is shown and for the 'London' patients only EMS is given and these two parameters are included in the same column in the table to save space. N/A = not available. For the 'Dublin' patients the correlation coefficients for the relationship between sLe^x and ESR, Ritchie, SJ and VAS were -0.39, -0.65, -0.66, and -0.33 respectively. For the 'London' patients, the correlation coefficients for the relationship between sLe^x and ESR, Ritchie, EMS and VAS were 0.07, 0.41, 0.40, and 0.64 respectively.

and unbound proteins were removed by washing 7 times with 2ml 25 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, 1 mM $CaCl_2$, 0.02% (w/v) Nonindet P40 and 0.05% (v/v) phenyl methyl sulphonyl fluoride. After briefly washing the beads with deionised water to remove the salts, the bound IgG was eluted with 200µl of 0.1 M trifluoroacetic acid. The concentration of purified IgG was determined by an ELISA using a peroxidase conjugate of an anti-IgG antibody (anti-human IgG (H+L), Catalogue code PP003, The Binding Site Ltd., Birmingham, UK) and purified commercial human IgG (Sigma -Aldrich Co Ltd., Poole, UK Catalogue number I-2511) was used as a standard.

Characterization of IgG

The purity of preparations was checked by SDS-PAGE (8% w/v gel) in a Laemmli buffer system and visualized by silver staining [10] and by immunoblotting with an anti-sLe^x monoclonal antibody (Clone CSLEX1, Lot. 50023, Becton Dickinson). The procedure for the immunoblotting was as previously described [20] and the antibody interaction was detected by enhanced chemiluminescence (ECL) using reagents from Amersham Life Sciences. The membrane was prewashed for 1h in a solution containing 0.05% Tween 20 and 0.25% bovine serum albumin (BSA) dispersed in a 1/10 dilution of 25mM Tris-HCl/100mM NaCl (pH = 7.5) (TTBS-10B), followed by treatment for 2h with the anti-sLe^x antibody diluted 1/1000 with TTBS-10B. The membrane was subsequently washed with TTBS-10B for 3h, treated for 2h with an anti-mouse 1gM goat antibody conjugated to HRP (diluted 1/1000 with TTBS-10B, Sera Lab), and finally washed for 3h in TTBS-10B, prior to detecting the binding with ECL. BSA conjugated with sLe^x (Catalogue number NGP 1403, Dextra laboratories, Reading, UK) and unconjugated BSA (Sigma -Aldrich Co Ltd., Poole, UK) were used as positive and negative controls in the blotting. Each molecule of BSA had on average 9.6 sLe^x groupings coupled to it via a 14 carbon spacer arm.

Measurement of sLe^x

Sialyl Lewis x was measured on IgG using a method previously developed to measure sLe^x on haptoglobin and synthetic glycoconjugates [10]. Multiwell plastic plates (Nunc) were coated with 100ng purified IgG in 25 mM Tris-HCl/100mM NaCl, pH7.5 (TBS); for each plate used, other wells were set up with 100ng of commercial IgG (Sigma), 50ng of 5% sLe^x coupled to polyacrylamide (PA-sLe^x) (Syntesome GmbH, Munchen, Germany), or uncoated wells as positive and negative controls respectively. To detect the sLe^x, the plates were exposed to 100ng of the anti-sLe^x monoclonal antibody in TTBS-10B for 1h at 37°C. The antibody binding was detected by incubating the plate for 1h at 37°C with anti-mouse IgM goat antibody (10ng/well conjugated with horseradish peroxidase, Sera Lab). The colour reaction was detected by adding 1mM 3,3′,5,5′ tetramethylbenzidine in 100 mM citrate buffer (pH 6.0) and 4.41 mM hydrogen peroxide and reading the absorbance of the wells at 450nm. All measurements were done in triplicate in duplicate assays. The mean background absorbance for each plate was subtracted from specimen absorbances and varied between 0.15 and 0.25. The mean absorbance values for the commercial IgG and PA-sLe^x were 0.69 ± 0.06 (4 assays) and 0.81 ± 0.05 (6 assays). All differences between groups were analysed statistically using the Mann-Whitney test. All measurements of sLe^x were done in a blind fashion.

Results and Discussion

Typical electrophoretic separations for 3 purified preparations of IgG are shown in Figure 1. The group of 5 specimens on the left were silver stained and the group of 5 specimens on the right are the same specimens after blotting. From the silver stained pattern it can be seen that all the preparations of IgG are very pure. Minor protein bands were detected in the IgG preparations, because the silver staining method used was very sensitive for detecting protein, but from the intensity of the staining of these bands it was concluded that they represented $< 1\%$ of the total protein. Furthermore, only a single band of reactivity was detected after treatment with the anti-sLe^x antibody and this was located in the region of the IgG heavy chain. The anti-sLe^x antibody reacted negatively and positively in the immunoblotting using the BSA and BSA-sLe^x controls, respectively, as might be predicted from the known specificity of the antibody (see Figure 1).

The sLe^x content of IgG isolated from healthy individuals, RA patients, and patients with a variety of rheumatic diseases is shown in Figure 2 (see legend for P values from the statistical analyses). The absorbance values of the HY group varied between 0.53 and 0.80, whereas all but one of the specimens in the two RA groups, gave values (0.76–1.53) that were higher than the normal range. For both RA groups, the difference between the HY and RA groups was very significant ($P < 0.0006$). There was no statistical difference between the values in the two RA groups. The sLe^x content of IgG in the OA, AS and SLE groups was less elevated compared to the HY group ($P < 0.02$).

The data obtained for the RAD group were analysed with respect to various parameters of disease activity (see Table 1 for individual values). Interestingly, sLe^x was inversely correlated with the 'Ritchie Score' ($r = -0.65$), the 'Swollen Joint Count' ($r = -0.66$) and 'Visual Analogue Score' ($r = -0.33$). In contrast, the sLe^x of IgG from RAL group was positively correlated with the 'Ritchie Score' ($r = +0.41$), with 'Early Morning Stiffness' ($r = +0.40$) and with the 'Visual Analogue Score' ($r = +0.64$). There was no correlation in any of the groups between sLe^x content and disease duration or age. There were insufficient specimens

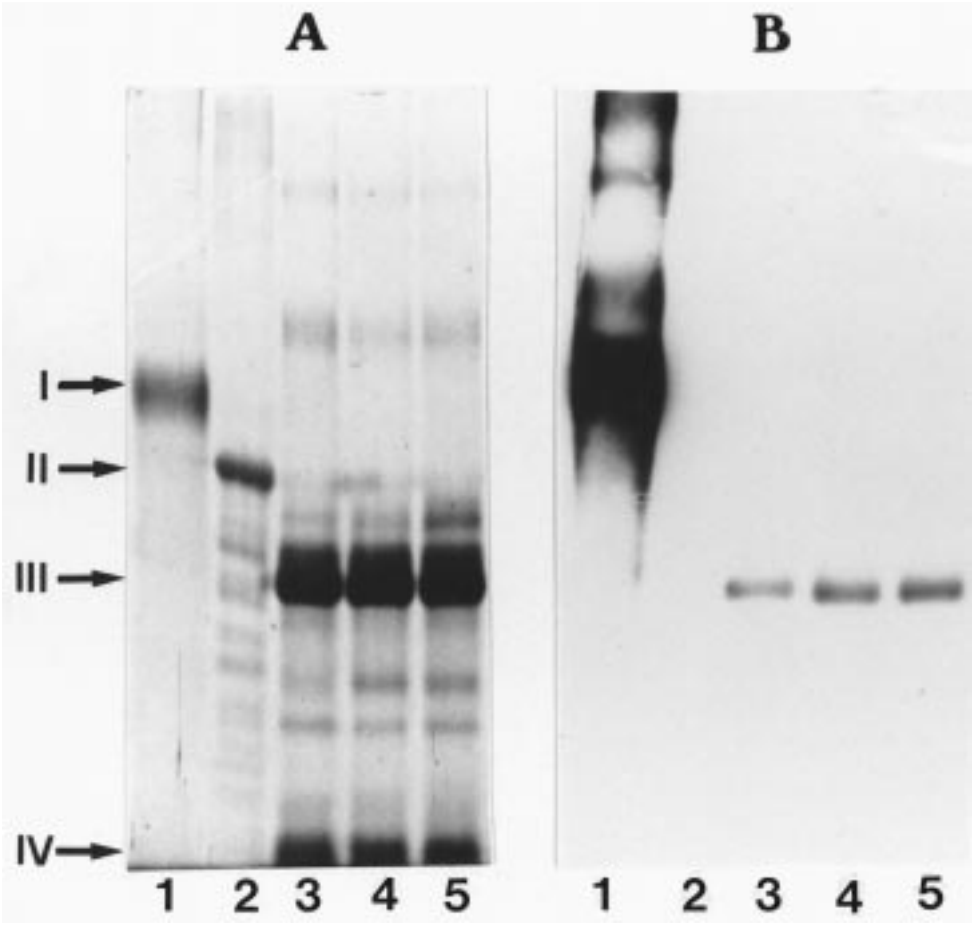

Figure 1. Electrophoretic separation of IgG isolated from blood serum and the expression of sLe^x. Lanes 1–5 in Figure 1A show the electrophoretic patterns for BSA-sLe^x conjugate, BSA, and 3 purified IgG samples after silver staining respectively. Lanes 1–5 in Figure 1B are the same specimens as in Figure 1A, but analysed by immunoblotting with an anti-sLe^x antibody (CSLEX). The position of BSA-sLe^x, BSA, IgG heavy chain, IgG light chain are indicated by Roman numerals I-IV respectively. Approximately 1–2μg were loaded in each lane

to statistically analyse the data with respect to therapy, although visual inspection did not suggest any relationship.

This study has clearly demonstrated that sLe^x expression on IgG is elevated in RA and to a lesser extent in some of the other rheumatic groups. Because some elevated values were observed in the non-RA groups, it could be argued that the changes observed in the RA groups were associated primarily with an inflammatory process and not linked per se to RA. However, in the 'early' RA group the content of sLe^x was inversely correlated with disease activity parameters and this argues against a non-specific process. With the 'later' RA patients this type of association was not observed, and may be therapy as well as the more advanced disease state was affecting sLe^x expression in these patients. Certainly, disease duration did not appear to be an important factor in the expression of sLe^x.

Another explanation for this difference in the relationship between the sLe^x content of IgG and disease activity in the 'early' and 'later' RA groups may be provided by a recent study of soluble E-selectin in serum of RA patients [21]. In the latter study it was found that the levels of E-selectin were, on average, twice as high in patients with disease duration of less than 12 months than in patients who had disease of longer duration. In our 'early' disease group, therefore, high levels of soluble E-selectin in some patients may be binding to IgG sLe^x and removing it from the circulation as a selectin complex. In our 'later' disease patients, however, the IgG sLe^x may not be removed to the same extent because the E-selectin levels are lower. In order to completely resolve this situation a longitudinal study needs to be done in which disease activity, sLe^x, and soluble E-selectin are measured in the same specimens.

It is still unclear which oligosaccharide chains of IgG carry the sLe^x antigen. IgG was reported to have 2.5 chains per molecule, two in the F_c region and the rest in the F_{ab} region [15]. The frequency of these chains in the F_{ab} region depends upon the presence of a consensus sequence for the attachment of N-glycan chains in the hypervariable regions

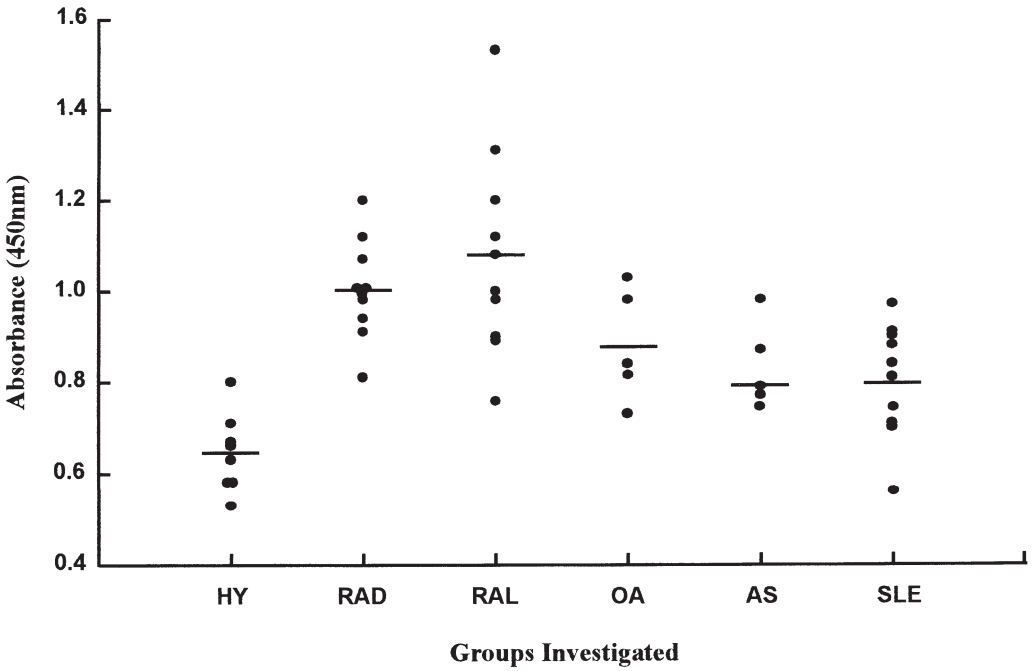

Figure 2. Sialyl Le^{X} expression on IgG isolated from healthy individuals (HY), rheumatoid arthritis sufferers (RAD, RAL) and patients with osteoarthritis (OA), ankylosing spondylitis (AS), or systemic lupus erythematosus (SLE). Sialyl Lewis^{X} expression was measured on IgG isolated from blood serum from the different groups (see text for description of the methods). The antigen content for each specimen is given as an absorbance value measured at 450nm. The mean values (± SD) for the HY, RAD, RAD, OA, AS and SLE groups were 0.65 ± 0.09, 1.00 ± 0.11, 1.08 ± 0.23, 0.88 ± 0.12, 0.81 ± 0.09, 0.80 ± 0.12 respectively. Mean values are shown on the plot as a horizontal lines. The P values for the statistical comparison of the HY group with the RAD, RAL, OA, AS, SLE groups were 0.0006, 0.0004, 0.01, 0.02, 0.01 respectively (Mann-Whitney).

[22]. It would be surprising if the sLe^{x} increase is occurring in the F_c region, because the degree of sialylation at these sites is very low [23] and the degalactosylation that is usually found in RA [23] would lead to even lower amounts of sialic acid. Alpha 2–6 linked sialic acid has been detected on IgG using the lectin *Sambucus nigra agglutinin;* however, it was shown that there was no difference in the binding of this lectin to IgG from healthy individuals and RA patients [23]. Nothing is known about the expression of alpha 2–3 linked sialic acid (one of the defining residues on the sLe^{x} tetrasaccharide antigen), although the above evidence would suggest that it may be present on a very restricted population of IgG molecules produced by a particular clone of B cells.

Understanding molecules involved in the adhesive interactions between soluble and cellular components in synovial fluid could be important for explaining some of the pathological changes in the RA joint. As the number of specimens and scope of this study were limited, one must be careful in drawing extensive conclusions from the results. However, they do show a clear increase in the expression of sLe^{x} on serum IgG in RA. Hence it might be speculated that if there were also high amounts of sLe^{x} on molecules in the synovial fluid in the joint this could affect interactions between inflammatory cells and selectins. Even if the change has no pathological significance, its presence as an early disease marker could be useful for monitoring disease progression. More extensive studies are needed to resolve such questions.

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