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Investigation into the Concanavalin A reactivity, fucosylation and oligosaccharide microheterogeneity of α_1-acid glycoprotein expressed in the sera of patients with rheumatoid arthritis

Moira A. Elliott[a,*], Heather G. Elliott[a], Kaye Gallagher[a], John McGuire[a], Max Field[b], Kevin D. Smith[a]

[a]*Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow G1 1XW, UK*
[b]*Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow G4, UK*

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Abstract

α_1-Acid glycoprotein (AGP) exists as an heterogeneous population of glycosylated variants (glycoforms) in plasma. The concentration of AGP increases some 2–5 fold in certain pathophysiological states exemplified by the chronic inflammatory disease, rheumatoid arthritis (RA). Moreover, the expressed glycosylation pattern alters in such conditions, indicating functional significance that is likely to be related to the oligosaccharide heterogeneity. We have investigated the heterogeneity of AGP glycosylation using the technique of high pH anion-exchange chromatography (HPAEC). AGP was isolated from the blood of RA sufferers, partially separated by Concanavalin A (Con A) affinity chromatography into bound and non-bound fractions and was enzymatically deglycosylated. Chromatography on the pellicular HPAE resin at pH 13 separated the released oligosaccharides and allowed a comparison of profiles in terms of branching and fucosylation. Results demonstrate an abnormal RA AGP glycosylation, with a tendency towards tri- and tetra-antennary oligosaccharides and enhanced fucosylation, in addition to the possible existence of penta-sialylated RA AGP glycoforms.

Keywords: α_1-Acid glycoprotein; Glycoproteins

1. Introduction

α_1-Acid glycoprotein (AGP or orosomucoid) is an extensively glycosylated serum protein with glycosylation accounting for some 42% of its molecular mass of 41 000 amu [1]. Five N-linked glycosylation sites at asparagines 15, 38, 54, 75 and 85 are occupied by covalently bound complex oligosaccharide chains which may exist as di-, tri- or tetra-antennary structures [2]. Classified as a positive acute phase reactant, the concentration of AGP is observed to increase some two- to five-fold in certain disease states [3].

A clearly defined function for AGP remains largely unknown, although studies have demonstrated an immunomodulatory role for the glycoprotein [4]. From in vitro experiments, the clear importance of the carbohydrate moiety of AGP in the regulation of immune function has been demonstrated [5]. Further in vitro work showed varying ability to

*Corresponding author.

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modulate immune responses by Con A reactive fractions of AGP [4]. Moreover, the expression of the tetrasaccharide adhesive antigen sialyl Lewis X (SLeX) on oligosaccharides of AGP has been shown in acute inflammation and thereafter throughout the inflammatory response [6].

This study examined oligosaccharide variance in AGP expressed in the blood of patients suffering from the chronic inflammatory condition, rheumatoid arthritis. The analysis of oligosaccharide chains is complicated by the existence of, and the need to distinguish between, subtle monosaccharide linkage variations from one isomer to another, in addition to the potential for multiple branching and sialylation. The requirements for sensitivity, high resolution, reproducibility and speed for analysis are all satisfied by high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [7–9].

Using an HPAEC–PAD system, we were able to analyse the oligosaccharides and monosaccharides released from rheumatoid arthritis AGP. We demonstrate that the oligosaccharide patterns of rheumatoid AGP vary considerably from that expressed in normal subjects, in addition to highlighting the possible existence of penta-sialylated rheumatoid arthritis (RA) AGP glycoforms. Using lectin affinity chromatography with the complex di-antennary oligosaccharide-specific lectin, Concanavalin A (Con A), we show a tendency towards tri- and tetra-antennary oligosaccharides in rheumatoid AGP.

2. Experimental

2.1. Materials

The XK26 chromatography columns, Q Sepharose Fast Flow and Red Sepharose column packings were all purchased from Pharmacia (Milton Keynes, UK). The Reactive Blue 2 Sepharose, Concanavalin A Sepharose 4B and Dowex-50W strongly acidic cation-exchanger were from Sigma (Poole, UK). Econo-Pac 10DG disposable chromatography columns packed with Bio-Gel P6 and Poly-Prep columns were purchased from Bio-Rad (Hemel Hempstead, UK). The peptide-N-glycosidase F (PNGase F), was purchased from Oxford GlycoSystems (Oxford, UK). HPLC/spectral-grade trifluoroacetic acid (TFA) was purchased from Pierce and Warriner (Chester, UK). Sodium hydroxide solution (50%, w/v) was from BDH (Poole, UK). Rathburn Chemicals (Murrayburn, UK) were the suppliers of HPLC-grade water. All other chemicals were of reagent grade and were purchased from Sigma (Poole, UK).

OligoStandards PI05 (di-sialylated di-antennary), GP03 (di-sialylated di-antennary, core fucosylated), FT07 [sialylated tri-antennary, 2× (α2,6)], FT07a [sialylated tri-antennary, 2× (α2,3)] and GP01 (tetra-sialylated, tetra-antennary, mixture of four isomers) were all purchased from Dionex (Camberley, UK). Fucose, mannose, galactose and glucosamine monosaccharide standards were purchased from Sigma (Poole, UK).

Units of freshly frozen normal plasma, tested negative for the presence of hepatitis B surface antigen and antibodies to HIV and HCV, were kindly supplied by Dr. Robin Fraser of the Scottish National Blood Tranfusion Service (Law Hospital, Carluke, UK). The whole blood samples from individual patients with rheumatoid arthritis were a kind gift from Dr. Max Field, Centre for Rheumatic Diseases at the Glasgow Royal Infirmary (Glasgow, UK).

2.2. AGP purification

AGP was isolated from freshly frozen normal plasma and from the whole blood of individual patients suffering from rheumatoid arthritis using the method developed in our laboratory, as previously reported by Smith et al. [10]. For individual rheumatoid patients, blood was supplied as 5–18 ml volumes in heparinised tubes. Briefly, an initial precipitation step of whole blood with poly(ethylene glycol) (PEG) 3350 to a final concentration of 40% (w/v) PEG, followed by centrifugation at 10 000 *g* separated soluble plasma proteins into the supernatant fraction. A series of three low pressure column chromatography steps using dye ligand (Reactive Blue 2 Sepharose and Red Sepharose) and anion-exchange (Q Sepharose Fast flow) chromatography purifies AGP with intact oligosaccharide structure, avoiding desialylation and degradation. AGP yield for this procedure has been calculated at 90%.

2.3. Desalting

Desalting of each purified AGP sample, dissolved in sodium acetate buffer from the final low-pressure chromatography column step, was carried out using Econo-Pac 10DG chromatography columns, pre-packed with Bio-Gel P6 size exclusion desalting gel, according to the BioRad stated method. The salt-free sample was collected, freeze-dried and stored at 4°C until use. Recovery of applied sample to this size exclusion desalting column was 100%.

2.4. Monosaccharide release from whole glycoprotein

A 10-μl solution of 5 μg/μl whole AGP was added to 100 μl of 2 *M* TFA and heated at 100°C for 4 h. The hydrolysate was then passed down a 5×0.5 cm column that was packed with Dowex-50W strongly acidic cation-exchanger, which had been washed previously with several column volumes of HPLC water at a flow-rate of 0.5 ml/min at room temperature. After the application of 1 ml of sample, 2 ml of eluent were collected as a single fraction and were dried to yield monosaccharide sample (J. McGuire, unpublished method).

2.5. Glycosidase digestions for the release of oligosaccharide

Intact oligosaccharides from 0.5 mg of AGP were released enzymically using the asparagine amidase peptide-N-glycosidase F (PNGase F). The successful release of oligosaccharide required prior protein denaturation by boiling in 160 μl of HPLC-grade water [10]. After boiling, 40 μl of five times concentrated buffer was added to yield a final solution (200 μl) of denatured AGP in 20 m*M* sodium phosphate buffer, pH 7.5. Addition of 2 U of PNGase F enzyme followed, with incubation at 37°C for 24 h. Following this time period, a further 1 U of PNGase F was added and an additional 24-h incubation allowed. Separation of the released oligosaccharides from the enzyme and remaining protein was achieved by precipitation with two 500-μl volumes of ice-cold ethanol, each ethanol addition being followed by centrifugation [11]. Decanting and drying of the supernatant yielded the oligosaccharide sample.

2.6. High-pH anion-exchange chromatography (HPAEC)

Monosaccharide and oligosaccharide analyses were carried out using the Dionex DX 300 chromatography system, where the oxyanion forms of monosaccharides and oligosaccharides were separated at pH 13 on a micropellicular anion-exchange resin with PAD. Typically, 10 μg of a monosaccharide mixture and 30–100 μg of released oligosaccharides were analysed. The HPAEC system consisted of an advanced gradient pump and pulsed electrochemical detector (PED-2), controlled via an advanced computer interface by a Vtech 486SX 25 computer. Mono- and oligosaccharide samples were chromatographed on a CarboPac PA-100 column (25×0.4 cm I.D.; Dionex).

PAD involved the following potentials and durations (for both mono- and oligosaccharide analyses): time=0 s, E=+0.05 V; 0.29 s, +0.05 V; 0.49 s, +0.05 V; 0.50 s, +0.05 V; 0.51 s, +0.6 V; 0.6 s, +0.6 V; 0.61 s, −0.6 V; 0.65 s, −0.6 V; 0.66 s, +0.05 V.

For monosaccharides, the gradient applied was as follows: equilibration with 16% solvent A (1 *M* sodium hydroxide)–84% solvent B (HPLC-grade water) at a flow-rate of 0.5 ml/min, an isocratic run of 16% A–84% B over a period of 20 min after sample injection, followed by a wash at 50% A–50% B for 10 min and concluding with a return to the intial conditions of 16% A–84% B over a period of 5 min.

For oligosaccharides: equilibration with 10% A (1 *M* sodium hydroxide)–5% B (1 *M* sodium acetate)–85%C (HPLC-grade water) at a flow-rate of 1.0 ml/min. After sample injection, the starting conditions are maintained for 10 min, followed by application of a linear gradient run from 10% A–5% B–85% C to 10% A–20% B–70% C developed over 40 min. The limit conditions are maintained for a further 5 min before a change over 1 min to 50% A–50% B, which is held for 10 min before returning to the initial conditions of 10% A–5% B–85% C.

Determination of the elution position of specified oligosaccharide charged species on the chromato-

gram was achieved from runs of the standards detailed in Section 2.1. Similarily, monosaccharide elution positions were determined from runs of monosaccharide standards as in Section 2.1.

2.7. Lectin affinity chromatography with Concanavalin A Sepharose 4B

A Poly-Prep column was packed with Concanavalin A (Con A) Sepharose 4B to a bed volume of 2 ml and was washed with 0.1 *M* NaCl–0.01 *M* Tris, pH 7.5 [12]. The column was washed with several column volumes of buffer to achieve equilibration at room temperature, evidence of which was given by two low and concordant readings of UV absorbance at 225 nm (denoted as B1 and B2 in each of A, B and C in Fig. 2). Use of 225 nm for detection had been determined to be more sensitive for AGP solutions than the use of 280 nm. Usually 1–2 mg of AGP sample were dissolved in 3 ml of buffer and applied to the column without elution and left at room temperature for 15 min. After this time, eighteen 1.5-ml fractions, representing the Con A unbound fraction, were collected at a flow-rate of 0.5 ml/min at room temperature and their UV absorbance at 225 nm was determined. A 100 m*M* solution of α-methyl-D-mannopyranoside in buffer was prepared and 10 ml were added to the column and left for 15 min; seven fractions (1.5 ml each), which represented the Con A bound molecules, were then collected and their UV absorbance was determined. Further washing of the column with increased concentrations of α-methyl-D-mannopyranoside did not elute further protein fractions, indicating that all of the protein had been eluted and collected. The collected and combined samples were then desalted as before and were freeze-dried prior to storage at 4°C.

3. Results

3.1. HPAEC analysis of monosaccharides

The addition of TFA to whole glycoprotein breaks all glycosidic bonds to give an amino acid–monosaccharide mixture [12] that can be effectively separated by Dowex resin, where amino acids are retained by virtue of their charge, while monosaccharides elute unhindered. Monosaccharides at a pH of 13 in HPAEC act as weak acids, due to their high pK_a values (≈12), subtle differences in which allow the effective separation of a mixture into individual monosaccharide peaks. In comparison with the standards (Fig. 1), the individual peaks of an AGP TFA hydrolysate mixture are identified as component monosaccharides. Using this system, detection at picomolar sensitivities is achievable.

Chromatographic runs of a range of concentrations of each monosaccharide reveals a linear response in HPAEC in terms of increasing area under each peak for increasing monosaccharide concentration. De-

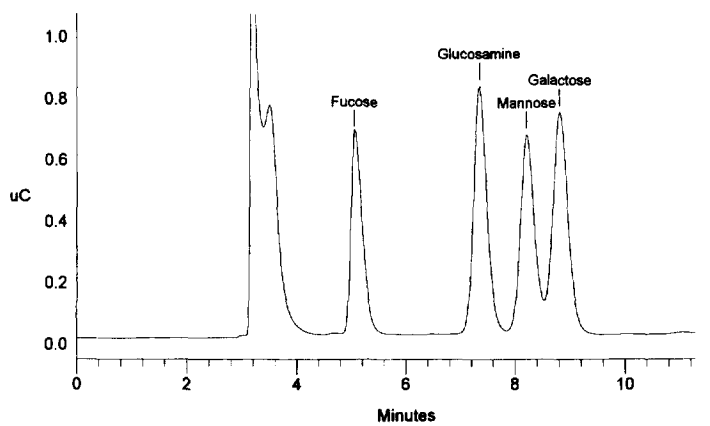

Fig. 1. HPAEC profile of 1 μg of each standard monosaccharide injected as a mixture in a final volume of 25 μl. To identify individual monosaccharide elution positions, runs of each individual standard were performed. For digests of rheumatoid AGP monosaccharides, 10 μg of total digest was run.

termination of the equation of the line for each monosaccharide standard concentration versus the corresponding chromatographic peak area allows one to calculate the amount of monosaccharide released from a known amount of glycoprotein monosaccharide hydrolysate. Using this system, values for the fucose and mannose content for each rheumatoid patient were calculated.

The molar amount of mannose in each sample was equated, by ratio, to represent 3 mol as for each oligosaccharide chain, no matter the number of antennae, there is only three mannose expressed, always in the tri-mannosyl core [1]. Therefore, calculating the amount of fucose in relation to 3 mol mannose gives information on the degree of fucosylation on each AGP oligosaccharide chain. For the rheumatoid arthritis patients tested, all differed from normal in the level of expressed fucosylation (Table 1), although there was a wide range in the degree of fucosylation within the rheumatoid population, from only 6% of side chains carrying fucose in RA 3, to RA 6 where 1.48 fucose molecules are found per tri-mannosyl core. Interestingly, for RA 3, AGP was isolated from a sample of synovial fluid, in the same manner as from blood, and showed an increased expression of fucose, i.e. 80% compared with 6% in blood.

Table 1
Ratio of reactivity with Con A and fucose content of normal plasma and rheumatoid arthritis AGP

Patient	Age	Con A reactivity ratio[a]	Fucose content[c] (mol/3 mol mannose)
Normal	–	0.59[b]	–
RA 1	54	0.60	0.77
RA 2	65	0.66	0.50
RA 3	25	0.96	0.06
RA 4	73	1.45	0.21
RA 5	43	1.52	0.14
RA 6	53	2.04	1.48

[a] The Con A reactivity ratio is taken from the ratio of unbound/bound molecules (this is calculated from the protein content of each fraction on desalting).
[b] Normal plasma Con A reactivity ratio was calculated from an average of three runs, standard deviation=0.08.
[c] For fucose analysis, 50 μg of whole glycoprotein was used in each case.

3.2. HPAEC analysis of released oligosaccharides

Fig. 2 contains examples of typical rheumatoid HPAE chromatograms. The absence of peaks in any of the three chromatograms at 7 min, corresponding to released sialic acid, shows no method-induced desialylation of the oligosaccharide structures.

Rheumatoid patient RA 1 (Fig. 2B) overall is unlike the normal subject. The multiple peaks, particularly within the tri-sialylated charge band, are indicators of rheumatoid oligosaccharide microheterogeneity. This is due to the expression of variously fucosylated and/or sialylated rheumatoid glycosylated variants (or glycoforms). Di-sialylated character is still present, although to a lesser extent.

The profile of rheumatoid patient RA 6 (Fig. 2C) also illustrates the importance of the tri-sialylated region to the apparent detriment of di-sialylation. As for patient RA 6, no evidence of a peak at 7 min, representing sialic acid, is evident from the chromatogram. Thus, the appearance of peaks at 12 and 15 min indicate the presence of mono-sialylated structures in this patient and do not merely represent the effect of method-induced oligosaccharide degeneration. Interestingly, both patients RA 1 and RA 6 display peaks at 45 min, the retention time of which exceeds those usually associated with tetra-sialylated structures.

3.3. Lectin affinity chromatography with Concanavalin A Sepharose 4B

Using a mini column procedure [13] with the lectin Con A, whole AGP was fractionated into Con A unbound molecules, eluted in buffer, and Con A bound molecules, eluted with 100 m*M* α-methyl-D-mannopyranoside (Fig. 3). The reactivity of each patient's AGP with Con A was expressed as the fraction of unbound molecules divided by the bound molecules (Table 1), determined from a calculation of the area under the curve for each fraction. Of the six rheumatoid patients tested, two showed a Con A pattern that was similar to the normal pattern (for example, RA 2; Fig. 3B). The remaining four patients all showed a greater tendency towards tri- and tetra-antennary oligosaccharides, with a reduced affinity for Con A compared to normal (for example, RA 5; Fig. 3C).

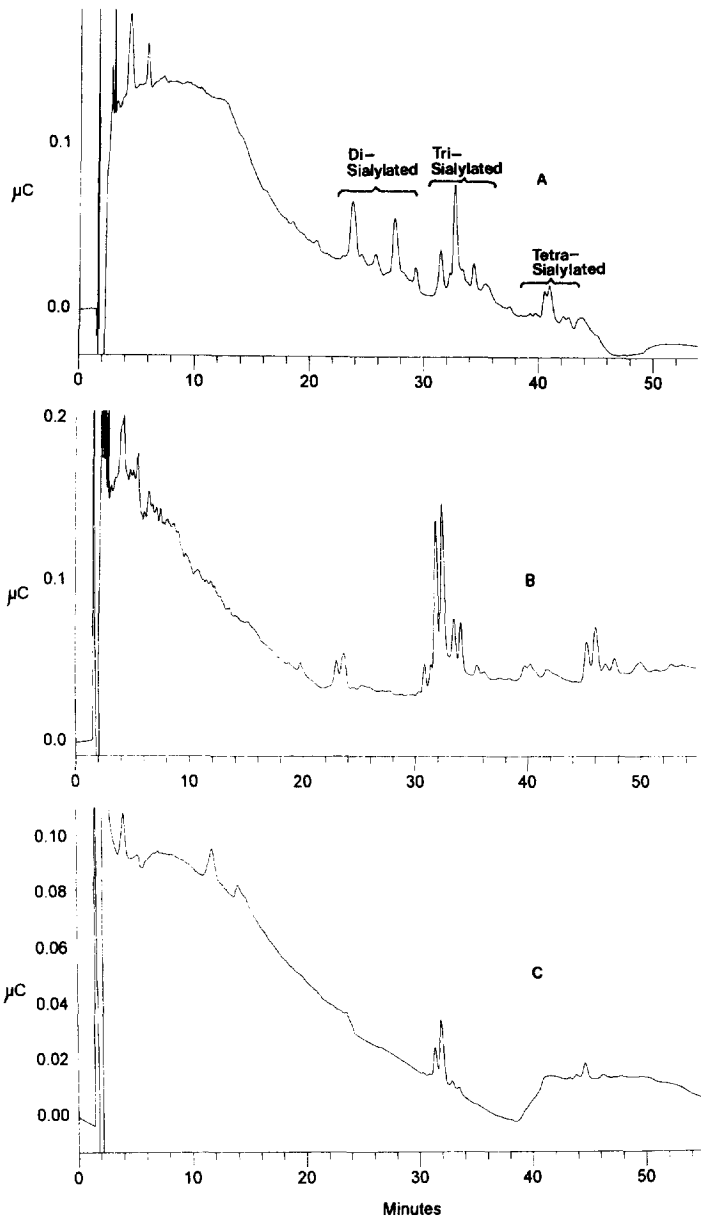

Fig. 2. Representative HPAEC profiles of oligosaccharide released from (A) pooled normal plasma AGP, (B) rheumatoid patient RA 1, (C) rheumatoid patient RA 6 (the positions of the sialylation bands shown were determined from runs of commercially available fully characterised standards, see Section 2.1).

4. Discussion

In TFA monosaccharide analyses, normal plasma samples showed no detectable fucose, which stood in marked contrast to the rheumatoid patients where all had evidence of fucosylation. This is likely to be significant in the disease state, since fucose occurs on N-linked chains in a variety of linkages, several of which are important antigenic structures. Fucose has never been reported in the α-1,6-linkage to the proximal N-acetylglucosamine in human AGP, thus the fucosylation presented is therefore assumed to be

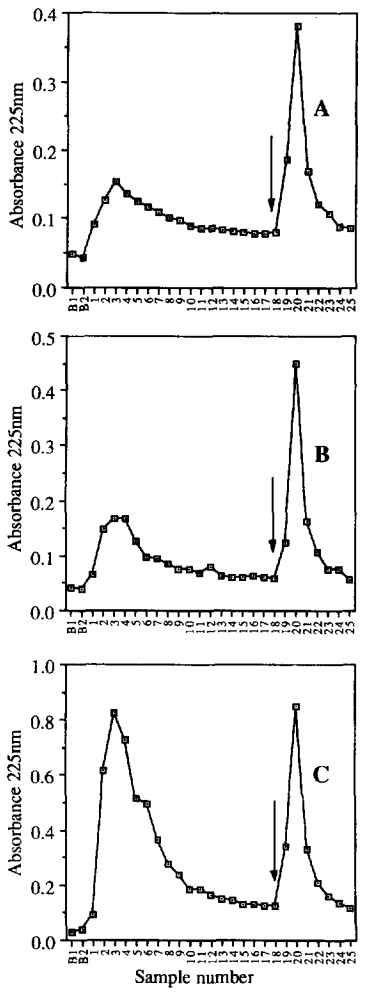

Fig. 3. Representative traces of AGP reactivity with the lectin, Concanavalin A. (A) Pooled normal plasma, (B) rheumatoid patient RA 2, (C) rheumatoid patient RA 5. The first peak represents the Con A unbound fraction. ↓ in each plot represents the addition of 100 m*M* α-methyl-D-mannopyranoside in buffer. BI and B2 in each figure represents blank readings 1 and 2 taken from column washings alone prior to sample application.

that of the outer branch sugars. The significance of outer branch fucose in an α-1,3-linkage to N-acetylglucosamine, if expressed on the same branch as sialic acid α-2,3-galactose, is in the potential for expression of the tetrasaccharide antigen, sialyl Lewis X (SLeX; sialic acid (α-2,3) galactose (β-1,4) [fucose(α-1,3)] N-acetylglucosamine (β1-)).

The expression of SLeX has been demonstrated in AGP in acute inflammation and thereafter throughout the inflammatory response [6]. E-selectin, an adhesion molecule expressed on cytokine-activated, inflamed endothelium, has the greatest affinity of the three selectin molecules for SLeX, whose expression on leucocytes is involved in the recruitment and extravasation of white cells to an inflammatory focus [14]. The binding of myeloid cells to E-selectin has been shown to be inhibited by glycoproteins expressing SLeX [15]. It has been suggested that through SLeX expression on AGP oligosaccharides, AGP may in some way be involved as part of a negative feedback mechanism, restricting leucocyte travel to an inflamed site [6,15,16].

These results, demonstrating an enhanced inflammatory AGP oligosaccharide fucosylation, also may indicate the potential for extensive SLeX expression on inflammatory AGP oligosaccharides, if the fucosylation appears on chains that are concomitantly α-2,3-sialylated.

From oligosaccharide profiling using HPAEC, variations in rheumatoid AGP from that expressed in normal plasma are displayed [10]. HPAEC separates, in the first instance, on the basis of formal negative charge, which in AGP oligosaccharides is carried by chains terminating in N-acetylneuraminic acid (sialic acid). Further separation within each charge band is then made on the basis of differences in the monosaccharide linkage, e.g. sialic acid α-2,3- or α-2,6 linked to terminal galactose, the presence or absence of fucosylation, or for example under sialylation of a higher branching structure (e.g. a tri-sialylated tetra-antennary chain does not coelute with a fully sialylated tri-antennary chain) [7–9].

The presence of the tri-sialylated AGP oligosaccharides in rheumatoid patients at the expense of other sialylation bands is perhaps an indicator of the importance of tri-sialylated triantennary (or indeed under sialylated tetra-antennary) oligosaccharides in the diseased state. This is in agreement with the observed glycosylation pattern of AGP produced from the HepG2 cell line inflammatory model [17]. Moreover, the presence of peaks outwith the area of the chromatogram usually associated with tetra-sialylation, appeared to be, if not a rheumatoid-specific, then an inflammatory-induced effect on AGP oligosaccharides. In repeated runs of tetra-sialylated standard oligosaccharides, the elution position did not appear at this 45-min point. This may in

fact tentatively suggest the existence of inflammatory-induced penta-sialylated AGP glycoforms that are not associated with a normal AGP oligosaccharide profile.

The lectin, Con A, from Jack bean *Canavalia ensiformis* has the ability to recognise and bind complex di-antennary oligosaccharide chains [18]. In normal serum, AGP may be categorised into fractions that are reactive and unreactive to this α-mannosyl binding lectin. In certain disease and pathophysiological states, the normal reactivity ratio is known to alter and abnormal AGP glycoforms, showing an altered reaction with Con A, have been identified [4]. Work by Mackiewicz et al. [19] in patients with RA, related the oligosaccharide microheterogeneity expressed by AGP in RA to their disease activity. Whilst those having inactive disease were observed to display no significant difference from healthy patients, those experiencing mildly to severely active disease were shown to have statistically significant differences in their AGP glycosylation pattern compared with those from healthy controls. On examination of AGP oligosaccharide microheterogeneity in RA in relation to disease duration [20], the results demonstrated a progression in RA from increased Con A binding in the early stages to reduced lectin binding in the chronic patient. Thus, it would appear that tri- and tetra-antennary structures are significant in the later stages of the disease, in agreement with the results from HPAEC presented here.

In a disease characterised by attacks and remissions, the apparent "normality" of two patients in relation to the Con A reactivity ratio is perhaps to be expected (RA1 and RA 2, Table 1). However, despite an apparent "normality" in this area, fucosylation is among the highest noted of the six patients studied. For RA 3, a sample of synovial fluid AGP (isolated in an identical manner to the blood sample) showed a Con A reactivity ratio of 1.81, i.e. greater tri- and tetra-antennary chains on AGP in the synovial fluid than in blood.

The results presented here of the apparent microheterogeneity of sialylated charge bands displayed in RA AGP HPAE chromatograms (due to a variation in α-2,3/α-2,6 sialylation and/or fucosylation), together with enhanced fucosylation, adds to the earlier presented evidence of RA SLeX expression [6]. This adds weight to the theory of AGP's involvement in a feedback mechanism to limit leucocyte extravasation through oligosaccharide SLeX expression [10].

5. Conclusion

Rheumatoid AGP shows marked differences in oligosaccharide pattern from that expressed in the normal population. Increased fucosylation is a consistent feature, accompanied by a general trend towards molecules expressing tri- and tetra-antennary oligosaccharide chains, to the detriment of di-antennary chains. We have highlighted the possibilty of penta-sialylated rheumatoid AGP glycoforms. Additionally, in a one patient comparison of blood and synovial fluid AGP, we have observed significantly increased fucosylation and tri- and tetra-antennary oligosaccharide character in the synovial sample, which may merit further investigation.

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