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Glycosylation of α1-acid glycoprotein in inflammatory disease: analysis by high-pH anion-exchange chromatography and concanavalin A crossed affinity immunoelectrophoresis

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High-pH anion-exchange chromatography with pulsed amperometric detection is a highly sensitive technique that can be used for detecting changes in sialylation and fucosylation, as well as different branching patterns of N-linked oligosaccharides in glycoproteins. We examined the N-glycans of α1-acid glycoprotein obtained from twelve patients with various inflammatory conditions with this technique, as well as traditional concanavalin A crossed affinity immunoelectrophoresis. We found the chromatographic profiles of N-glycans in all patients with rheumatoid arthritis to be very similar, but significantly different from normal controls. N-glycans from patients with ulcerative colitis also showed specific alterations in their chromatographic profiles. However, some heterogeneity was found between these patients, perhaps reflecting changes in glycosylation secondary to certain states of the disease, or to medical treatment. We conclude that this technique is useful for detailed mapping of glycosylation changes in α1-acid glycoprotein in clinical samples, and that it may be used to further increase our knowledge about glycosylation changes in response to inflammatory disease.

***Keywords*: α1-acid glycoprotein, concanavalin A, crossed affinity immunoelectrophoresis, fucosylation, high-pH anion-exchange chromatography, inflammation**

***Abbreviations*: AC, acute cholangitis; AGP, α1-acid glycoprotein; CAIE, crossed affinity immunoelectrophoresis; Con A, concanavalin A; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; IEC, ion exchange chromatography; RA, rheumatoid arthritis; SLe^x, sialyl Le^x; UC, ulcerative colitis**

Introduction

α1-Acid glycoprotein (AGP, orosomucoid), is built up of 181 amino acids and five N-linked complex type oligosaccharide side chains. It is one of the major acute phase reactants, yet its physiological function is not well understood [1]. Glycosylation of AGP has been studied with respect to fetal development, hormonal influence, liver diseases, renal failure, acute and chronic inflammatory conditions, and malignant diseases [2–9]. Crossed affinity immunoelectrophoresis (CAIE) with the lectin concanavalin A (Con A) has been used to separate differently glycosylated AGP fractions by reducing the electrophoretic mobility of AGP fractions rich in biantennary N-linked oligosaccharides, or by separation of Con A-binding fractions on a Con A affinity column [10, 11]. In Con A CAIE, separated AGP fractions are commonly visualized by immunoprecipitation in a second dimension gel electrophoresis, as originally described by Bøg-Hansen and co-workers [10]. However, the Con A CAIE pattern of AGP gives no detailed information on the carbohydrate structures of the N-linked oligosaccharide side chains in AGP. It may also be very similar in different pathological conditions, which reduces its value in diagnostic procedures.

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a highly sensitive method which allows detailed mapping of oligosaccharides released from glycoproteins [12, 13]. In the present study we compared HPAEC-PAD with Con A CAIE for analysing the glycosylation pattern of AGP isolated from blood donors and patients with various inflammatory diseases, in order to investigate if HPAEC-PAD would add any valuable information on glycosylation changes of AGP in individuals with different types of inflammation.

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Materials and methods

Chemicals

AGP, concanavalin A, and methyl-α-D-glucopyranoside were from Sigma (USA); N-glycosidase F from Boehringer Mannheim (Germany); DEAE Sepharose CL-6B and the HiTrap SP column from Pharmacia (Sweden); agarose LE and ME from SeaKem (USA); rabbit anti-human AGP immunoglobulins, rabbit anti-human serum immunoglobulins, and human serum protein calibrator (HSPC) from DAKO (Denmark); Ag50W-X8, and Bio-Gel P-4 (Extra Fine) from Bio Rad (USA); tritiated sodium borohydride (11.6 Ci $mmol^{-1}$) from Amersham (USA), and polyethylene glycol (PEG) 6000 and 20000 was obtained from Kebo (Sweden). All other reagents were analytical grade chemicals.

Patient samples

EDTA-plasma was obtained from blood donors (and pooled), and from twelve patients with inflammatory diseases. Five patients had rheumatoid arthritis (RA), six had ulcerative colitis (UC), and one patient suffered from acute cholangitis presenting clinically as sepsis (AC/sepsis). Samples were drawn from patients admitted to hospital because of increasing symptoms of a previously known disease (RA or UC). The patient with AC/sepsis was admitted to hospital with sepsis of unknown cause when the sample was drawn. The patient was subsequently found to suffer from acute cholangitis. All patients included in this study had elevated plasma concentrations of AGP and/or CRP, confirming the presence of a systemic inflammatory disease. Patient data are briefly summarized in Table 1. Four patients were taking oral corticosteroids (two with RA and two with UC, Table 1), but patients receiving any other systemic immunosuppressive treatment were excluded from the study. The samples were stored at $-70\,^{\circ}C$ until purified and analysed. The AGP concentration was measured by electroimmunoassay, using monospecific antiserum for precipitation, and HSPC as a standard. We also included commercially available lyophilized AGP (Sigma) in the study for comparison.

AGP purification

A modification of the ion exchange chromatography (IEC) method in two steps, as described by Succari and co-workers [14] was used for purification of AGP. In short, 10 ml plasma was dialysed against starting buffer (20 mM citrate-phosphate buffer, pH 4.0) over night. After centrifugation at $500 \times \mathbf{g}$ for 10 min, the supernatant was applied to a column of DEAE Sepharose CL-6B (12×2.6 cm) equilibrated with the starting buffer. The column was washed with starting buffer until no absorbance at 280 nm was recorded, the buffer was then changed to 20 mM citrate-phosphate buffer, pH 7.0, which eluted AGP together with a few other proteins, predominantly α-1-antichymotrypsin and haptoglobin. Fractions of 2 ml were collected and AGP-containing fractions were pooled, dialysed against distilled water over night, and concentrated by dialysing against PEG 20000. The concentrated sample was applied on a HiTrap SP column (5 ml) equilibrated with the same starting buffer as previously. AGP was eluted as a single peak with 20 mM citrate-phosphate buffer at a pH of 4.8. Both chromatographic steps were carried out at room temperature, the first

Table 1. Clinical data on the patients studied, including AGP concentration, approximate duration of disease, and any immunosuppressive treatment (prednisolone or equivalent) taken by the patient on admission.

Patient	*Sex*	*Age*	*AGP ($g\,l^{-1}$) ref range 0.4–1.1 $g\,l^{-1}$*	*Duration of disease*	*Prednisolone ($mg\,d^{-1}$)*
RA 1	Female	63	1.9	1 month	0
RA 2	Female	47	1.5	10 years	0
RA 3	Female	75	1.9	> 10 years	5
RA 4	Male	67	1.6	> 10 years	17.5
RA 5	Female	85	1.7	> 10 years	0
UC 1	Female	24	1.2	1 week	0
UC 2	Female	29	> 3	1 year	40
UC 3	Female	26	1.9	3 years	0
UC 4	Male	46	2.6	> 10 years	40
UC 5	Female	32	1.1	14 years	0
UC 6	Male	62	1.1	> 20 years	0
AC/sepsis	Male	68	1.9	1 week	0

step with a flow rate of 150 ml h^{-1}, and the second step with a flow rate of 300 ml h^{-1}. No traces of other plasma proteins could be detected by immunoelectrophoresis with rabbit anti-human serum immunoglobulins.

Con A CAIE

The Con A CAIE method used was modified from Bøg-Hansen [10]. For the first dimension gel, 30 ml 1% agarose LE in electrophoresis buffer (barbital 0.075 M with calcium lactate 0.9 mM) containing 56 mg Con A was used. The sample (2.5 µl; AGP concentration approximately 1 gl^{-1}) was separated at 25 V cm^{-1} for 50 min. Gel strips were then transferred to the second dimension gel; 30 ml 1% agarose ME in electrophoresis buffer with 125 µl rabbit anti-human AGP immunoglobulins, 0.16 mg methyl-α-D-glucopyranoside, and 3 ml 30% PEG 6000 incorporated in the gel. The separation was performed at 8 V cm^{-1} for 12 h. After electrophoresis the gel was washed several times with 0.15 M NaCl, dried, and stained with Coomassie brilliant blue.

Release and purification of N-linked oligosaccharides

The N-linked oligosaccharides were released by endoglycosidase digestion as described by Landberg and coworkers [15]. Samples of purified AGP (0.5 mg) were dissolved in 150 µl 0.1 M *di*-sodium hydrogen phosphate containing 0.1% SDS. After addition of 10 µl 0.1 M EDTA and 5 µl mercaptoethanol, samples were boiled for 4 min. The samples were then incubated with 1 U (5 µl) N-glycosidase F (Boehringer Mannheim) at 37 °C for 48 h. After enzymatic digestion, 17 µl 5% acetic acid was added, and the samples were incubated at room temperature for 2 h. The samples were evaporated to dryness under a stream of nitrogen. The released N-glycans were reduced by adding 300 µl 100 mM sodium hydroxide containing 2.5 mCi tritiated sodium borohydride. After incubation for 15 h at room temperature, 3 mg of cold sodium borohydride was added and the samples were incubated for 3 h at room temperature. The samples were neutralized by adding acetic acid, and desalted on a cation-exchange column (Ag50W-X8, H + form, 1ml gel volume). The samples were then evaporated with methanol under a stream of nitrogen. The released N-linked oligosaccharides were purified on a Bio-Gel P-4 column (1.6 × 50 cm) and eluted with 0.1 M ammonium acetate at a flow rate of 0.3 ml min^{-1}. Fractions (1.5 ml) were collected and examined for radioactivity by liquid scintillation. Fractions containing released N-linked oligosaccharides were pooled and lyophilized.

Desialylation of oligosaccharides

Sialic acid was released by hydrolysis with 2 M acetic acid at 80 °C for 3 h [15]. The desialylated oligosaccharides were further purified by anion exchange chromatography (Ag-X4A, OH^- form), and lyophilized.

HPAEC-PAD

The lyophilized oligosaccharides were dissolved in distilled water and analysed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, USA). *N*-acetyl and *N*-glycolylneuraminic acid were included as internal standards. The enzymatically released N-glycans were separated on a Dionex Carbopack PA-100 column maintained at 20 °C, with a flow rate of 1 ml min^{-1}. Samples of 25 µl were injected and the HPAEC-PAD measurements were performed using either the 'S' gradient for sialylated N-glycans or the 'D' gradient for the desialylated oligosaccharides (Table 2). The 'D' gradient was as described by Hermentin and coworkers [12]. Fractions (0.4 ml) were collected and examined for radioactivity by liquid scintillation.

Statistics

Results from the patients with RA and UC were compared with normal and tested for significance using the two-sided Student's *t*-test.

Table 2. Gradients used for HPAEC-PAD analyses of sialylated (gradient 'S') and desialylated (gradient 'D') N-glycans enzymatically released from normal and patient AGP.

Gradient '*S*'			*Gradient 'D'*		
Min	*Eluent 1 (0.1 M NaOH)*	*Eluent 2 (0.5 M NaAc in 0.1 M NaOH)*	*Min*	*Eluent 1 (0.2 M NaOH)*	*Eluent 2 (0.6 M NaAc in 0.2 M NaOH)*
0	100	0	0	100	0
0.1	100	0	5	100	0
70	60	40	35	80	20
90	0	100	45	0	100
95	100	0	50	0	100
105	100	0	51	100	0
			60	100	0

Results

HPAEC-PAD analysis of N-glycans from normal AGP

N-glycans from normal AGP were released by endoglycosidase treatment. The released oligosaccharides were analysed by HPAEC-PAD (Figure 1a). In the HPAEC system used, the number of sialic acid and fucose residues in an oligosaccharide greatly affects its retention time. Therefore it is possible to assign the peaks in the HPAE-chromatogram to groups corresponding mainly to mono-, di-, tri, and tetra-sialylated oligosaccharide structures with or without

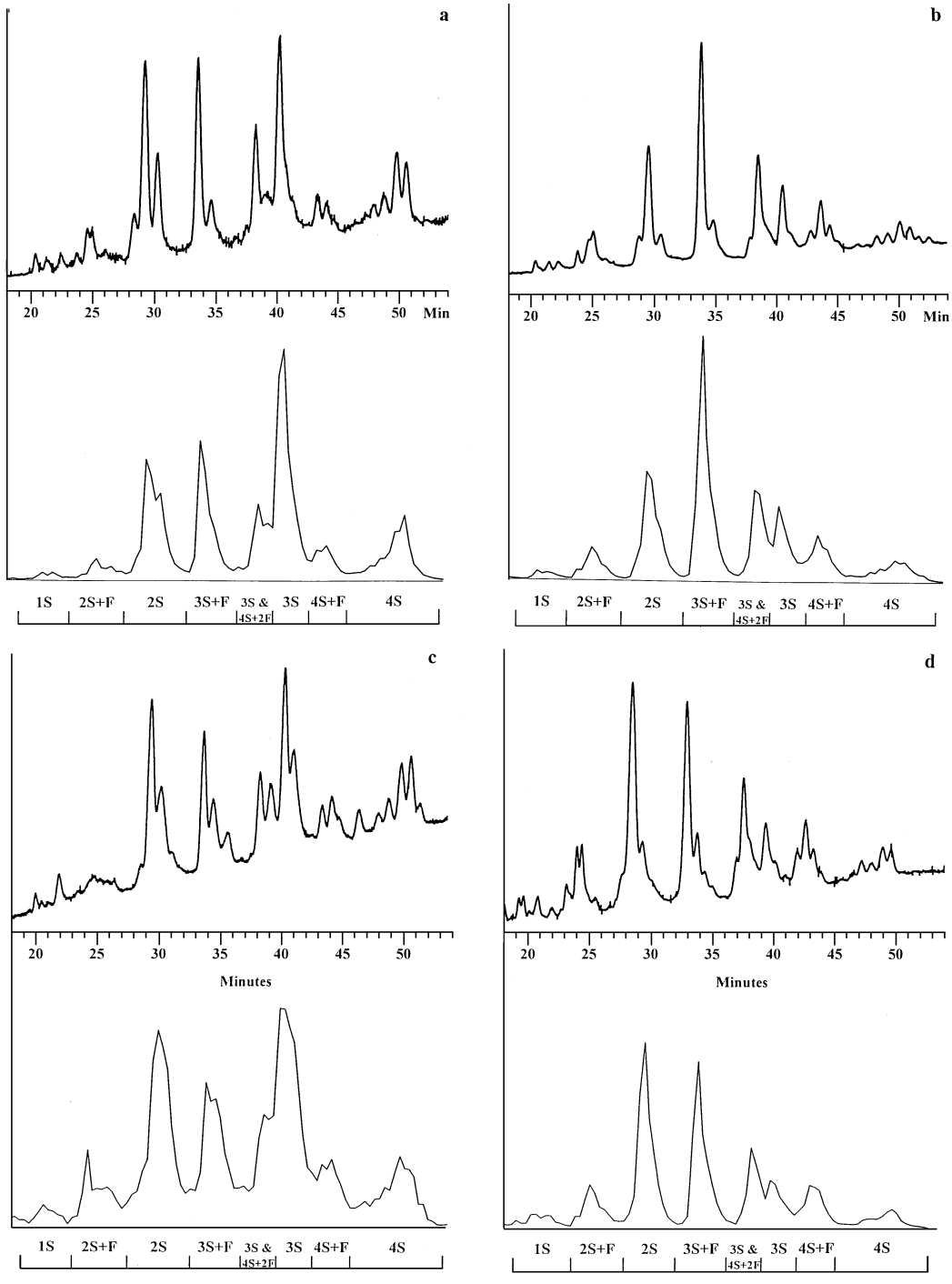

Figure 1. HPAEC-PAD chromatograms of N-glycans enzymatically released from AGP (top), and the corresponding plots from liquid scintillation (bottom). Bars denote groups of peaks corresponding to N-glycans with a certain number of sialic acid and additional fucose residues (where present). 1S, 2S, 3S, 4S: N-glycans with one, two, three, and four sialic acids respectively. F, 2F: N-glycans with one and two additional fucose residues respectively. (a) normal pool; (b) a patient with RA; (c) a patient with UC; (d) a patient with AC/sepsis.

fucose substitution [12]. These groups are indicated by bars in Figure 1a.

HPAEC-PAD analysis of N-glycans from patient AGP

The HPAEC-PAD analysis of the released N-glycans from patients with RA, UC, and AC/sepsis showed different profiles for the different inflammatory diseases (Figure 1b–d). The HPAEC-PAD profiles of AGP N-glycans obtained from the patients with RA and AC/sepsis were both strikingly different from the normal pool. All chromatograms of AGP N-glycans from patients with RA revealed a sharp increase in size of the group of peaks representing fucosylated, trisialylated oligosaccharides, while the groups of peaks representing non-fucosylated, tri- and tetrasialylated oligosaccharides were decreased compared to the chromatograms of N-glycans from normal AGP (Figure 1b). In contrast, the sialylation and fucosylation of AGP N-glycans in most patients with UC were similar to N-glycans from normal AGP (Figure 1c), but more heterogeneous profiles were noted, with several additional peaks. The HPAEC-PAD profile of the AGP N-glycans from the patient with AC/sepsis showed a relative increase in the group of peaks corresponding to di-sialylated oligosaccharides, and a relative decrease in the groups of peaks representing non-fucosylated tri- and tetra-sialylated oligosaccharides (Figure 1d). Sialic acid eluted at 14.5 min in all investigated samples, indicating that retention times were identical between HPAEC runs.

HPAEC analysis with radiochemical detection

The PAD response depends to some degree on the composition of the oligosaccharide analysed [16]. To obtain a relative quantitation of the AGP N-glycans the released oligosaccharides were treated with ^{3}H-$NaBH_4$, specifically labelling the reducing termini of the oligosaccharides. Thus, by measuring the radioactivity in the differently glycosylated fractions separated by HPAEC, a more careful quantitation could be made compared to using PAD response only. The peaks representing mono-, di-, tri-, and tetra-sialylated oligosaccharides with or without fucose substitution are indicated by bars in Figure 1a–d. The relative amounts of differently glycosylated fractions were calculated for patient and normal AGP, as percent of the total scintillation count from the separated N-glycans in each HPAEC run. The results are shown in Table 3. The sialylation and fucosylation of AGP N-glycans from normal plasma was identical to commercially obtained AGP. In AGP N-glycans from the patients with RA, the amount of fucosylated di- and tri-sialylated oligosaccharides were significantly increased compared to AGP N-glycans from the normal pool ($p < 0.01$), while the amount of non-fucosylated tri- and tetrasialylated structures was decreased (Table 3, Figure 1b). All patients with RA showed very similar AGP N-glycan profiles. The glycosylation profiles of AGP from the patients with UC however, were in most cases similar to the normal pool, though more heterogeneous with a slight increase in fucosylated, di-sialylated oligosaccharides (Table 3, Figure 1c). In contrast to the patients with RA, the inter-individual variations in the N-glycan profiles were larger among the patients with UC (Table 3). In AGP N-glycans from the patient with AC/sepsis, fucosylation was increased, while sialylation was decreased, with a shift to mono- and di-sialylated N-glycans (Table 3, Figure 1d).

HPAEC-PAD analysis of desialylated AGP N-glycans

To obtain further structural information on the AGP N-glycans, the released oligosaccharides were desialylated by acid hydrolysis before HPAEC-PAD analysis. Analysing desialylated oligosaccharides with the HPAEC-PAD method gives more information on the degree of fucosylation, with separation of di-fucosylated N-glycans. In addition, information was obtained on the branching of the N-glycans [12]. The HPAEC-PAD profile of desialylated N-glycans from normal AGP is shown in Figure 2a. Desialylated AGP N-glycans from patients with RA showed a relative increase in fucosylated N-glycans, and

Table 3. The different N-glycan composition in normal and patient AGP, with reference to the content of sialic acid and fucose residues; given as percent of total amount of N-glycans. For RA and UC, mean values ± SD are given. (1S, 2S, 3S, 4S: N-glycans with one, two, three, and four sialic acid respectively; F, 2F: N-glycans with one and two additional fucose residues.)

	1S	*2S + F*	*2S*	*3S + F*	*3S & 4S + 2F*	*3S*	*4S + F*	*4S*
Normal pool	2	5	20	17	12	27	6	12
AGP Sigma	1	4	21	17	16	23	6	13
RA ($n = 5$)	3 ± 1.5	10 ± 2.6**	23 ± 2.6	27 ± 3.2**	12 ± 3.0	11 ± 2.5*	7 ± 1.1	7 ± 1.1**
UC ($n = 6$)	3 ± 1.1	7 ± 1.6*	22 ± 3.8	18 ± 6.7	10 ± 3.3	21 ± 6.0	7 ± 1.5	11 ± 4.9
AC/sepsis	4	9	28	23	13	8	9	6

*Significantly different from normal pool: $p < 0.05$; **$p < 0.01$.

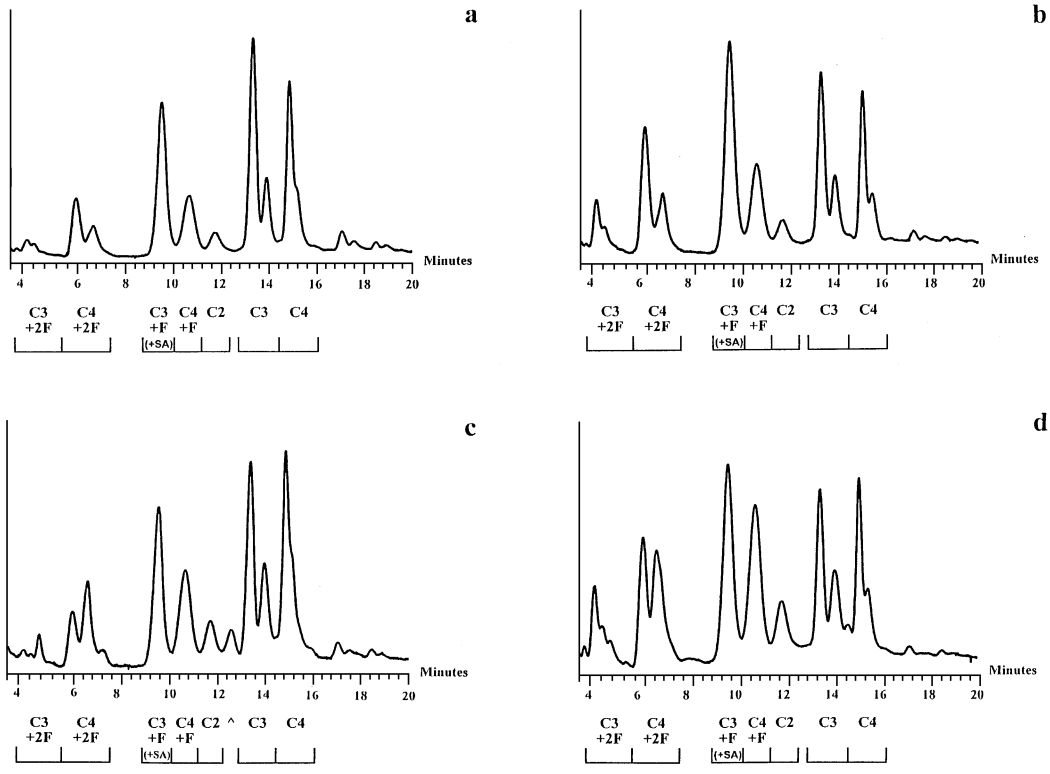

Figure 2. HPAEC-PAD chromatograms of desialylated N-glycans enzymatically released from AGP. Bars denote groups of peaks corresponding to N-glycans with a certain number of antennae and additional fucose residues (where present). C2, C3, C4: N-glycans with two, three, and four antennae respectively. F, 2F: N-glycans with one and two fucose residues respectively. SA: residual free sialic acid that may be left in a sample after desialylation. (a) normal pool; (b) a patient with RA; (c) a patient with UC (^ denotes additional peak); (d) a patient with AC/sepsis.

consequently, a relative decrease in non-fucosylated oligosaccharides (Figure 2b). The glycosylation profiles of desialylated AGP N-glycans from the patients with UC were similar to normal. However, the distribution of the individual peaks within the groups of peaks representing tri- and tetra-antennary N-glycans with two additional fucose residues in the patients with UC was different from the chromatograms of N-glycans from normal AGP, and in one patient with UC, an additional peak eluted in between the peaks representing biantennary and non-fucosylated triantennary N-glycans (Figure 2c). In the patient with AC/sepsis, fucosylation, as well as the relative amount of biantennary AGP N-glycans, were increased compared to normal (Figure 2d). *N*-glycolyl-neuraminic acid eluted at 29.5 min. in all investigated samples, indicating that retention times were identical between HPAEC runs.

Analysis of AGP by Con A CAIE

AGP purified from pooled donor plasma, as well as commercially obtained AGP separated into two major fractions corresponding to Con A non-reactive AGP and AGP reacting weakly with Con A (Figure 3a). In patients with RA, the fraction representing Con A non-reactive AGP was increased (Figure 3b); in patients with UC, there was a slight increase in fractions reacting weakly with Con A, as well as an additional third fraction corresponding to AGP reacting more strongly with Con A (Figure 3c), while in the patient with AC/sepsis, a considerable increase in fractions corresponding to Con A-reactive AGP was seen, compared to normal AGP (Figure 3d).

Discussion

Con A CAIE has been used in several reports to detect glycosylation changes in AGP in response to different inflammatory conditions. The method primarily detects an increase in AGP glycoforms containing biantennary N-glycans. As a complement to measuring only the concentration of AGP, Con A CAIE analysis may be useful in a clinical setting to distinguish an acute intercurrent inflammatory process in patients with chronic inflammation [8]. However, no information is gained on the changes in fucosylation and sialylation that occur in some types of inflammation. HPAEC-PAD has been widely used as a highly sensitive method for analysis of complex carbohydrates [16]. Recently, Hermentin and co-workers showed that HPAEC-PAD could be used for the analysis of AGP

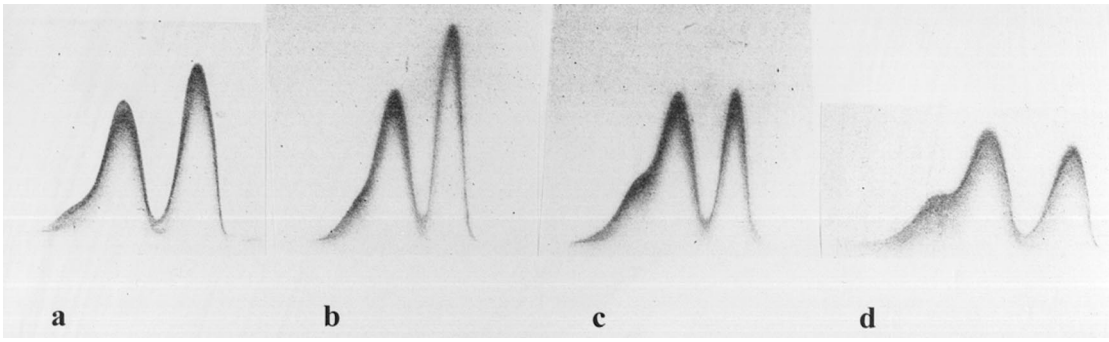

Figure 3. The patterns of Con A CAIE performed on AGP. Only the second dimension gels are shown. In the first dimension electrophoresis the anode was to the right, and in the second dimension the anode was at the top. (a) normal pool; (b) a patient with RA; (c) a patient with UC; (d) a patient with AC/sepsis.

N-glycans. Although the exact oligosaccharide structures cannot be determined from HPAEC retention times only, the AGP N-glycans could be separated into groups of peaks representing structures with variable numbers of sialic acid and fucose residues [12]. By further analysing the released AGP N-glycans after desialylation, information on branching was obtained.

In the present study HPAEC-PAD was used, in addition to Con A CAIE analysis, to acquire a more detailed profile of the glycosylation of AGP associated with various inflammatory diseases. In AGP N-glycans from the patients with RA, we found a characteristic and reproducible HPAEC-PAD profile, with increased fucosylation; the most conspicuous change being a significant increase in tri-sialylated N-glycans with one additional fucose. There was also a relative decrease in sialylation, which in fact may be considered a lack of increase of sialylated structures, when taking into account that the total plasma concentration of AGP was elevated in the patients studied (Table 1). In contrast, sialylation and fucosylation of AGP N-glycans from the patients with UC were in most cases similar to N-glycans from normal AGP, though with more heterogeneous profiles with several additional peaks. In addition, variation in N-glycan profiles between different patients with UC was larger than for patients with RA. Desialylated AGP N-glycans from patients with UC showed different profiles within the groups of peaks containing di-fucosylated tri- and tetra-antennary N-glycans, and in one patient with UC, an additional structure not present in normal AGP was noted. We aimed primarily to investigate different types of chronic inflammation, but included one patient with acute inflammation (AC/sepsis) for comparison. In AGP N-glycans from this patient, increased fucosylation and a shift to less sialylated N-glycans were detected. When analysing the desialylated N-glycans, an increase in biantennary N-glycans was seen, consistent with the results from the Con A CAIE analysis, as well as minor peaks not present in normal AGP, perhaps indicating abnormal glycan structures. An increase in fucosylated and biantennary N-glycans have been described previously in sepsis [17]. Previous studies have also indicated changes in sialylation and fucosylation of AGP in patients with RA, compared with normal [8, 18, 19]. De Graaf and co-workers showed in a longitudinal study that increased fucosylation, as well as decreased sialylation of AGP occurred in RA, consistent with our results. It was also shown that fucosylation decreased following a successful treatment with methotrexate [18]. However, it is not known whether this change depends on the activity of the disease or if the drug itself exerts a direct effect on fucosylation in the hepatocytes. In our study, no patients received any immunosuppressive treatment other than corticosteroids when samples were drawn (Table 1). Two patients with UC had started taking high daily doses of prednisolone before admission to hospital, and it remains to be investigated whether such medical treatment can affect the glycosylation profile of AGP. Further studies including a larger amount of patient samples are needed to establish the specific clinical value of analysing AGP glycosylation profiles.

Glycosylation changes of AGP in inflammatory conditions have been described as being a direct consequence of the increased activity of cytokines, independent of the concurrent increase in polypeptide synthesis leading to increased plasma concentrations of the acute phase proteins [6]. In many studies, immunomodulative functions have been attributed to AGP, and changes in the way AGP interacted with leukocytes has been described as being dependent on changes in glycosylation, *eg* branching and sialylation [20, 21]. Increased fucosylation and the resulting increase in sialyl Lewis^x (SLe^x) structures on AGP has recently been postulated to be a feed-back mechanism, for inhibition of the interaction between leukocytes and inflamed endothelium mediated by SLe^x structures on leukocytes and E-selectin on the inflamed endothelium [22, 23]. This is perhaps the most intriguing hypothesis to

date concerning the biological role of AGP in the acute phase response.

In the present study HPAEC-PAD has proved to be a useful method for detailed mapping of glycosylation changes in AGP from individual patients, providing information on sialylation and fucosylation, not revealed by Con A CAIE. One draw-back of the HPAEC-PAD method in clinical use is the laborious preparation of samples. On the other hand, detailed studies of AGP glycosylation may provide new tools for diagnosis and monitoring of inflammatory disease. Further studies are needed to investigate the clinical importance of glycosylation changes in inflammatory bowel disease (UC and Crohn"s disease), and the possible interference of corticosteroids and other anti-inflammatory drugs.

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References

1 Schmid K (1975) In *The plasma proteins, structure, function, and genetic control*, vol 1 (Putnam FW, ed) pp 184–228. New York: Academic Press.
2 Seta N, Tissot B, Forestier F, Feger J, Daffos F, Durand G (1991) *Clin Chim Acta* **203**: 167–76.
3 Succari M, Foglietti M-J, Percheron F (1990) *Clin Chim Acta* **187**: 235–42.
4 Biou D, Chanton P, Konan D, Seta N, N'Guyen H, Feger J, Durand G (1989) *Clin Chim Acta* **186**: 59–66.
5 Freyman NH, van de Velde EJ, Belpaire FM, Lameire NH (1989) *Clin Chim Acta* **181**: 47–54.
6 Pos O, van der Stelt M, Wolbink G-J, Nijsten MWN, van der Tempel GL, van Dijk W (1990) *Clin Exp Immunol* **82**: 579–82.
7 Fassbender K, Zimmerli W, Kissling R, Sobieska M, Aeschlimann A, Kellner M, Müller W (1991) *Clin Chim Acta* **203**: 315–28.
8 Pawlowski T, Mackiewicz S, Mackiewicz A (1989) *Arth Rheum* **32**: 347–51.
9 Hansen J-E, Larsen VA, Bøg-Hansen TC (1984) *Clin Chim Acta* **138**: 41–47.
10 Bøg-Hansen TC (1973) *Anal Biochem* **56**: 480–88.
11 Bierhuizen MFA, De Wit M, Govers CARL, Ferwerda W, Koeleman C, Pos O, van Dijk W (1988) *Eur J Biochem* **175**: 387–94.
12 Hermentin P, Witzel R, Doenges R, Bauer R, Haupt H, Patel T, Parekh RB, Brazel D (1992) *Anal Biochem* **206**: 419–29.
13 Smith KD, Elliott MA, Elliott HG, McLaughlin CM, Wightman P, Wood GC (1994) *J Chromatography* **661**: 7–14.
14 Succari M, Foglietti M-J, Percheron F (1985) *J Chromatography* **341**: 457–61.
15 Landberg E, Påhlsson P, Lundblad A, Arnetorp A, Jeppson J-O (1995) *Biochem Biophys Res Commun* **210**: 267–74.
16 Lee YC (1990) *Anal Biochem* **189**: 151–62.
17 Brinkman-van der Linden ECM, van Ommen ECR, van Dijk W (1996) *Glycoconjugate J* **13**: 27–31.
18 De Graaf TW, Van Ommen ECR, van der Stelt ME, Kerstens PJSM, Boerbooms AMT, van Dijk W (1994) *J Rheumatol* **21**: 2209–16.
19 Moule SK, Peak M, Thompson S, Turner GA (1987) *Clin Chim Acta* **166**: 177–85.
20 Pos O, Oostendorp RAJ, van der Stelt ME, Scheper RJ, van Dijk W (1990) *Inflammation* **14**: 133–41.
21 Bennett M, Schmid K (1980) *Proc Natl Acd Sci USA* **77**: 6109–13.
22 van der Linden ECM, de Graaf TW, Anbergen MG, van Ommen ECR, van der Stelt ME, van Dijk W (1993) *Glycoconjugate J* **10**: 316–17.
23 De Graaf TW, van der Stelt ME, Anbergen MG, van Dijk W (1993) *J Exp Med* **177**: 657–66.

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