

# Inflammation-induced Expression of Sialyl Lewis X-containing Glycan Structures on $\alpha_1$ -Acid Glycoprotein (Orosomucoid) in Human Sera

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## Summary

The glycosylation of the acute phase glycoprotein  $\alpha_1$ -acid glycoprotein (AGP) in human sera is subject to marked changes during acute inflammation as a result of the cytokine-induced hepatic acute phase reaction. The changes described thus far comprise alterations in the type of branching of the carbohydrate structures as revealed by increased reactivity of AGP with concanavalin A. We now report on acute inflammation-induced increases in  $\alpha_1 \rightarrow 3$ -fucosylated AGP molecules, as detected by the reactivity of AGP towards the fucose-binding *Aleuria aurantia* lectin (AAL) in crossed affino-immunoelectrophoresis of human sera. Laparotomy of women, for the removal of benign tumors of the uterus, was used as a model for the development of the hepatic acute phase response. Huge increases were detected in the amounts of strongly AAL-reactive fractions of AGP, presumably containing three or more fucosylated *N*-acetylglucosamine units. At least part of these Lewis X-type glycans (Gal $\beta$ 1 $\rightarrow$ [Fuc $\alpha$ 1 $\rightarrow$ 3]GlcNAc-R) appeared to be substituted also with an  $\alpha$ 2 $\rightarrow$ 3-linked sialic acid residue. This was revealed by the laparotomy-induced abundant staining of AGP with an antisialyl Lewis X monoclonal antibody (CSLEX-1) on blots of sodium dodecyl sulfate-polyacrylamide gels containing AGP isolated from the sera of a patient at various days after operation. It is concluded that acute inflammation induces a strong increase in sialyl Lewis X-substituted AGP molecules that persists at a high level throughout the inflammatory period. We postulate that these changes represent a physiological feedback response on the interaction between leukocytes and inflamed endothelium, which is mediated via sialylated Lewis X structures and the selectin endothelial-leukocyte adhesion molecule 1.

Characteristic changes in the glycosylation of  $\alpha_1$ -acid glycoprotein (AGP)<sup>1</sup> and other acute phase glycoproteins occur during the acute phase of inflammation with respect to the degree of branching of the N-linked glycans on the molecules (1–6). Cytokines, like IL-1, IL-6, and TNF, involved in the induction of the inflammatory reaction have been shown to be responsible for these changes by affecting the glycosylation process in the liver (5, 7–10). The function of the changes in glycosylation is not known, although effects on immunomodulative properties of AGP and  $\alpha_1$ -protease

inhibitor (PI) have been described (11–14). In liver cirrhosis and in cancer sera the occurrence has been described of AGP and other serum glycoproteins expressing fucosylated and sialylated glycans of the type sialyl Lewis X (SLeX; Neu-Ac $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4[Fuc $\alpha$ 1 $\rightarrow$ 3]GlcNAc-R) (15–18). In normal serum, however, only a low expression of SLeX on serum glycoproteins has been reported (15, 17, 18). The Lewis X structure in its sialylated form is of special interest because this structure, when present on leukocytes, is the ligand for the cell adhesion molecules E-Selectin (the endothelial-leukocyte adhesion molecule [ELAM-1]) and P-Selectin (GMP140/PADGEM/CD62), involved in the inflammation-dependent adhesion of neutrophils, monocytes, or resting T cells to vascular endothelium or platelets (19–23). E-Selectin and P-Selectin are normally not expressed on the cell surface of the cells, but can be induced rapidly upon stimulation with inflammatory mediators. Walz et al. (19) have described that AGP has an affinity for E-Selectin and that this affinity can be substantially increased by *in vitro* fucosylation of AGP increasing most probably the expression of SLeX on the

<sup>1</sup> Abbreviations used in this paper: AAL, *Aleuria aurantia* lectin; AGP,  $\alpha_1$ -acid glycoprotein; CAIE, crossed affino-immunoelectrophoresis; ELAM, endothelial-leukocyte adhesion molecule; PI,  $\alpha_1$ -protease inhibitor; PSA, *Pisum sativum* agglutinin; SLeX, sialyl Lewis X.

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glycans. They suggested that in vivo an increased serum level of SLeX-expressing AGP, as found in liver cirrhosis (15, 16), could have a feedback inhibitory effect on the extravasation of leukocytes, by competition for the E-Selectin adhesion molecules.

To investigate the probability of such a type of humoral response, we decided to study the occurrence of changes in the level of SLeX-substituted AGP in human plasma during the acute phase reaction. Laparotomy of women for the removal of benign tumors of the uterus was used as a model for the development of the hepatic acute phase response. Crossed affino-immunoelectrophoresis (CAIE) of AGP in patient sera before and after operation with fucose-specific lectins as affino-component, and staining with a mAb against SLeX (CSLEX-1) of electroblots of SDS-polyacrylamide gels containing isolated AGP, were used for detection.

## Materials and Methods

**Sources of Sera.** Control sera were obtained from apparently healthy individuals. Serum samples of women, subjected to laparotomy either for the removal of benign tumor(s) of the uterus or for primary Caesarean section, were taken 24 h before operation and at several days after operation until they were released from the hospital. Serum samples of previously healthy burn patients were taken within hours of the injury and at regular intervals within the following 2 mo, as detailed elsewhere (5).

**Isolation of *Aleuria aurantia* Lectin (AAL).** Fruiting bodies of *Aleuria aurantia* were collected locally and stored frozen at  $-80^{\circ}\text{C}$  in 200-g portions. Fruiting bodies (200 g) were homogenized in 1.5 vol (vol/wt) 10 mM phosphate buffer (pH 7.2), 0.14 M NaCl, 0.02%  $\text{NaN}_3$  (PBS) using a Polytron homogenizer (Kinematica GmbH, Kriens/Luzern, Switzerland). The lectin was extracted by stirring the homogenate for 4 h at  $4^{\circ}\text{C}$  followed by centrifugation for 30 min at 14,000 rpm ( $4^{\circ}\text{C}$ ; 6X300 rotor, MSE 21), and was subsequently precipitated by ammonium sulphate (80% saturation). The precipitate was resuspended in 150 ml PBS, followed by dialysis against PBS for 24 h with three changes of the buffer. Residual insoluble materials were removed from the retentate by centrifugation (40 min; 40,000 rpm at  $4^{\circ}\text{C}$ ; 8X50 rotor, MSE 65). AAL was isolated from the crude preparation by affinity chromatography according to Debray and Montreuil (24) using an L-fucose-agarose column (1.8  $\times$  12 cm; F7379; Sigma Chemical Co., St. Louis, MO) at a flow rate of 10 ml/h. The column was washed with PBS until the absorption at  $A_{280\text{ nm}}$  of the effluent was negligible. Specific elution of the lectin was performed with two-column volumes of PBS, 0.15 M  $\alpha$ -L-fucose (Sigma Chemical Co.). The eluate was dialyzed against 10 times diluted PBS for 4 d with six changes of the buffer. The hemagglutination titer of AAL was determined using a 3% suspension of human red blood cells in PBS in V-shaped microtiter plates and a twofold well-to-well dilution. The AAL preparation was concentrated to a titer of 512 by using hygroscopic powder (Aquacide II; Calbiochem-Behring Corp., La Jolla, CA) applied at the outside of a dialysis tube containing the preparation. The final preparation was stored at  $4^{\circ}\text{C}$  and remained stable for at least 3 mo. The purity of the final preparation was checked by SDS-PAGE under denaturing conditions (see below). Only one protein band was observed with an apparent molecular mass of 30 kD in accordance with the report of Debray and Montreuil (24).

**Characterization of the Binding Specificity of AAL.** The binding

specificity of AAL was determined after immobilization of the isolated lectin on activated CH-Sepharose 4B (4 mg/ml) (Pharmacia LKB, Uppsala, Sweden). Chromatography on AAL-Sepharose 4B was performed using a 1.3-ml column (0.3  $\times$  18 cm; 4 ml/h; 0.2-ml fractions;  $20^{\circ}\text{C}$ ) and PBS, 0.02%  $\text{NaN}_3$  as starting buffer. Radioactively labeled standard glycopeptides or oligosaccharides were applied in 0.1 ml of starting buffer. Nonbound and weakly retarded compounds were eluted with five-column volumes of starting buffer. Retarded or bound glycans were eluted with 10 mM L-fucose in starting buffer. The radioactivity in each fraction was determined by liquid scintillation. The AAL-Sepharose column was regenerated with 10-column volumes of starting buffer.

All standard structures used were characterized previously by  $^1\text{H-NMR}$  or capillary gas chromatography/mass fragmentography.  $[2\text{-}^3\text{H}]\text{Man}$ -labeled  $(\text{Man})_6\text{-GlcNAcOH}$  [ $\text{Man}\alpha 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 3(\text{Man}\alpha 1 \rightarrow 3[\text{Man}\alpha 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 6]\text{Man}\alpha 1 \rightarrow 6)\text{-Man}\beta 1 \rightarrow 4\text{GlcNAcOH}$ ] was a kind gift from Dr. R. Geyer (Biochemisches Institut am Klinikum der Universität Giessen, Giessen, Germany) (25). Asialo complex type biantennary glycopeptide GP2 ( $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 3[\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 6]\text{-Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow \text{Asn-Lys}$ ), asialo complex type triantennary glycopeptide GP3 ( $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4[\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2]\text{Man}\alpha 1 \rightarrow 3[\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 6]\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow \text{Asn-Lys}$ ), asialo complex type tetraantennary glycopeptide GP4 [ $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4(\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2)\text{Man}\alpha 1 \rightarrow 3(\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6[\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2]\text{Man}\alpha 1 \rightarrow 6)\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow \text{peptide}$ ], and GP4-MF (a mixture of GP4 with L-fucose  $\alpha 1 \rightarrow 3$  linked to a GlcNAc residue of one of the  $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}$  branches [Lewis X-type structure]) were originally prepared from human AGP and labeled with  $[^{14}\text{C}]\text{acetate}$  in the peptide part as described previously (26). GP4-MF1, GP4-MF2, and GP4-MF3 were prepared from GP4-MF as described previously (26), and represent Lewis X-type glycopeptides fucosylated at the  $\beta 1 \rightarrow 4\text{Man}\alpha 1 \rightarrow 3$ -, the  $\beta 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow 6$ -, and the  $\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 6$ -linked  $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}$  branches, respectively. Core-fucosylated complex-type monoantennary glycopeptide MS,  $\text{NeuAc}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{-Man}\alpha 1 \rightarrow 3(\text{Man}\alpha 1 \rightarrow 6)\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4(\text{Fuc}\alpha 1 \rightarrow 6)\text{GlcNAc}\beta 1 \rightarrow [^{14}\text{C}]\text{peptide}$  was a kind gift from Dr. D. H. van den Eijnden (Department of Medical Chemistry, Vrije Universiteit, Amsterdam, The Netherlands).

**CAIE.** CAIE with Con A or *Pisum sativum* agglutinin (PSA) as affino-component in the first-dimension gel was performed according to Bøg-Hansen (27), as previously described (5), using 2 mg Con A (Type V; Sigma Chemical Co.) or 5 mg PSA (crude preparation; EY Labs, San Mateo, CA)/ml gel. The first-dimension gel for CAIE with AAL was prepared from a mixture of 300  $\mu\text{l}$  AAL preparation (hemagglutination titer 512), 75  $\mu\text{l}$  five times concentrated electrophoresis buffer, and 625  $\mu\text{l}$  1.6% agarose (Standard Low m, Agarose; Bio-Rad Laboratories, Richmond, CA)/ml. 0.5–2  $\mu\text{l}$  serum was applied to the gel and electrophoresis was performed at 10 V/cm for 90 min at  $15^{\circ}\text{C}$ . Cibron blue bound to BSA was included in the sample as an internal standard of the electrophoretic mobility. The second-dimension gel containing monospecific rabbit anti-human AGP IgG or anti-PI IgG (1:300; vol/vol) (Dakopatts, Copenhagen, Denmark) was separated from the first-dimension gel by a 1-cm-wide intermediate gel containing 0.1 M methyl- $\alpha$ -D-glucopyranoside (Sigma Chemical Co.) without (in the case of Con A) or with 0.01 M  $\alpha$ -L-fucose in (in the case of AAL) to decompose the complexes with the lectin. Electrophoresis in this dimension was carried out for 18 h at 2 V/cm and  $15^{\circ}\text{C}$ . Gels were stained with coomassie

brilliant blue R 250 (5). The area of each AGP or PI form under the precipitation line was measured using planimetry and the reactivity coefficient (RC) was calculated for each pattern. The RC was defined as the ratio between the sum of the areas of lectin-reactive forms and the area of the nonreactive form. The total amounts of AGP and PI as present in the various samples were determined by circular immunodiffusion (28).

**Partial Purification of AGP from Serum.** Isolation of AGP from serum was performed by immunoaffinity chromatography over an anti-AGP-Sepharose 4B column (prepared from CH-Sepharose 4B [Pharmacia-LKB] and rabbit anti-human AGP IgG according to the instructions of the supplier). 0.5 ml of serum was applied to the anti-AGP-Sepharose 4B column (2.4 × 0.8 cm; 5 ml/h). The Sepharose column was subsequently washed with PBS until the absorption at  $A_{280\text{ nm}}$  of the effluent was negligible. Specific elution of the bound AGP was performed with 0.05 M diethylamine (pH 11.5), 0.15 M NaCl, 0.02%  $\text{NaN}_3$  under immediate neutralization of the eluted fractions. AGP-containing fractions (as detected by rocket immunoelectrophoresis) were pooled, dialyzed extensively against 0.1 M ammonium bicarbonate (pH 8.0), lyophilized, and stored at  $-20^\circ\text{C}$ . The amount of AGP in each preparation was determined by radial immunodiffusion (28).

**Isolation of Various Con A- or AAL-reactive Fractions of AGP.** Various lectin-reactive fractions of sera were obtained by preparative CAIE, as detailed elsewhere (29). In short, the method involved the electrophoretic separation of the various lectin-reactive AGP forms in a Con A- or AAL-containing agarose gel in which 75  $\mu\text{l}$  of serum was applied in various holes. Gel lanes containing the nonretarded and the lectin-retarded forms were cut out perpendicular to the electrophoresis direction, after determination of the positions of the various forms in small control lanes by immunoelectrophoresis in the second dimension using rabbit anti-human AGP IgG. The glycoprotein forms were recovered from the lectin-containing gels by electrophoresis into a second-dimension agarose gel without additions. A small intermediate gel containing the appropriate sugar(s) was used to dissociate lectin-glycoprotein complexes. The various lectin-reactive forms of AGP were collected from the second-dimension gels by electroelution (422 Electro-Eluter; Bio-Rad Laboratories).

**SDS-PAGE Blotting, and Immunoassay of AGP.** 10% SDS-PAGE was performed according to Laemmli (30) using the Mini-Protean II dual slab gel apparatus (Bio-Rad Laboratories). Gels were loaded with equal amounts of AGP as present in patient sera or in partially purified preparations of AGP from the sera of one of the patients. AGP isolated from pooled normal human serum (4) was used as a standard. Protein bands were detected by staining of the gels with coomassie brilliant blue R250. Proteins were blotted onto nitrocellulose by electrophoretic transfer using the Mini Trans-Blot Cell (Bio-Rad Laboratories). AGP was detected on the blots using rabbit anti-human AGP IgG and peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunology, London, UK). SLeX determinants on AGP were detected by incubating AGP-containing nitrocellulose strips with mouse monoclonal anti-SLeX IgM (CSLEX-1, 20  $\mu\text{g}/\text{ml}$  in 10 times diluted PBS; Tissue Typing Laboratory, Department of Surgery, UCLA School of Medicine, Los Angeles, CA) (31), followed by alkaline phosphatase-conjugated goat anti-mouse IgM (1:250; vol/vol; Zymed Laboratories, San Francisco, CA) for detection. The specificity of the binding of the CSLEX-1 antibody was checked by desialylation of AGP preparations with neuraminidase (*Vibrio cholerae*; Boehringer, Mannheim, Germany) (0.1 U/ml, 2 h,  $37^\circ\text{C}$ ) before SDS-PAGE.

## Results

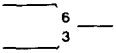
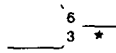
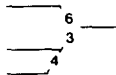
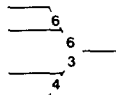
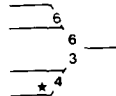
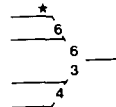
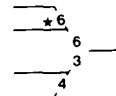
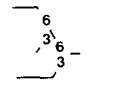
**Control of the Specificity of Binding of AAL.** The AAL preparations exhibited the same binding characteristics for  $\alpha 1\rightarrow 3$ - and  $\alpha 1\rightarrow 6$ -linked complex type glycans as reported by Debray and Montreuil (24) when the lectin was immobilized on CH-Sepharose 4B (AAL-Sepharose; Table 1). N-linked complex-type glycopeptides substituted with an  $\alpha 1\rightarrow 3$ -linked Fuc residue (the GP4MF isomers) were retarded by 0.5 (GP4MF3) to one-column volume of PBS (GP4MF-1 and -2). The Fuc $\alpha 1\rightarrow 6$ -substituted glycopeptide MS was strongly retarded on the AAL-Sepharose column since it was eluted with 5–10-column volumes of PBS or (specifically) with one-column volume of PBS containing 0.05 M L-Fuc. Non-fucosylated bi-, tri-, and tetraantennary complex-type glycans as well as the oligomannose saccharide  $(\text{Man})_8\text{GlcNAcOH}$  were neither bound nor retarded.

**Reactivity of AGP in Control Human Sera with the Fucose-specific Lectins AAL and PSA.** AGP present in pooled normal human sera was fractionated in a nonreactive and four reactive fractions upon CAIE with AAL as affino-component (Fig. 1). All fractions were recovered on the same positions upon reanalysis by CAIE with AAL, after fractionation of serum in different AAL-reactive fractions by preparative CAIE (not shown). Analyses of 16 different sera of apparently healthy volunteers revealed that  $40 \pm 13\%$  of AGP did not interact at all with AAL (A0), and  $14 \pm 2\%$  (A1),  $16 \pm 3\%$  (A2),  $13 \pm 5\%$  (A3), and  $17 \pm 8\%$  (A4) of AGP were retarded by AAL to different extents. No retardation was detectable for AGP when sera were subjected to CAIE with the Fuc $\alpha 1\rightarrow 6$ -specific lectin PSA as affino component (not shown).

**Acute Phase-induced Increases in the Reactivity of AGP with AAL.** Laparotomy of women for the removal of benign tumors of the uterus was used as a model of the acute phase response in serum. The development of the acute phase reaction after laparotomy is illustrated by the changes in the total amount and in the Con A reactivity of AGP (Fig. 2 and Table 2). All three patients displayed a strong increase in the AAL reactivity of AGP after the onset of acute inflammation. The increase in AAL reactivity especially regarded forms A3 and A4 of AGP, as is illustrated in Fig. 2. The highest increases in AAL reactivity were noted at the end of the period studied. The changes in reactivity with Con A and AAL occurred independently from each other and from the changes in the total amount of AGP.

Like in control sera (see above), no retardation of AGP was detectable in the sera of the various patients when analyzed by CAIE with the Fuc $\alpha 1\rightarrow 6$ -specific lectin PSA. This indicates that the increased AAL reactivity of AGP did not concern the de novo expression of  $\alpha 1\rightarrow 6$ -linked Fuc residues, at least with regard to biantennary glycans (32, 33). Since, in addition, the common type of fucosylation of AGP is in an  $\alpha 1\rightarrow 3$  linkage (4, 33, 34), the observed change in AAL reactivity was most likely evoked by an increased substitution of the glycans with  $\alpha 1\rightarrow 3$ -linked Fuc residues. PSA did react with PI revealing the presence of  $\alpha 1\rightarrow 6$ -linked Fuc residues. However, no acute phase-induced changes could be detected in the reactivity of this Fuc $\alpha 1\rightarrow 6$ -substituted acute

**Table 1.** Characterization of Carbohydrate Binding Specificity of AAL-Sepharose

Glycopeptide or oligosaccharide	Schematic structure	Elution behavior
GP2		Not retarded
MS		Eluted with 0.05 M $\alpha$ -L-Fuc in PBS
GP3		Not retarded
GP4		Not retarded
GP4MF1		Retarded by one-column volume PBS
GP4MF2		Retarded by one-column volume PBS
GP4MF3		Retarded by 0.5-column volume PBS
(Man) <sub>6</sub> GlcNAcOH		Not retarded

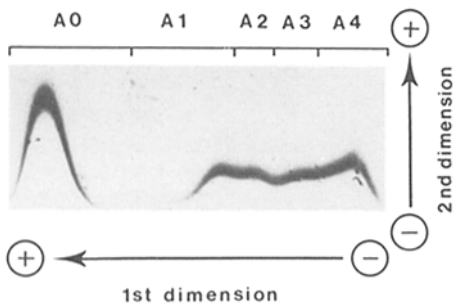
Not retarded glycans were eluted in the void volume with the washing buffer PBS. The structures are depicted in a schematic form, with numbers indicating the positions of substitution at the branching points. See Materials and Methods for the definition of the standard structures and for details of the chromatographic procedure used.

\* Fucose residues.

phase glycoprotein with either PSA or AAL in the patient sera (not shown).

**Acute Phase-induced Increase in the Expression of SLeX Antigens on AGP.** To establish whether the acute phase-induced change in AAL reactivity of AGP involved an increased occurrence of fucosylated and sialylated *N*-acetylglucosamine units of the type NeuAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4(Fuc $\alpha$ 1 $\rightarrow$ 3)GlcNAc-R (SLeX), the expression of this type of antigen on AGP was studied during the development of the acute phase using anti-SLeX monoclonal IgM. The presence of SLeX antigens on AGP could not be unequivocally established on nitrocellu-

lose blots of SDS-PAGE gels of total sera, because of both comigrating other serum glycoproteins and the low sensitivity of the method. It was decided, therefore, to perform the analyses on immunoaffinity-purified AGP. For obvious reasons this effort was performed only for one set of sera (patient 1 of Table 2). SDS-PAGE of equal amounts of purified AGP fractions revealed broad protein bands at 43 kD (4) that comigrated with standard AGP and reacted with anti-human AGP antibodies (Fig. 3 A). A weak positive staining with anti-SLeX was observed for AGP isolated from serum before operation (Fig. 3 B). Strongly enhanced staining was observed

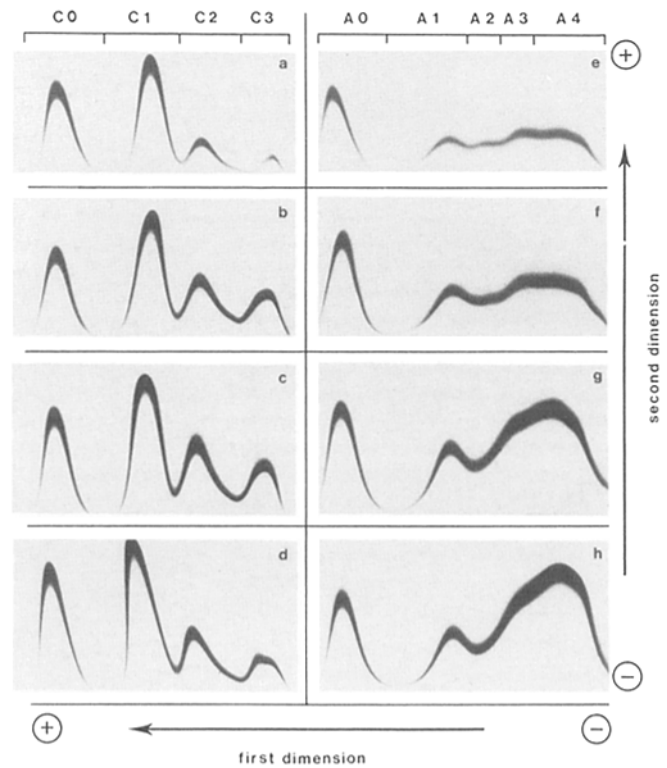


**Figure 1.** Reactivity of AGP in control human serum with AAL. The reactivity of AGP with AAL was determined by CAIE as described in Materials and Methods using 1  $\mu$ l of pooled human sera and rabbit anti-human AGP IgG for detection. Only the second-dimension gel is shown; the application site in the first-dimension gel coincides with the right hand side of the figure. (A0) AGP fraction nonreactive with AAL; (A1–A4) reactive fractions with AAL in increasing order of reactivity.

at 2 and 4 d after operation (note that the same amount of AGP was applied in each lane [Fig. 3 A]). The binding of CSLEX-1 was neuraminidase sensitive (not shown). These findings strongly suggest that the fraction of AGP and/or the number of glycans per AGP molecule expressing SLeX determinant(s) were elevated after the onset of the inflammation.

**Increase in AAL Reactivity of AGP in Other Models of Acute Inflammation.** To study the ubiquity of the acute phase-induced change in AAL reactivity of AGP, serum samples of two other groups of patients, women subjected to laparotomy for primary Caesarean section and patients suffering from severe burns (Table 3 and Fig. 4), were analyzed by CAIE. In all patients an increase in AAL reactivity of AGP became clearly manifest to the end of the period studied.

**Effect of Biantennary Glycan Content on the AAL Reactivity of AGP.** During the acute phase the fraction of AGP con-

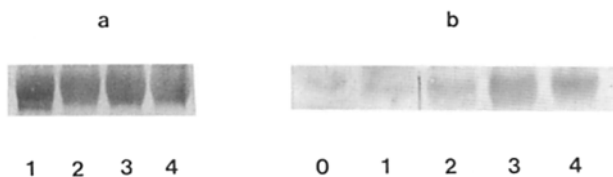


**Figure 2.** Reactivity of AGP with Con A (a–d) and AAL (e–h) before and at various days after laparotomy. Sera were obtained 1 d before (a and e), and 1 (b and f), 2 (c and g), and 4 d (d and h) after laparotomy for the removal of a benign tumor of the uterus from an otherwise healthy women (patient 1 in Table 2). The reactivity with Con A or AAL was determined by CAIE as described in Materials and Methods using 1  $\mu$ l of serum, and rabbit anti-human AGP IgG for detection. Only the second-dimension gels are shown; the application site in the first-dimension gel coincides with the right hand side of each figure. (A0 and C0) AGP fraction nonreactive with AAL, respectively, Con A; (A1–A4 and C1–C3) AGP fractions reactive with AAL, respectively, Con A in increasing order of reactivity.

**Table 2.** Effect of Laparotomy-induced Acute Inflammation on the Reactivity of AGP with AAL and Con A

Day	Patient 1			Patient 2			Patient 3		
	AGP	RC		AGP	RC		AGP	RC	
	mg/ml	Con A	AAL	mg/ml	Con A	AAL	mg/ml	Con A	AAL
-1	1.6	1.9	2.1	1.4	1.4	0.7	2.1	1.1	1.6
+1	1.8	3.0	2.5	1.4	1.8	0.9	2.0	2.0	4.1
+2	1.9	2.9	3.7	-	-	-	-	-	-
+4	2.1	2.2	4.5	2.4	2.0	1.8	-	-	-
+5	-	-	-	-	-	-	1.7	1.4	6.5

The sera of women subjected to laparotomy for the removal of benign tumors of the uterus were analyzed before and at various days after operation for acute phase-induced changes in concentration (Mancini) and in lectin reactivity of AGP (CAIE). RC (reactivity coefficient), ratio of the sum of the lectin-retarded fractions over the nonretarded fraction of AGP. See Materials and Methods for details.



**Figure 3.** Inflammation-induced increase in the expression of SLeX antigens on AGP. Immunoaffinity-purified preparations of AGP from the four sera of the laparotomy patient 1 (compare Fig. 2 and Table 2) were subjected to SDS-PAGE, followed by blotting and detection of AGP (a) or SLeX (b) with specific (monoclonal) antibodies. Only the part of the blots containing the AGP bands is reproduced. Care was taken that the same amount of AGP (8  $\mu$ g) was applied in each lane of the various gels, as is demonstrated by the specific detection of AGP in a. (Lane 1) 1 d before operation; (lanes 2, 3, and 4), respectively, 1, 2, and 4 d after laparotomy. (Lane 0 in b) AGP isolated from pooled human serum. See Materials and Methods for further details.

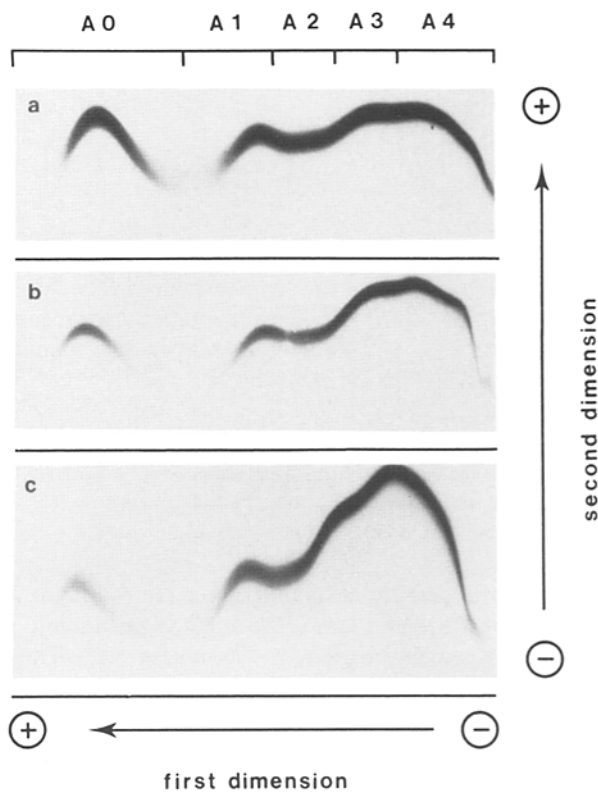
taining one or more biantennary glycans increased, as indicated by the reactivity with Con A (Table 2). To investigate the relationship with the increase in AAL reactivity, fractions of AGP reacting differently with Con A were prepared from acute phase sera and subjected to CAIE with AAL as affino-component. Fractions expressing only tri- or tetra-antennary glycans (AGP-C0), or in which one (AGP-C1) or two of these glycans are replaced by biantennary glycans (AGP-C2) (4), were obtained from sera of a burn patient (patient

**Table 3.** Effect of Acute and Chronic Inflammation on the Reactivity of AGP with AAL

Source of sera	Day after injury or operation	Patient 1		Patient 2		Patient 3	
		AGP	AAL	AGP	AAL	AGP	AAL
		<i>mg/ml</i>		<i>mg/ml</i>		<i>mg/ml</i>	
Burns*	1	0.8	2.7	0.8	3.0	1.8	2.3
	3	2.1	3.4	-	-	-	-
	4	-	-	1.9	3.0	2.1	3.1
	16	-	-	7.3	3.7	-	-
	19	-	-	-	-	4.6	4.4
	30	4.5	5.1	-	-	-	-
PS	-1	1.3	1.0	1.0	0.2		
	+1	1.3	1.1	1.0	0.3		
	+7	2.0	2.6	1.7	1.9		

Acute-phase induced changes in concentration (Mancini) and in AAL reactivity of AGP (CAIE) were analyzed in sera of patients suffering from severe burns (Burns) or patients subjected to laparotomy for primary Caesarean section (PS) at various days during hospitalization. RC (reactivity coefficient), ratio of the sum of the lectin-retarded fractions over the non-retarded fraction of AGP. See Materials and Methods for details.

\* Part of the values for the concentration of AGP in the sera of burn patients were published earlier (5).

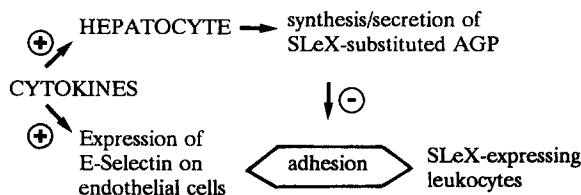


**Figure 4.** AAL reactivity of AGP at various days after injury by burning. Sera were obtained at the first day (a), and 3 (b) and 30 d (c) after the injury by burning (burn patient 1 in Table 3). 2.5 (a) or 1  $\mu$ l of serum (b and c) was analyzed by CAIE for the reactivity of AGP with AAL. See Fig. 2 for further explanation and Materials and Methods for details.

1 in Table 3) via fractionation of the sera by preparative CAIE with Con A as affino-component. The AAL reactivity of all three AGP fractions appeared to increase from day 1 to 30, like in the unfractionated sera: the RC values for AGP-C0, -C1, and -C2, respectively, were 1.7, 2.1, and 2.2 on the first day, and 5.0, 5.4, and 5.0 on the 30th day after the injury by burning (compare with Table 3, patient 1).

## Discussion

In this study we have utilized the fucose-specific lectin AAL and a mouse mAb to investigate the inflammation-induced expression of sialylated Lewis X structures, NeuAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4(Fuc $\alpha$ 1 $\rightarrow$ 3)GlcNAc-R, on the acute phase glycoprotein



**Figure 5.** Simplified model of cytokine-induced feedback inhibition of E-Selectin-mediated cell adhesion during inflammation.

AGP in human sera. The absolute amount of fucosylated AGP molecules as well as the number of Fuc residues per molecule were enhanced under the various acute inflammatory conditions studied (Tables 2 and 3). The amount of AGP molecules expressing three or more Fuc residues was especially increased. This is concluded from the huge increases in the strongly retarded fractions A3 and A4 (see Figs. 2 and 4), under the assumption that the number of Fuc residues per fraction is the sole determinant for the extent of retardation by AAL (see references 27 and 35 for theoretical background of the method). The laparotomy-induced abundant staining of AGP with a SLeX-specific mAb (Fig. 3) demonstrates that a significant part of the fucosylated *N*-acetylglucosamine units of the glycans were substituted also with an  $\alpha 2 \rightarrow 3$ -linked sialic acid residue. So, acute inflammation appears to induce a strong increase in the SLeX-substituted glycans per AGP molecule, which persists at a high level throughout the whole period studied. Increases in SLeX-bearing glycans on acute phase glycoproteins have thus far only been reported in chronic inflammation and in sera from patients with cancer (15–17).

Fucosylated glycans recognized by AAL might express  $\alpha 1 \rightarrow 3$ -,  $\alpha 1 \rightarrow 6$ -, and/or  $\alpha 1 \rightarrow 2$ -linked glycans. The reactivities of the AGP molecules with AAL, however, only reflect the interaction with Fuc residues in an  $\alpha 1 \rightarrow 3$  linkage to type II *N*-acetylglucosamine units (Lewis X) of the asparagine-linked glycans for the following arguments. (a) Fuc residues on AGP molecules isolated from normal human sera have been shown to occur only in an  $\alpha 1 \rightarrow 3$  linkage to the GlcNAc residue of one of the *N*-acetylglucosamine branches of the glycans (4, 34, 36, 37). (b) The *N*-acetylglucosamine units of AGP are of the type II (Gal $\beta 1 \rightarrow 4$ GlcNAc) under normal (4, 34, 36, 37), inflamed (16), and tumorigenic conditions (33). (c) No interaction could be established between AGP and PSA, a lectin specific for Fuc $\alpha 1 \rightarrow 6$ -substituted biantennary glycans (see Results). This type of glycan was only reported to be expressed on AGP isolated from human metastatic livers (33). PSA did react with PI, another acute phase glycoprotein synthesized by the liver, being indicative for the presence of  $\alpha 1 \rightarrow 6$ -linked Fuc residues on PI. This type of linkage most probably was responsible also for the reactivity of PI with AAL, since during acute inflammation no changes were found in the reactivity of PI with either PSA or AAL. This further suggests that the hepatic biosynthesis of  $\alpha 1 \rightarrow 6$ -linked Fuc residues was not affected by the inflammatory conditions. (d) The lack of change in AAL reactivity of PI also makes it very unlikely that  $\alpha 1 \rightarrow 2$ -linked Fuc residues were introduced on serum glycoproteins during the hepatic acute phase reaction. Such a type of abnormal fucosylation has only been suggested to occur during liver diseases on PI and other acute phase glycoproteins (38, 39).

According to literature, 30–50% of the bi-, tri-, and tetraantennary *N*-linked glycans of normal AGP contain one Fuc residue (34, 36, 37), and a minor part of the tetraantennary glycans contains two Fuc residues (36). At least part of the fucosylated glycans were not fully substituted with  $\alpha 2 \rightarrow 3$ - or  $\alpha 2 \rightarrow 6$ -linked sialic acid residues (37), resulting in a low expression of SLeX on normal AGP (15). Since five glycans

are present on each AGP molecule, it should be expected that all molecules would contain at least one fucosylated glycan. In this study, however,  $\sim 40\%$  of the AGP molecules present in normal human sera apparently were not fucosylated at all, since they did not react with AAL in CAIE. This indicates that in normal AGP the fucosylated structures are not distributed at random over the various molecules.

In previous studies it was established that at least three molecular forms of AGP occur in normal sera that differ in the degree of branching of the glycans, and that can be distinguished by their reactivities with Con A (4, 6). Con A-reactive AGP, containing one or two biantennary glycans, is increased during acute inflammation (1–7). Our present experiments exclude that a direct relationship exists between the inflammation-induced increases in biantennary glycan content and the extent of fucosylation of AGP. First, the changes in Con A and AAL reactivity differ both in magnitude and in time. For example, the AAL reactivity in burn sera increased steadily over a period of 30 d (Fig. 4), whereas the Con A reactivity reached maximal values in the first 16 d after injury and rapidly returned to control values thereafter (5). Second, the AAL reactivities of isolated Con A-reactive and nonreactive fractions of AGP were found to be comparable throughout the inflammation period studied, and were shown to increase to the same extent (see Results). So, it can be concluded that the change in fucosylation and in the type of branching of AGP are differently regulated.

We and others have supported evidence that changes in glycosylation of acute phase glycoproteins result from cytokine-induced variations in their biosynthesis in the liver (5–7, 10). It can be supposed, therefore, that the liver is also involved in the changes in glycosylation described in this study. The synthesis of SLeX-bearing glycans is inducible in the liver, since the occurrence has been described during liver cirrhosis, both on membrane-bound glycoproteins and on secreted AGP (15, 16, 40). Furthermore, the liver  $\alpha 1 \rightarrow 3$ -fucosyltransferase is a likely candidate for the regulation of the enhancement of fucosylation, since the enzyme can transfer Fuc residues to  $\alpha 2 \rightarrow 3$ -sialylated *N*-acetylglucosamine units of the glycans (41). Fucosylation of  $\alpha 2 \rightarrow 6$ -sialylated *N*-acetylglucosamine units is prohibited by the structural requirements of the enzyme (41). However, it can be speculated that the acute phase-induced secretion of the  $\alpha 2 \rightarrow 6$ -sialyltransferase (42–44) will lead to a decreased substitution of the glycans of AGP with  $\alpha 2 \rightarrow 6$ -linked sialic acid, and consequently, will allow both the resident  $\alpha 2 \rightarrow 3$ -sialyltransferase and the  $\alpha 1 \rightarrow 3$ -fucosyltransferase to introduce a SLeX type of structure on the corresponding *N*-acetylglucosamine units of the glycans. Such a type of regulation for the expression of SLeX has been suggested to occur in human myeloid cells during maturation (45). An acute phase-induced change in the substitution of AGP with  $\alpha 2 \rightarrow 3$ -linked sialic acid residues is suggested from our results. A decrease, or at least a stabilization, in the expression of the  $\alpha 2 \rightarrow 3$ -sialylated LeX structures on AGP is suggested from Fig. 3 at the fourth day of the laparotomy-induced inflammatory reaction, whereas the degree of fucosylation of AGP on the fourth day was higher

than on the second day (Table 2). Studies are in progress to further substantiate these differences.

The inflammation-induced increase in SLeX-substituted glycans on AGP might represent a humoral mechanism provided by the liver for feedback inhibition of granulocyte extravasation into inflamed tissues (Fig. 5). Such a mechanism was proposed by Walz et al. (19) recently. They reported that increased fucosylation of AGP, by means of treatment with  $\alpha 1 \rightarrow 3$ -fucosyltransferase, substantially increased its affinity for ELAM-1 or E-Selectin. E-Selectin mediates the primary interaction of inflamed endothelial cells with, e.g., granulocytes and memory T cells expressing the SLeX-containing glycans (19–23). Cytokines are involved in the expression of the E-Selectin on the endothelial cells, and, as is discussed above, are likely to be involved in the induction of the SLeX expression on AGP (Fig. 5). The possibility that an increase in the plasma level of SLeX-bearing AGP molecules will competitively inhibit the primary interaction of leukocytes with E-Selectin is in accordance with our finding that the changes in fucosylation reach a maximum to the end of the acute phase periods studied. The proposed inhibitory function of SLeX-expressing AGP can be extended to the cellular adhesion process mediated by P-Selectin (GMP140/PADGEM/CD62). SLeX-expressing molecules have also been reported to be ligands for this adhesion molecule, which

mediates the adhesion between leukocytes and inflammation-induced endothelial cells or platelets (20, 21). SLeX-substituted AGP molecules, furthermore, are good candidates to serve as soluble binding proteins for Selectin molecules that have been shed from endothelial cells. It could be speculated that inflammation induces an expression of SLeX on a variety of acute phase glycoproteins, because acute phase-induced changes in glycosylation (Con A reactivity) are known to occur for all these molecules (2, 5, 7, 12). Our results, however, do not support a general phenomenon because no significant inflammation-induced changes in the AAL reactivity were detected for the acute phase glycoprotein PI. In future studies the above-mentioned hypotheses will be tested in in vitro cell adhesion assays using purified AGP isolated from patient sera before and after induction of the acute phase reaction.

The degree of sialylation and/or type of branching of the glycans of AGP have been reported to affect immunomodulatory properties of AGP, like lymphocyte proliferation (11, 14), the induction of IL-1-inhibiting activity in macrophages (13), and also the aggregation of platelets (46). The presence of fucose or of SLeX-substituted glycans has not been considered in these studies. To further understand the role(s) of AGP in the acute phase response, it seems necessary to reinvestigate the immunomodulatory properties of AGP as a function of the degree of substitution with SLeX.

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