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# Inflammation-induced expression of sialyl Lewis$^x$ is not restricted to $\alpha_1$-acid glycoprotein but also occurs to a lesser extent on $\alpha_1$-antichymotrypsin and haptoglobin

Els C.M. Brinkman-van der Linden**, Patricia F. de Haan, Ellen C. Havenaar and Willem van Dijk*

*Department of Medical Chemistry, Faculty of Medicine, Vrije Universiteit, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands*

**Acute and chronic inflammation-induced expression of sialyl Lewis$^x$ has already been shown to occur on $\alpha_1$-acid glycoprotein. We now demonstrate that this phenomenon is not restricted to $\alpha_1$-acid glycoprotein but also occurs on two other acute-phase proteins. *ie* on $\alpha_1$-antichymotrypsin and on haptoglobin. The level of expression of sialyl Lewis$^x$ on these proteins was lower than on $\alpha_1$-acid glycoprotein, in all likelihood because $\alpha_1$-acid glycoprotein is the only acute-phase protein containing tetraantennary glycans. No expression of sialyl Lewis$^x$ was detectable on $\alpha_1$-protease inhibitor, a protein with a high diantennary glycan content. Non-sialylated Lewis$^x$ was not detectable on these major acute-phase proteins in any of the conditions studied. This indicates that the majority of the $\alpha$3-linked fucose residues are present as sialyl Lewis$^x$ on $\alpha_1$-acid glycoprotein, $\alpha_1$-antichymotrypsin and haptoglobin. The absolute contribution to the total phenotype in plasma of protein containing this determinant in a multivalent form was highest for $\alpha_1$-acid glycoprotein. This leads us to propose that $\alpha_1$-acid glycoprotein is, among the acute-phase proteins studied, the one with the highest potential for interference with the extravasation of leukocytes by binding to the selectins.**

***Keywords*: acute-phase proteins, *Aleuria aurantia* lectin, fucosylation, orosomucoid, sialyl Lewis$^x$, $\alpha_1$-acid glycoprotein**

***Abbreviations*: AAL, *Aleuria aurantia* lectin; ACT, $\alpha_1$-antichymotrypsin; AGP, $\alpha_1$-acid glycoprotein; APP, acute-phase protein; APR, acute-phase reaction; CAIE, crossed affinoimmunoelectrophoresis; Con A, Concanavalin A; HG, haptoglobin; HSPC, human serum protein calibrator; PI, $\alpha_1$-protease inhibitor; RA, rheumatoid arthritis; (S)LeX, (sialy) Lewis$^x$**

## Introduction

The cytokine-induced acute-phase response results in an increase in hepatic synthesis of various plasma glycoproteins, the so-called positive acute-phase proteins (APPs). It is generally accepted that these proteins have a function in limiting the damaging effects of the inflammatory response [1]. Besides the increase in synthesis, changes in glycosylation occur in various (patho)physiological conditions on the APPs (reviewed in [2]). The changes in glycosylation on $\alpha_1$-acid glycoprotein (AGP), a strongly glycosylated protein containing five N-linked glycans, have been extensively investigated in our and other laboratories (reviewed in [2] and [3]). The branching of the glycans on AGP is transiently decreased in acute inflammation and the sialyl Lewis$^x$ (SLeX) expression on AGP is increased in both acute and chronic inflammation [2, 3]. We have postulated that the increase in SLeX expression on human AGP could interfere with selectin-mediated rolling of leukocytes on endothelium in inflamed areas [4]. Changes in glycosylation of AGP also occur in pregnancy and after oestrogen treatment. Remarkably, these changes are opposite to the changes found in inflammation [5–8].

Inflammation-induced changes in glycosylation are not only found on AGP, but also on other major APPs, containing like AGP only complex-type N-linked glycans. For instance, in acute inflammation for $\alpha_1$-antichymotrypsin (ACT) and $\alpha_1$-protease inhibitor (PI) similar changes in degree of branching were found as for AGP [6, 9]. Furthermore, increases in the extent of fucosylation have been described to occur in RA, a chronic inflammatory condition, on ACT, haptoglobin (HG) and PI [10–14]. However, it is not known in which type of linkage the fucose residues are

*To whom correspondence should be addressed. Tel: 31.20.4448159; Fax: 31.20.4448144; E-mail: W.van_Dijk.medchem@med.vu.nl.
**Present address: Dept Med, Cancer Center, 0607, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92098, USA.

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present on these APPs, although for HG at least part of it can be due to the SLeX-type of configuration [15]. We have now studied, therefore, whether the increased expression of SLeX is a general phenomenon in the hepatic acute-phase response. This was determined on the major APPs, ACT, HG and PI in various (patho)physiological conditions in relation to the expression on AGP. SLeX expression was determined on Western blots using the mouse monoclonal anti-SLeX IgM CSLEX-1. The extent of fucosylation and degree of branching were analysed with crossed affinoimmunoelectrophoresis using, as affinocomponents, the lectins *Aleuria aurantia* (AAL) and concanavalin A (Con A), respectively.

## Materials and methods

### Materials

*Aleuria aurantia* mushrooms were collected locally and AAL was isolated as detailed earlier [4]. CNBR-activated Sepharose beads were obtained from Pharmacia-LKB (Uppsala, Sweden). Con A (Type V), Coomassie Brilliant Blue R250, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside and α-L-fucose were obtained from Sigma (St Louis, MO, USA). Human serum protein calibrator (HSPC) and rabbit anti-human AGP, ACT, HG, and PI IgG were purchased from Dakopatts (Glostrup, Denmark), and polyacrylamide and agarose M from BioRad (Richmond, CA, USA). Mouse anti-SLeX IgM CSLEX-1 (ATCC, HB 8580) prepared from mouse ascites fluid was a kind gift from Dr D. Dus from the Ludwik Hirzfeld Institute, Wroclaw, Poland. Mouse anti-LeX IgM anti-CD15 and the poolserum of healthy individuals were purchased from the Central Laboratory of Bloodtransfusion (CLB, Amsterdam, The Netherlands). Alkaline phosphatase-conjugated goat anti-mouse IgM was obtained from Zymed (San Francisco, CA, USA), and *Vibrio cholerae* neuraminidase from Boehringer (Mannheim, Germany). All other materials used were of analytical grade and were obtained from commerical sources.

### Source of sera and determination of protein concentration of the various APPs

Sera were pooled from healthy individuals ($n = 75$) (CLB), from patients suffering from severe trauma in the second week of the trauma ($n = 6$), from patients suffering from rheumatoid arthritis ($n = 15$), and from male-to-female transsexuals receiving oral oestrogen treatment ($n = 8$). Sera were stored at $-20\,^{\circ}C$ until analysis. The concentration of the various APPs in these sera were determined by radial immunodiffusion according to Mancini [16]. HSPC consisting of pooled sera from healthy blood donors was used as a standard.

### Detection of expression of (S)LeX

To detect the expression of SLeX on the APPs, enriched preparations of these proteins were obtained from the indicated sera by immunoaffinity chromatography over anti-APP-Sepharose columns. The anti-APP-Sepharose columns ($0.8 \times 3.0$ cm) were prepared from CNBR-activated Sepharose and rabbit anti-human-AGP, ACT, HG or PI IgG, according to the instructions of the supplier. One hundred μl of serum was applied to each column, which was subsequently washed with PBS ($2\,ml\,h^{-1}$) until the absorption at $A_{280nm}$ of the effluent was zero. Bound proteins were eluted with 0.05 M diethylamine (pH 11.5), 0.15 M NaCl under immediate neutralization and were subsequently dialysed extensively against tri-distilled water. SDS-PAGE was performed according to Laemmli [17] with equal amounts of APP (5 μg) in each lane. The proteins were subsequently blotted onto nitrocellulose by electrophoretic transfer. SLeX determinants were detected by incubating the nitrocellulose blots with the mouse monoclonal anti-SLeX IgM CSLEX-1, as previously described [18]. AGP desialylated with neuraminidase (*Vibrio cholerae*) was used as a negative control [4]. LeX determinants were detected by incubating the nitrocellulose blots of protein preparations with the monoclonal anti-LeX IgM anti-CD15 ($15\,\mu g\,ml^{-1}$) following the same procedures as described for CSLEX-1. AGP isolated from a patient suffering from severe trauma was desialylated with neuraminidase (*Vibrio cholerae*) and used as a positive control.

### Crossed affino-immunoelectrophoresis (CAIE)

CAIE was performed according to the Bøg-Hansen method [19]. The different glycosylated glycoforms of AGP, ACT and PI were separated by electrophoresis of sera (0.3–9.0 μl) through a Con A ($1\,mg\,ml^{-1}$) or AAL ($2.5\,mg\,ml^{-1}$ of an AAL preparation with a haemagglutination titre of 1024) [4] containing polyacrylamide slab gel [20]. The glycoforms of HG could not be separated in a polyacrylamide slab gel because of the high molecular weight and therefore these were separated conventionally through a Con A ($1\,mg\,ml^{-1}$) or AAL ($2.5\,mg\,ml^{-1}$) containing agarose gel [12]. Immunoelectrophoresis in the second perpendicular dimension in the presence of a precipitating monospecific antiserum in a 1% agarose gel, the subsequent staining of the precipitation curves of the separated glycoforms with Coomassie Brilliant Blue R250, as well as the determination of the areas under the curves representing the relative amounts of glycoprotein, were performed as described previously [12]. Means ± SD were calculated from three independent assays.

## Results

### Expression of (sialyl) Lewis$^x$ on APPs

In order to analyse the level of expression of SLeX on AGP, ACT, HG and PI, partially purified APPs from the indicated pooled sera were subjected to SDS-PAGE, blotting and subsequent staining with a monoclonal antibody directed against SLeX (CSLEX-1). In Figure 1 the expression

of SLeX on AGP, ACT and HG is shown. On PI no expression of SLeX could be detected in any of the conditions studied. In healthy conditions plus or minus oestrogen treatment the expression of SLeX was low on AGP and ACT and hardly detectable on HG. Conversely, an increase in expression of SLeX was found in trauma and RA on AGP, ACT and HG. However, the level of expression was most prominent on AGP. Desialylation abolished the staining with CSLEX-1 (not shown). In all these conditions and on all four proteins studied no expression of LeX could be detected with a monoclonal antibody directed against LeX (anti-CD15) (not shown).

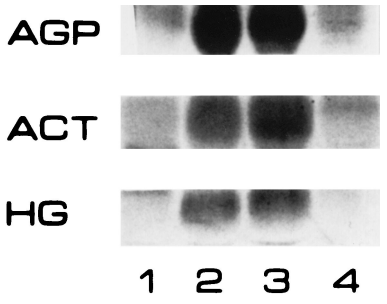


**Figure 1.** Expression of SLeX on AGP, ACT and HG isolated in various conditions. Partially purified APPs (5 μg) were subjected to SDS-PAGE, subsequent blotting and detection of SLeX with a specific monoclonal antibody (CSLEX-1), as described in Materials and methods. Care was taken that an equal amount of APP was applied in each lane and this was checked by specific detection of the proteins on a separate blot (not shown, see Materials and methods). Only the part of the blot containing APP-bands is reproduced. 1, healthy condition; 2, severe trauma, 3, RA; and 4, oral oestrogen treatment.

## Extent of fucosylation of APPs

The extent of fucosylation of AGP, ACT, HG and PI was determined by the CAIE of the various sera with the fucose-specific lectin AAL. This lectin fractionates the various APPs into a non-reactive glycoform A0, with no fucose residues, and into the reactive glycoforms A1–A3/4 containing fucose residues in increasing amount. The precipitation curves are given in Figure 2. The extent of fucosylation was increased in trauma and RA for all four APPs in comparison to the healthy condition, as indicated by the relative increase in their strongly fucosylated glycoforms as well as by the relative decrease in the A0 glycoforms lacking fucose (Figure 2). The relative increase in extent of fucosylation was

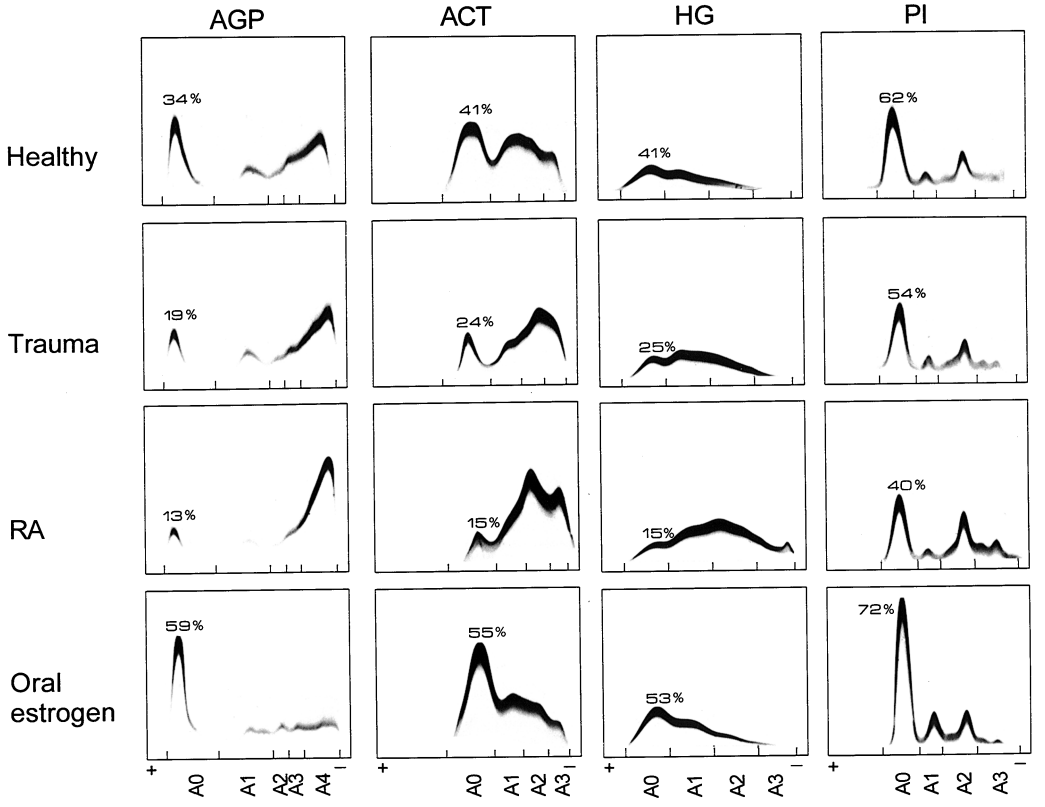


**Figure 2.** Reactivity of AGP, ACT, HG and PI with AAL in healthy individuals plus and minus oestrogen treatment, in patients suffering from severe trauma, and in patients suffering from RA. Sera were subjected to CAIE with AAL as described in Materials and methods. Only the second dimension gels are shown. The application site is located at the cathodic site, indicated by –. Electrophoresis was performed from right to left for the first dimension, and from bottom to top for the second dimension. A0: APP-fractions that are non-reactive with AAL, A1–A4: APP-fractions reactive with AAL and containing fucose residues in increasing amount. The percentage of A0 as determined by planometry (see Materials and methods) is given for each APP in all conditions.

more pronounced in RA than in trauma for all four APPs (*cf.* trauma and RA panel in Figure 2). Oral oestrogen treatment induced an opposite effect on the extent of fucosylation of all four APPs studied to that found during inflammatory conditions. This is clear from the precipitation curves obtained with the sera of male-to-female transsexuals treated orally with oestrogens in comparison to non-treated healthy individuals (Figure 2).

The absolute levels of α3-fucosylated glycoforms of AGP, ACT and HG were calculated from the total concentration in serum of these APPs in the various conditions and their relative distribution as determined from the areas under the curves of A1–A4 (see Materials and methods). It is clearly demonstrated that acute and chronic inflammation induced high increases in the plasma levels of the fucosylated glycoforms of these proteins (Figure 3A). However, AGP appeared to be the most predominant protein when only the fucosylated glycoforms containing presumably more than two α3-linked fucose residues (A3/4) were taken into account (Figure 3B). Although inflammation also induced an increase in α3-fucosylation of PI, this could not be quantified because this protein also contains α6-linked fucose residues [21, 22], which contributes to the retardation of PI by AAL [23, 24].

## Degree of branching of APPs

In order to determine the relative degree of branching of the glycans of AGP, ACT, HG and PI the various sera were subjected to CAIE with Con A. Since the proteins studied contain only complex-type N-linked glycans, Con A will fractionate the APPs into a non-reactive glycoform C0, with a higher than diantennary degree of branching, and into the reactive glycoforms C1–C3/4 containing diantennary glycans in increasing amount. In all conditions studied AGP appeared to be the APP with the most predominant C0 fraction, *ie* with the highest amount of tri- and/or tetraantennary glycans. The C0 fraction of ACT was much lower, whereas for HG and PI this fraction was hardly detectable, as is illustrated in Figure 4 for RA. The changes in degree of branching of the glycans on the APPs in the various conditions were in accordance with earlier findings (not shown) [2, 3, 6, 8–10, 14, 25, 26].

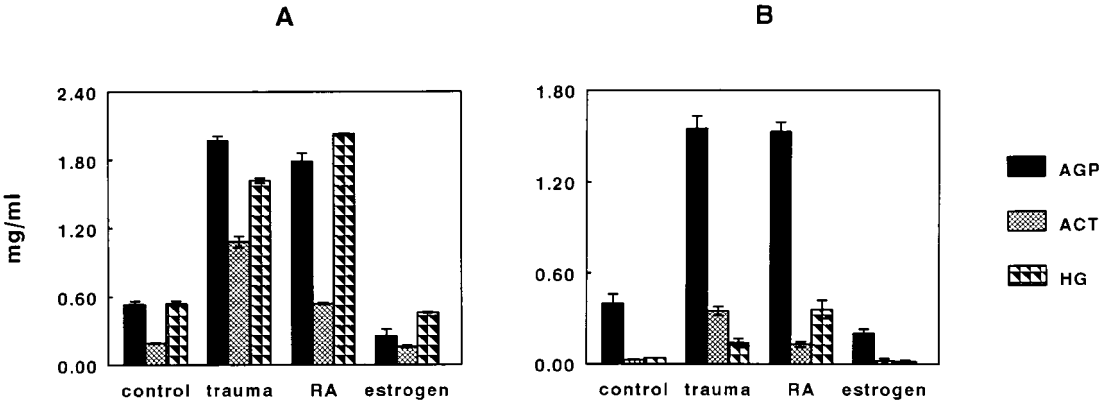


**Figure 3.** Serum levels of fucosylated APP-glycoforms. (A) The absolute levels of all *a*3-fucosylated glycoforms of AGP, ACT and HG in plasma as calculated from the total concentration in plasma determined by radial immunodiffusion according to Mancini [16] (not shown), and the relative distribution of these glycoforms in CAIE (see Figure 2) determined by planometry (see Materials and methods); (B) The absolute levels of glycoforms containing more than two *a*3-linked fucose residues of these proteins in plasma, calculated as in (A).

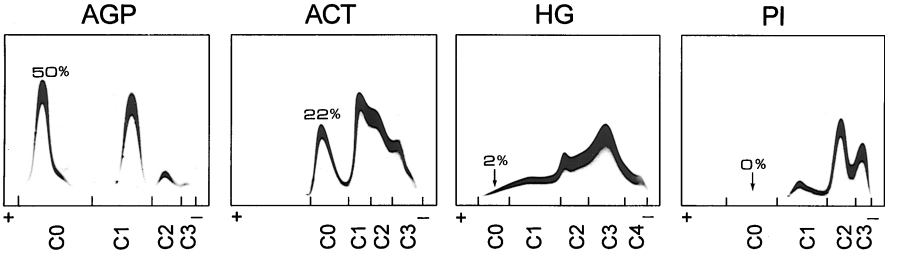


**Figure 4.** Reactivity of AGP, ACT, HG and PI with Con A in patients suffering from RA. Sera were subjected to CAIE with Con A as described in Materials and methods. Only the second dimension gels are shown. The application site is located at the cathodic site, indicated by − . Electrophoresis was performed from right to left for the first dimension, and from bottom to top for the second dimension. C0: APP-fractions that are non-reactive with Con A, C1–C4: APP-fractions reactive with Con A and containing diantennary glycans in increasing amount. The percentage of C0 as determined by planometry (see Materials and methods) is given for each APP.

## Discussion

In this study we have shown that the hepatic acute-phase response in acute and chronic inflammation induces increased expression of SLeX not only on AGP, but also on ACT and HG. For AGP, ACT and HG the level of expression of SLeX correlated with the changes in extent of retardation with AAL in CAIE (*cf.* Figures 1 and 3). This indicates that changes in AAL-reactivity can be interpreted as changes in α3-fucosylation, and not α6-fucosylation, of these three APPs. This was already known for AGP [4] and is in agreement with our findings, *ie* no retardation by AAL of ACT and HG was observed in the CAIE of sera from individuals lacking the fucosyltransferase-VI activity which is responsible for the α3-fucosylation of APPs in the liver ([24], Brinkman-Van der Linden ECM, Mollicone R, Oriol R, Van Dijk W, unpublished observations). LeX was not detectable on the APPs studied, indicating that the majority of α3-linked fucose residues are present as SLeX on AGP, ACT and HG. No expression of (S)LeX was detectable on PI in any of the conditions studied. PI has a relatively low fucose content (Figure 2), part of which is present in an α6-linkage to the chitobiose core of the glycan [21, 22]. Nevertheless, a low level of expression of (S)LeX cannot be excluded since inflammation-induced increases in fucosylation of PI were detected by the highly sensitive technique of CAIE with AAL. This concerned the fucosylated fractions A2 and A3 (Figure 2), which have previously been shown to be partly substituted with α3-linked fucose residues [24].

An increase in level of expression of SLeX on HG in RA was suggested by Katnik *et al.* [15] from the analysis of sera of RA patients using a CSLEX-1 based ELISA assay. Our study shows that the level of expression of SLeX on AGP was higher than on ACT and especially than on HG, as detected by immunoblotting with the same anti-SLeX antibody CSLEX-1. A direct relationship between reactivity with the fucose specific lectin AAL and SLeX expression was previously established by us [18]. Therefore, a higher SLeX content of AGP is also indicated by the very strong retardation of AGP relative to ACT and HG in CAIE with AAL (Figure 2). It is conceivable that the underlying higher content of α3-linked fucose in AGP is brought about by the higher degree of branching of the glycans of AGP. This is the only APP also containing tetraantennary glycans, whereas diantennary glycans predominate on ACT and especially on HG as well as on PI [21, 22, 27–30]. This is illustrated in Figure 4 by the difference in Con A reactivity of the various APPs in RA. A dependency of SLeX expression on the degree of branching is supported by the absence of antibody-detectable (S)LeX on PI. This APP appeared to have an even higher diantennary glycan content than HG. Furthermore, it is conceivable that a decreased complexity of the glycans will result in a lower SLeX expression, since the ratio of α2-3-linked to α2-6-linked sialic acid residues is decreased concomitantly on the glycans as a consequence of the specificities of the glycosyltransferases involved [31–33]. Indeed, for the diantennary glycans of PI all sialic acid residues were found to occur in an α2-6-type of linkage [21, 22].

In conclusion, as a result of the hepatic inflammatory response the total number of circulating SLeX-expressing molecules increases dramatically (Figure 3A), thereby affecting the phenotype of plasma. Various immunomodulatory properties of APPs have been shown to be dependent on their type of glycosylation [30, 34 –38]. The large changes in glycosylation of plasma proteins can, therefore, be considered to have functional implications in the inflammatory response [4]. It can be assumed that effects of increased expression of SLeX on APPs are most predominant for AGP because this appeared to be the only APP exhibiting a strong increase in multivalent expression of SLeX in inflammation (Figure 3B). Such a multivalency has been shown to be of importance for binding between selectins and its counterreceptors [39–41]. We are now studying a possible involvement of SLeX-expressing glycoforms of AGP in various immunomodulatory events, with emphasis on selectin-mediated interaction of leukocytes with endothelium in inflamed areas.

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