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Elevated Serum Level and Altered Glycosylation of α_1-Acid Glycoprotein in Hyperimmunoglobulinemia D and Periodic Fever Syndrome: Evidence for Persistent Inflammation

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Crossed affinoimmunoelectrophoresis using concanavalin A and *Aleuria aurantia* lectin as diantennary glycan- and fucose-specific affinocomponents, respectively, was applied to study changes in the concentration and glycosylation of the acute phase protein α_1-acid glycoprotein (AGP) in sera obtained from patients with hyperimmunoglobulinemia D and periodic fever syndrome. Increases in concentration of AGP compared to control values were found not only during attacks, but also during remissions. Compared to healthy controls, the presence of diantennary glycan-containing glycoforms of AGP also increased during febrile attacks, while no changes were found during remissions. A continuous high degree of $\alpha 1 \rightarrow 3$ fucosylation was accompanied by a continuous high expression of sialyl Lewis^x on AGP. Despite the clinical picture of recurrent febrile attacks with asymptomatic intervals, these studies indicate that hyperimmunoglobulinemia D should be considered a condition of persistent inflammation.

INTRODUCTION

Hyperimmunoglobulinemia D and periodic fever (hyper IgD syndrome)[1] is a syndrome characterized by recurrent febrile attacks, usually of very early onset (during the first year of life) and a constantly elevated polyclonal serum IgD level. Since the first description of the hyper-IgD syndrome (1), the diagnosis has been made in up to 56 patients mainly from Europe in a collaborative study by the Hyperimmunoglobulinemia D Study Group (2–4). The attacks are characterized by an acute onset of high spiking fever, often preceded by cold chills. Abdominal complaints, vomiting, diarrhea, headache, and arthralgias are associated with the episodes. During the attacks, swollen, tender lymph nodes are often found. Especially in the young age group, splenomegaly may be found. Nondestructive recurrent arthritis can be demonstrated in more than 60% of the patients (4). During the attacks, most patients have skin lesions due to cutaneous vasculitis (3). Typical attacks occur every 4 to 8 weeks and last 3 to 7 days each. Laboratory testing during attacks reveals an acute phase response (leukocytosis, neutrophilia, and increasing erythrocyte sedimentation rate (ESR)). The diagnosis is based on the clinical picture and the elevated serum IgD level (>100 U/ml). The pathogenesis of the syndrome remains to be elucidated and no treatment is known.

During remissions the concentration of C-reactive protein, a well-known marker of an acute phase reaction, returns to normal levels, although it is elevated during attacks (4). It is unclear as yet whether the syndrome is characteristic for an acute state of inflammation, or whether there is a state of persistent inflammation. It has been shown in several types of inflammation that the glycosylation of acute phase proteins changes during acute and chronic inflammatory processes (5). α_1-Acid glycoprotein (AGP, orosomucoid) is a positive acute phase protein with five N-linked glycans. At least 12 glycoforms of AGP can be detected in normal human serum differing in degree of branching, fucosylation, and sialylation of the glycans. The glycoforms containing one or more diantennary glycans are increased in concentration during acute inflammation. This occurs after acute trauma (6–9) or can appear with intercurrent infections, as have been described in rheumatoid arthritis (10, 12). In chronic inflammation such as rheumatoid arthritis, however, only minor changes could be established (10, 11). Cytokines like IL-1, IL-6, and TNF are involved in the inflammatory response and have been shown to affect the glycosylation process in the liver (12–14). Besides the changes in diantennary glycan content, we have recently reported

[1] Abbreviations used in this paper: AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; AGP; α_1-acid glycoprotein; CAIE, crossed affinoimmunoelectrophoresis; SLeX, sialyl Lewis X; hyper-IgD syndrome, hyperimmunoglobulinemia D; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; AT, α_1-antitrypsin; IL-1, interleukin 1; IL-6, interleukin-6; TNF, tumor necrosis factor.

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increases in the $\alpha 1 \rightarrow 3$ fucosylation of AGP, during both acute (15) and chronic inflammation (16, 17). The high degree of $\alpha 1 \rightarrow 3$ fucosylation in patients with rheumatoid arthritis could be returned to a lower level by means of treatment with methotrexate (17). This increase in fucosylation has also been described for haptoglobin (17–19) and α_1-antitrypsin (AT) (17). The increased $\alpha 1 \rightarrow 3$ fucosylation was accompanied by an increased expression of blood group determinant sialyl Lewis^X (SLeX; NeuAc$\alpha 2 \rightarrow$ 3Gal$\beta 1 \rightarrow$ 4 (Fuc$\alpha 1 \rightarrow$ 3)-GlcNAc-R) (16). Therefore, the type of glycosylation of the acute-phase protein AGP can give more information about the state of inflammation and can serve as a better marker for discrimination between acute and chronic inflammatory states than the CRP concentration alone.

To gain a better understanding of the nature of the inflammatory response occurring in the hyper-IgD syndrome, the serum concentration of the acute-phase protein AGP and its type of glycosylation including the expression of SLeX were investigated in sera from patients with this syndrome, during both febrile attacks and remissions. Crossed affinoimmunoelectrophoresis (CAIE) was used to determine the presence of diantennary containing glycoforms of AGP using concanavalin A (Con A) as affinocomponent in the first-dimension gel. The presence of glycoforms with $\alpha 1 \rightarrow 3$ fucosylated glycans was determined using CAIE with *Aleuria aurantia* lectin (AAL) as affinocomponent. AAL is the only lectin to date that will react with the fucosylated lactosamine units present on AGP (15, 20, 21).

MATERIALS AND METHODS

Source of sera. Eleven patients (nine males, two females) suffering from the hyper-IgD syndrome were studied. The diagnosis was in accordance with the guidelines of the "International Hyper-IgD Study Group," and these patients represented the numbers 2, 5, 9, 11, 18, 21, 24, 26, 27, 35, and 36 in a recent clinical review (4). The mean age at the time of the attacks and collecting of blood was 30 years (range 13–69 years) and all patients were Dutch. Sampling of serum was performed during attacks and remissions. Attacks were defined when the patients were febrile >38°C (not caused by infection) and experiencing symptoms compatible with the hyper-IgD syndrome. Remission was defined as the complete absence of symptoms for at least 2 weeks. No medical treatment was allowed during the study period. In addition, sera from two patients suffering from the hyper-IgD syndrome, who had been asymptomatic for at least 2 years, were studied. Sera were stored at –20°C until analysis. Sera from healthy individuals were used for the determination of control values.

(Glyco)protein concentration. Concentrations of CRP were determined by rocket immunoelectrophoresis according to Laurell (22), using rabbit anti-human CRP IgG (RAH-CRP-IgG) for precipitation. AGP concentrations were determined by single radial immunodiffusion, according to Mancini *et al.* (23), using monospecific anti-human AGP IgG (RAH-AGP-IgG) for precipitation. All antibodies were purchased from Dakopatts, Glostrup, Denmark. Human serum C-reactive protein (HS-CRP) calibrator, consisting of a delipidated pool of human serum enriched in CRP, was used as a standard for the determination of the CRP concentration; according to the manufacturer's information the CRP concentration in this preparation was 0.162 mg/ml. Human serum protein calibrator (HSPC), consisting of pooled sera from healthy blood donors, was used as a standard for the determination of the AGP concentration; according to the manufacturer's information that AGP concentration in this preparation was 0.76 mg/ml. Both HSPC and HS-CRP calibrator were from Dakopatts.

Crossed affinoimmunoelectrophoresis (CAIE). CAIE was performed according to the method of Bøg-Hansen (24), using 2.5 mg/ml (with a hemagglutination titer of 512) *Aleuria aurantia* lectin (AAL) as the fucose-specific affino component, or 2 mg/ml concanavalin A as the diantennary-specific affinocomponent as described previously (15). AAL was isolated from fruiting bodies of the *Aleuria aurantia* mushroom as detailed earlier (15). In short, 0.5–2.0 μl of serum was electrophoresed through a lectin containing 1% agarose gel, resulting in a separation of the differently glycosylated AGP forms. In the second, perpendicular dimension, these glycoprotein forms were immunoelectrophoresed against the precipitating monospecific antiserum (RAH-AGP-IgG). The resulting precipitation curves were visualized by staining with Coomassie brilliant blue R250 (Sigma, St. Louis, MO). The areas under the curves indicate the relative amounts of glycoprotein, which were determined using a Summagraph (ACECAD D-9000) coupled to a 386 SX PC equipped with an area measurement program (developed by D. Philips, Department of Physiology, Faculty of Medicine, Vrije Universiteit, Amsterdam, The Netherlands).

Partial purification of AGP from serum and detection of SLeX-content of AGP. In order to obtain preparations enriched with AGP for a better interpretation of immunoblots, AGP was isolated from serum by immunoaffinity chromatography, as described (25), using an anti-AGP-Sepharose 4B column. Serum (75–100 μl) was applied to the anti-AGP-Sepharose 4B column (0.8 $\times$ 1.3 cm; 2 ml/hr). The glycosylation profiles of AGP before and after affinity purification were identical, indicating that no glycosidases were present during the procedure. The concentration was determined by radial immunodiffusion, according to Mancini *et al.* (23). Ten percent SDS–PAGE was performed according to

Laemmli (26) using the Mini Protean II dual slab gel apparatus from Bio-Rad. Gels were loaded with equal amounts of partially purified AGP; AGP isolated from Cohn fraction V from pooled normal human serum, a kind gift from Dr. D. H. van den Eijnden, was used as a standard. Proteins were blotted onto nitrocellulose by electrophoretic transfer using the Mini Transblot Cell from Bio-Rad. SLeX determinants on AGP were detected as previously described (15) by incubating AGP containing nitrocellulose strips with mouse monoclonal anti-SLeX IgM [CSLEX-1 (27) 25 μg/ml in 10 times diluted PBS; ATCC HB8580], followed by alkaline phosphatase-conjugated goat anti-mouse IgM (1:250 dilution, Zymed, San Francisco, CA) for detection. An AAL-nonreactive fraction isolated from pooled normal human serum by means of preparative affinity electrophoresis (25) was used as a negative control.

Statistics. All values were tested for significance using the Student t test.

RESULTS

Concentrations of CRP and AGP in hyper-IgD sera. The CRP concentrations in the sera of hyper-IgD patients were below 0.02 mg/ml during remission and increased to 0.10–0.30 mg/ml during an attack. In contrast, the AGP concentrations during remission were higher than those in control sera (1.98 mg/ml compared to 0.79 mg/ml in control sera, $P < 0.05$), with large interindividual differences. These concentrations increased to an average value of 2.31 mg/ml during a febrile attack, also with large interindividual differences (Table 1). For the two patients who had been asymptomatic for at least 2 years, all protein concentrations had returned to control values (results not shown).

Reactivity of AGP with Con A and AAL. To establish whether changes in the diantennary glycan content and $\alpha 1 \rightarrow 3$ fucosylation of AGP occurred in the sera of patients with the hyper-IgD syndrome during remission and febrile attacks, CAIE was performed with Con A and AAL as affinocomponents, respectively. In Figs. 1b, 1c, 1e, and 1f, precipitation curves of AGP are illustrated for one hyper-IgD patient, produced by CAIE with Con A and AAL, respectively. During a febrile attack, the reactivity with Con A was significantly increased compared to control and remission values ($P < 0.05$), since the percentage of the Con A nonreactive fraction (C0) was decreased from 40.6% during remission to 35.3% during an attack (Table 1). The degree of reactivity ((C2 + C3)/C1) was concomitantly increased from 0.37 during remission to 0.51 during an attack. During remission, the reactivity of AGP with Con A returned to the control value (Table 1).

The reactivity of AGP with AAL showed more distinct differences when compared to control values for both states of disease (Table 1). The degree of reactivity ((A3 + A4 + A5)/(A1 + A2)) of AGP with AAL for hyper-IgD patients was extremely high during both a febrile attack (3.64) and remission (3.51). Although the values expressed large interindividual differences, they were significantly different from the degree of AAL reactivity of the control (0.92, $P < 0.05$). These increases in degree of reactivity corresponded with low percentages of unreactive component (A0) for AGP during both remission (16.6%) and fever (15.8%) compared to the control value (38.0%). The glycosylation of AGP in the sera from patients who had been asymptomatic for at least 2 years had returned to control values, with respect to both the diantennary glycan content and the $\alpha 1 \rightarrow 3$ fucosylation (results not shown).

Expression of Sialyl Lewis^X (SLeX) on AGP in hyper-IgD sera. In order to determine if the increases in $\alpha 1 \rightarrow 3$ fucosylation also coincided with an increased expression of SLeX, partially purified AGP from 4 of the

TABLE 1

Concentrations of CRP and AGP and Reactivities with Con A and AAL of AGP in Hyper-IgD and Control Sera

				ConA		AAL	
	CRP (mg/ml)	AGP (mg/ml)	n	C0 (%)	DR (C2+C3)/C1	A0 (%)	DR (A3+A4+A5)/(A1+A2)
Control	< 0.02	0.79 ± 0.17	10	46.0 ± 8.0	0.33 ± 0.11	38.0 ± 13.0	0.92 ± 0.36
HIgD—remission	< 0.02 (11/0)	1.98 ± 0.72[a] (0/11)	11	40.6 ± 5.6 (7/4)	0.37 ± 0.10 (9/2)	16.6 ± 8.2[a] (1/10)	3.51 ± 2.17[a] (0/11)
HIgD—fever	0.18 ± 0.10[a,b] (0/11)	2.31 ± 1.10[a] (0/11)	11	35.3 ± 4.7[a,b] (2/9)	0.51 ± 0.15[a,b] (4/7)	15.8 ± 8.8[a] (3/8)	3.64 ± 2.06[a] (0/11)

Note. Values are given as the mean ± SD. DR, degree of reactivity; this is a measure for the intensity of the interaction between the lectin and AGP. The degrees of reactivity were determined by dividing the sum of the strongly reactive glycoforms by the sum of the moderately reactive glycoforms. C0 and A0, fraction of AGP nonreactive with Con A and AAL, respectively. Values given in parentheses are the ratio of values that fall within the control range over the values that fall outside it.

[a] Significantly different from control value $P < 0.05$.

[b] Significantly different from HIgD—remission value $P < 0.05$.

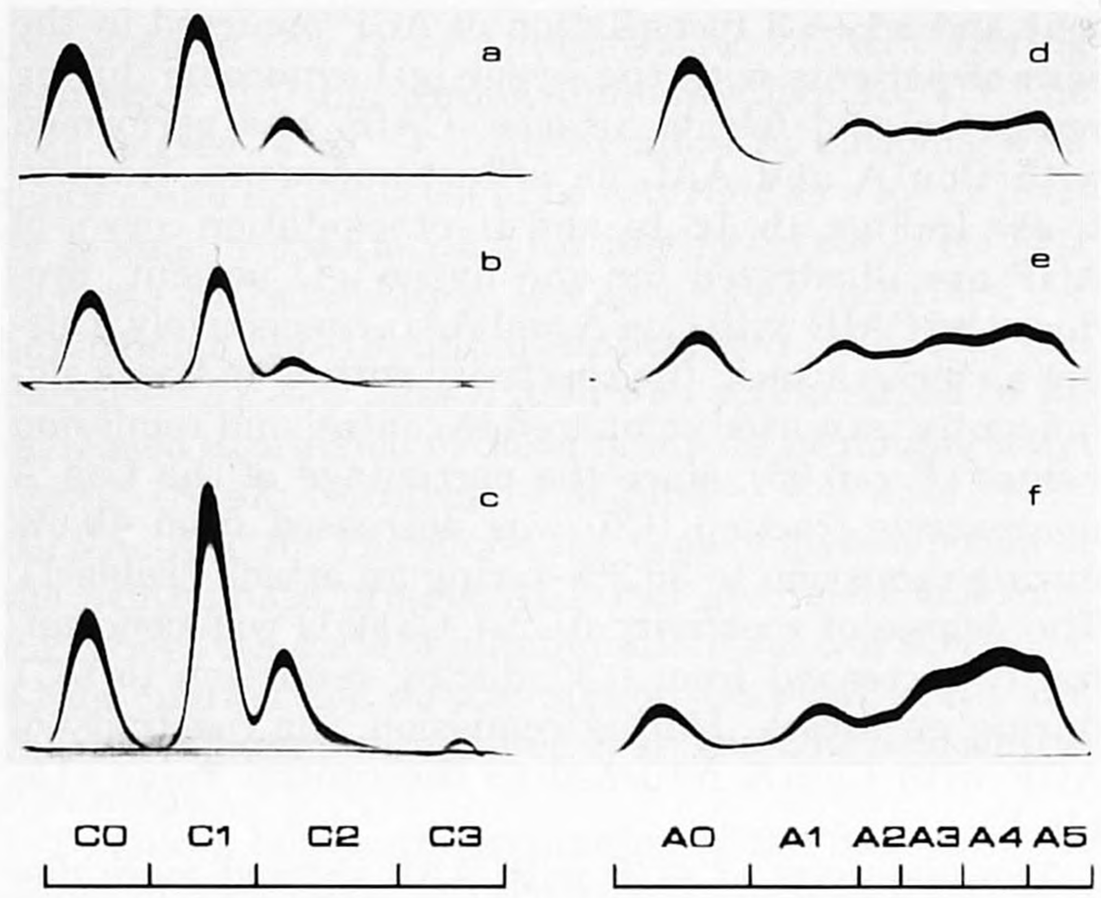

FIG. 1. Reactivity of AGP with Con A (a–c) and AAL (d–f) in control serum (a,d) and hyper-IgD serum during remission (b,e) and during a febrile attack (c,f). Serum (0.5–2.0 μl) from the same patient was subjected to CAIE as described under Materials and Methods. Only the second-dimension gels are shown. The lower right corner of each gel corresponds with the site of application in the first-dimension gel. Electrophoresis was performed in the directions right to left for the first dimension and bottom to top for the second dimension. CO and AO, AGP fractions that are nonreactive with Con A and AAL, respectively; C1–C3 and A1–A5, forms of AGP that are increasingly reactive with Con A and AAL, respectively.

11 different patient sera (chosen at random) was subjected to SDS–PAGE, blotted, and subsequently stained with a monoclonal antibody directed against SLeX (CSLEX-1). A representative Western blot of AGP isolated from one patient is shown in Fig. 2. Upon staining of AGP-containing nitrocellulose strips with CSLEX-1, a weak positive staining of AGP from sera obtained from healthy individuals was observed (Fig. 2, lane 1). A strongly enhanced positive staining was observed for AGP isolated from hyper-IgD patients during remission and febrile attack (Fig. 2, lanes 3 and 4, respectively), indicating a significant increase in the expression of SLeX on AGP in the hyper-IgD syndrome. No staining was observed for an AAL-nonreactive fraction isolated from the serum of healthy individuals (Fig. 2, lane 2), which was used as a negative control.

FIG. 2. Expression of SLeX on partially purified AGP from hyper-IgD sera. Equal amounts of affinity-purified AGP [3 μg as determined by radial immunodiffusion (23)] were subjected to SDS–PAGE, subsequent blotting, and detection of SLeX with a specific monoclonal antibody (CSLEX-1) as described under Materials and Methods. A representative Western blot of AGP from one hyper-IgD patient is shown; only the part of the blot containing AGP bands is reproduced. 1, AGP from control serum; 2, AGP nonreactive with AAL; 3, AGP from hyper-IgD serum during remission; 4, AGP from hyper-IgD serum during an attack.

DISCUSSION

Although the hyper-IgD syndrome is characterized by short febrile attacks followed by apparently asymptomatic intervals, the present study is indicative of both acute and chronic inflammatory responses within this disease. The concentration of the acute phase protein AGP is significantly increased during both febrile attacks and remissions, as compared to concentrations in serum of healthy individuals. The concentration of CRP is increased only during febrile attacks. The $\alpha 1 \rightarrow 3$ fucosylation of AGP, as indicated by the high reactivity with AAL and the CSLEX-1 staining, in serum of hyper-IgD patients is increased during both attacks and remissions. The increases in $\alpha 1 \rightarrow 3$ fucosylation are extreme compared to the value for healthy individuals, with a concomitant increased expression of SLeX. Previously, it has been shown that for chronic inflammation such as rheumatoid arthritis, the percentage of nonfucosylated AGP (A0) was approximately 18 (17), whereas for acute inflammation induced by laparotomy, this percentage was found to be approximately 30 (15). Therefore, the low percentages of nonfucosylated AGP (A0) continuously present in hyper-IgD patients are compatible with chronic inflammation. The continuously high $\alpha 1 \rightarrow 3$ fucosylation in rheumatoid arthritis can be decreased by treatment with methotrexate, and this coincides with a decrease in disease activity (17). Possibly, the relatively high frequency of febrile attacks (once every 4–8 weeks) in the hyper-IgD syndrome does not allow the $\alpha 1 \rightarrow 3$ fucosylation to return to a basic level, since after an asymptomatic period of 2 years, the concentrations of CRP and AGP and the glycosylation of AGP returned to control values. This would be in agreement with our previous finding that in the case of severe burns, the $\alpha 1 \rightarrow 3$ fucosylation can remain extremely high for at least 30 days after the event (15), indicating a persistent state of inflammation.

With respect to the Con A reactivity of AGP, significant increases occurred during a febrile attack, returning to control values during remission. These increases in Con A reactivity of AGP may be interpreted as increases in diantennary glycan content, since it has previously been shown that the glycans of AGP are of a complex type, in both healthy and diseased states (9). This supports the previous findings that the febrile attack is accompanied by an acute phase response, which is further corroborated by the steeply elevated CRP plasma concentrations found in these patients during attacks.

In conclusion, our studies indicate that the hyper-IgD syndrome seems to be a disorder of persistent inflammation. On the one hand the concentration of AGP continuously increases during attacks and remissions, as does the $\alpha 1 \rightarrow 3$ fucosylation of AGP, indicating a chronic state of inflammation. On the other hand the concentration of CRP increases only during attacks, as does the diantennary glycan content of AGP, indicating an acute state of inflammation within the persistently inflamed state. These patterns are also found during intercurrent infections in patients with rheumatoid arthritis (11), but are unique for a noninfectious disorder like the hyper-IgD syndrome. Proinflammatory cytokines (IL-1, IL-6, TNF) have been shown to modify the glycosylation of acute phase proteins such as AGP (12–14). The changes reported here for the hyper-IgD syndrome suggest a possible role for such cytokines in the pathogenesis of this syndrome.

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