Microheterogeneity of acute-phase glycoproteins in patients with pulmonary sarcoidosis

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Microheterogeneity of acute-phase glycoproteins in patients with pulmonary sarcoidosis. P. Hrycaj, K. Wurm, P. Mennet, W. Müller. ©ERS Journals Ltd 1996.
ABSTRACT: This study was designed to investigate qualitative changes in the carbohydrate side-chains of two acute-phase glycoproteins, α_1-acid glycoprotein (AGP) and α_1-antichymotrypsin (ACT), in 37 patients with pulmonary sarcoidosis.

The glycosylation profile of AGP and ACT was studied using affinity immunoelectrophoresis with the lectin concanavalin A (conA). Serum concentration of soluble receptor for interleukin-2 (sIL-2R) and activity of serum angiotensin converting enzyme (ACE) were measured by specific enzyme-linked immunosorbent assay (ELISA) and enzyme kinetic assay, respectively. Rocket immunoelectrophoresis and nephelometric assay were used to determine serum concentration of AGP, ACT and C-reactive protein (CRP).

In 11 patients with active disease, a decreased reactivity of AGP with conA was found as compared with controls (n=44) and patients with nonactive sarcoidosis (n=26). A similar tendency was seen with ACT. In the same group, increased concentrations of serum AGP and higher levels of sIL-2R were detected compared with patients with nonactive sarcoidosis. In the entire sarcoidosis group, there was a negative correlation between ACE activity and AGP and ACT affinity for conA (r= -0.6358, and r=-0.5019, respectively) and a positive correlation with sIL-2R level (r=0.8241). In nine patients with elevated concentrations of serum CRP, no differences were found in disease activity and glycosylation profile of AGP and ACT when compared to patients with normal serum CRP.

The results suggest that in active pulmonary sarcoidosis changes in the glycosylation pattern of acute-phase glycoproteins exist, which are similar in trend and magnitude to those found in other chronic inflammatory diseases. The synthesis and glycosylation of acute-phase proteins in pulmonary sarcoidosis are probably regulated independently.
Eur Respir J., 1996, 9, 313–318.

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Most acute-phase proteins, with only few exceptions, carry N-glycosidically linked oligosaccharides. The most characteristic acute-phase glycoprotein, α_1-acid glycoprotein (AGP), consists of a single polypeptide chain with five α-mannose-containing heteroglycan side-chains coupled to five asparagin molecules [1, 2]. Another typical member of this group, α_1-antichymotrypsin (ACT) possesses three carbohydrate side-chains. It has been shown that the carbohydrate moieties of acute-phase glycoproteins may vary significantly in their structure - a property which has been termed "microheterogeneity". Some of them have two branches attached to the common pentasaccharide core unit (biantennary heteroglycans), the other have three or four core-linked terminal chains (tri- and tetra-antennary carbohydrates, respectively) [3, 4].

To study the microheterogeneity of AGP and other acute-phase glycoproteins, affinity immunoelectrophoresis with the lectin concanavalin A (conA) as a ligand has been used [5]. The presence of either two α-linked nonreducing terminals or α-linked 2-O-substituted mannose residues with unmodified C-3, C-4 and C-6 hydroxyl groups in heteroglycan chains of particular glycoproteins is required for binding to conA [6]. Only biantennary carbohydrates of AGP react with the lectin and, therefore, various microheterogeneous forms of AGP containing different number of biantennary heteroglycans differ in their reactivity with conA [4]. Difference in affinity for the lectin results in the different mobility of particular AGP glycoforms in the conA-containing agarose gel during electrophoresis [7].

Inflammatory states are usually associated with changes in the glycosylation profile of acute-phase proteins. It has been demonstrated that there is an increased concentration of the conA-reactive microheterogeneous forms of acute-phase glycoproteins in patients with acute inflammations, such as acute bacterial infections, tissue necrosis or burns [8–10]. Conversely, a shift in the population of acute-phase glycoproteins towards those with a higher content of conA-nonreactive tri- and tetra-antennary

carbohydrates has been shown in the sera of patients with chronic inflammatory diseases (*e.g.* chronic bacterial infections, rheumatoid arthritis, ankylosing spondylitis) [8, 11, 12].

Little is known of the acute-phase response in patients with pulmonary sarcoidosis. In a recent work, it was suggested that serum concentration of acute-phase proteins is usually normal or only slightly elevated in most patients with sarcoidosis and does not relate to disease stage or activity [13]. There is no report dealing with the glycosylation profile of acute-phase glycoproteins in patients with sarcoidosis.

In this work, we used affinity immunoelectrophoresis to study the microheterogeneity of serum AGP and ACT in patients with pulmonary sarcoidosis. The main question that we addressed was whether there are any changes in the glycosylation profile of these proteins. Additionally, we looked for an eventual relationship between the glycosylation profile of acute-phase glycoproteins and two markers of disease activity, serum activity of angiotensin converting enzyme (ACE) and serum concentration of soluble receptor for interleukin-2 (sIL-2R) [14–16].

Patients and methods

Patients

Thirty seven patients with pulmonary sarcoidosis were studied. All of them were in-patients of sarcoidosis clinic in Höchenschwand, Germany. No patients with extrapulmonary manifestations were included, to ensure homogeneity of the study group. The characteristics of the study group regarding sex, smoking status, age, and disease duration have been shown in table 1. In all cases, the diagnosis of sarcoidosis was established according to clinical and laboratory findings, and was confirmed by biopsy. Diseases different from sarcoidosis but similar in their clinical appearance (*e.g.* tuberculosis) were excluded. Chest radiographic images were made to assess the stage of the disease. At entry, there were 21 patients with stage I, 10 patients with stage II, and 6 patients with stage III disease. Chest radiograms were then repeated two times (after 6 and 12 months) to confirm an eventual disease progression. For purpose of the study,

Table 1. – Characteristics of the patients with pulmonary sarcoidosis and of the controls

	Patients (n=37)	Controls (n=44)
Sex M/F	19/18	19/25
Smokers/nonsmokers		
Males	5/14	7/12
Females	3/15	7/18
Age* yrs		
Males	48 (30–68)	50 (23–80)
Females	51 (27–69)	45 (23–84)
Disease duration* yrs	12 (1–35)	

patients were considered to have an "active" sarcoidosis if progression of pulmonary changes was seen on subsequent chest radiograms, and an increased activity of ACE was found in serum specimens. Blood samples were taken from all patients on the day they were included in the study, and two serum specimens for each patient were stored frozen at -90°C. No patients were receiving glucocorticoids for at least 3 months before and during the study.

Controls

The control group (n=44) consisted of 20 healthy individuals recruited from clinic staff and 24 patients with osteoarthritis. Since osteoarthritis is not associated with changes in the glycosylation pattern of acute-phase proteins, the patients with osteoarthritis were included as additional control of the affinity immunoelectrophoresis. See table 1 for data on sex, smoking status and age in the control group. The sex and age distribution in the control group did not differ from the sex and age distribution in the patient group. Also, both groups were similar regarding smoking behaviour. As expected, no significant differences were found in AGP and ACT affinity for conA, and serum concentration of AGP, ACT and C-reactive protein (CRP) between healthy controls and patients with osteoarthritis.

Affinity immunoelectrophoresis

Affinity immunoelectrophoresis with conA was carried out as described by Bøg-Hansen [5], with some minor modifications. Briefly, conA (Sigma type 4, C-2010 Sigma, St. Louis, USA) at a concentration of 40 mmol·L^{-1} was incorporated into the first dimension agarose gel and electrophoresis was carried out for 60 min at 10 V·cm^{-1}. Gel strips were then transferred onto the second dimension plate and the gel adjacent to the first dimension gel was poured out. It contained anti-AGP (or anti-ACT) antibodies (Dakopatts), and 7.5% of methyl-α-D-mannopyranoside (Sigma) to solubilize the conA-glycoprotein complexes. After electrophoresis (16–18 h, 1.5 V·cm^{-1}) gel was pressed, dried and stained using Coomassie brilliant blue R-250 (Sigma). The areas enclosed by the precipitates representing microheterogeneous variants of AGP and ACT (fig. 1) were measured planimetrically. The results were expressed as reactivity coefficients (AGP-RC and ACT-RC, respectively) calculated according to the formula: total area under the peaks of the conA-reactive variants (1–3) divided by the area enclosed by the peak representing the conA-nonreactive variant (0).

Determination of serum AGP and ACT concentrations

Serum AGP and ACT concentrations were determined by rocket immunoelectrophoresis according to Laurell [17]. Serum protein calibrator (Dako, code No. X908, lot 010) was used to construct the standard curve. The concentrations were calculated using linear regression and expressed in g·L^{-1}.
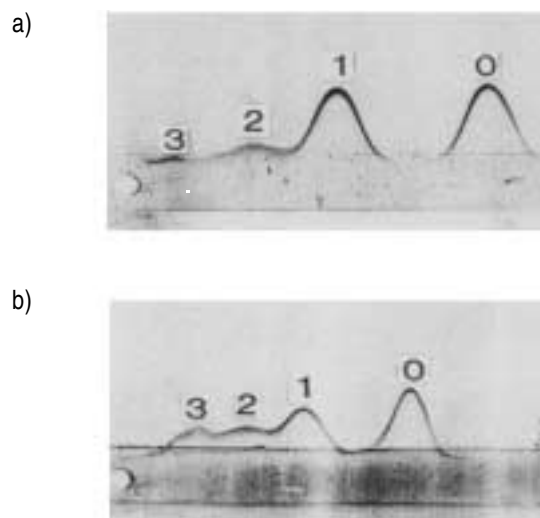
a)

b)

Fig. 1. – Normal glycosylation pattern of: a) α_1-acid glycoprotein (AGP); and b) α_1-antichymotrypsin (ACT). Affinity immunoelectrophoresis with conA was carried out as described in text. 0: conA nonreactive variant; 1–3: conA reactive variants. conA: concanavalin A.

Determination of serum concentration of CRP

Serum CRP levels were measured using a routine nephelometric method. The results were expressed in $mg \cdot L^{-1}$.

Determination of serum activity of ACE

ACE activity was determined using a routine automated enzyme kinetic assay. As a substrate, N-(3-[2-furyl]acryloyl)-L-phenylalanyl-glycylglycine (FAPGG) was used, at concentration of 1.0 $mmol \cdot L^{-1}$. The reaction was carried out in borate buffer (80 $mmol \cdot L^{-1}$, pH=8.2) at 37°C. Absorbance of the product was measured at 345 nm. The linearity range of the assay extended to 180 $U \cdot L^{-1}$ (25% of substrate hydrolysis). Results were expressed in $U \cdot L^{-1}$. Intra- and interassay variability did not exceed 5%.

Determination of serum concentration of sIL-2R

Serum level of sIL-2R was measured using commercial enzyme-linked immunosorbent assay (ELISA) kit (DAKO, code No. K004, lot No. 062). All samples were examined in duplicate and the sIL-2R concentration was calculated using the standard curve constructed from known sIL-2R levels, and corresponding optical densities obtained for standards provided with the kit. The coefficients of variation (CV%) did not exceed 10%. The results were expressed in $U \cdot mL^{-1}$.

Statistics

To analyse the data, descriptive statistics and standard nonparametric and parametric tests were used. Medians were compared using Mann-Whitney U-test. Chi-squared or Fisher's exact test were applied to compare frequencies of particular findings in the subsets of patients. Relationships between variables were studied using analysis of variance and regression. When applicable, 95% confidence intervals were assumed.

Results

The entire sarcoidosis group did not differ significantly from the controls in AGP-RC (1.20 vs 1.20), ACT-RC (3.71 vs 3.76) and concentration of AGP (1.27 vs 1.10 $g \cdot L^{-1}$), ACT (0.48 vs 0.49 $g \cdot L^{-1}$, and CRP (2.9 vs 2.25 $mg \cdot L^{-1}$), all values are medians. In 11 patients with active disease, however, median values of AGP-RC were decreased ($p < 0.01$) (fig. 2). A similar (but not significant) tendency was seen with ACT-RC (fig. 3). The patients
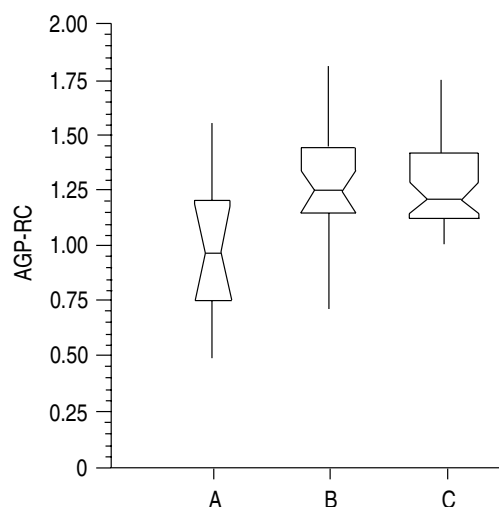

Fig. 2. – Notched box-and-whiskers plots showing statistical summaries of α_1-acid glycoprotein reactivity coefficient (AGP-RC) in patients with active sarcoidosis (A), nonactive sarcoidosis (B), and in controls (C). The box covers the middle 50% of the data values, between the lower and upper quartile. The notches indicate the 95% confidence limits. The "whiskers" extend out to the extremes (minimum and maximum values) that are within 1.5 times the interquartile range, whilst the central line represents median.

Fig. 3. – Notched box-and-whiskers plots showing statistical summaries of α_1-antichymotrypsin reactivity coefficient (ACT-RC) in patients with active sarcoidosis (A), nonactive sarcoidosis (B), and in controls (C). The box covers the middle 50% of the data values, between the lower and upper quartile. The notches indicate the 95% confidence limits. The "whiskers" extend out to the extremes (minimum and maximum values) that are within 1.5 times the interquartile range, whilst the central line represents median.
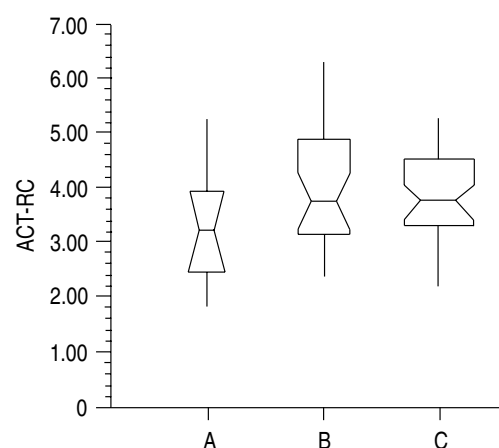
Table 2. – AGP-RC, ACT-RC, AGP, ACT, CRP, ACE, and sIL-2R in patients with pulmonary sarcoidosis in relation to the disease stage*

	Pt n	AGP-RC	ACT-RC	AGP g·L^{-1}	ACT g·L^{-1}	CRP mg·L^{-1}	ACE U·L^{-1}	sIL-2R U·mL^{-1}
Stage 1	21	1.21±0.06	3.73±0.22	1.39±0.19	0.48±0.04	3.3±1.0	38.5±6.8	375±195
Stage 2	10	1.18±0.12	4.61±0.39	1.07±0.08	0.47±0.05	2.0±1.3	31.0±16.0	405±102
Stage 3	6	1.15±0.15	3.27±0.48	1.22±0.23	0.37±0.05	2.0±0.9	37.2±13.1	365±168

Values are presented as median±SEM. AGP-RC: AGP reactivity coefficient; ACT-RC: ACT reactivity coefficient; AGP: α_1-acid glycoprotein; ACT: α_1-antichymotrypsin; CRP: C-reactive protein; ACE: angiotensin-converting enzyme; sIL-2R: serum interleukin-2 receptor. *: all differences were statistically nonsignificant.

with active disease also had elevated serum AGP (1.96 g·L^{-1}; p<0.02) but their median ACT (0.55 g·L^{-1}) and CRP (3.3 mg·L^{-1}) levels did not differ from those in the control group. When compared with patients with low disease activity (n=26), those with active sarcoidosis had lower values of AGP-RC (p<0.01) (fig. 2), increased concentrations of serum AGP (1.96 vs 1.12 g·L^{-1}; p<0.05), and raised sIL-2R levels (623 vs 320 U·mL^{-1}; p<0.01). Median ACT-RC was also lower in this group (fig. 3), but statistical significance was not reached. No differences in the serum level of CRP were found between active and nonactive patients (2.2 vs 3.3 mg·L^{-1}). There were no significant differences in AGP-RC, ACT-RC, AGP, ACT, CRP, ACE, and sIL-2R between the different disease stages (table 2).

In 9 out of 37 patients, increased levels of serum CRP were detected. Compared with patients with normal CRP, elevated serum AGP (1.63 vs 1.10 g·L^{-1}; p<0.02) and increased sIL-2R level (798 vs 344 U·mL^{-1}; p<0.05) were found in this group. There were no differences in the median ACE activity (44.0 vs 35.9 U·L^{-1}) and median values of AGP-RC (1.22 vs 1.19) and ACT-RC (3.96 vs 3.36) between the groups.

In the entire sarcoidosis group, regression analysis revealed an inverse linear relationship between ACE activity and both AGP-RC (r= -0.6358; p<0.0001) (fig. 4) and ACT-RC (r= -0.5019; p<0.005) (fig. 5). There was a correlation between ACE activity and both serum AGP and sIL-2R levels (r=0.4501; p<0.01 and r=0.8241; p<0.00001, respectively). Serum AGP concentration correlated with the sIL-2R concentration (r=0.5621; p<0.0005). There was only a weak association between serum levels of the three acute-phase proteins studied (serum CRP vs serum ACT: r=0.4177; p<0.02; serum AGP vs serum ACT: r=0.3539; p<0.05). AGP-RC and ACT-RC correlated well with each other (r=0.8365; <0.00001).

Discussion

The results presented here provide evidence that there are changes in the glycosylation pattern of acute-phase glycoproteins in patients with active sarcoidosis. These alterations are characterized by decreased reactivity of acute-phase glycoproteins with conA and indicate an increased serum concentration of glycoforms with conA-nonreactive tri- and tetra-antenary heteroglycan side-chains, and decreased concentration of those bearing biantennary carbohydrates. The changes in the glycosylation profile depended to some degree on disease activity;
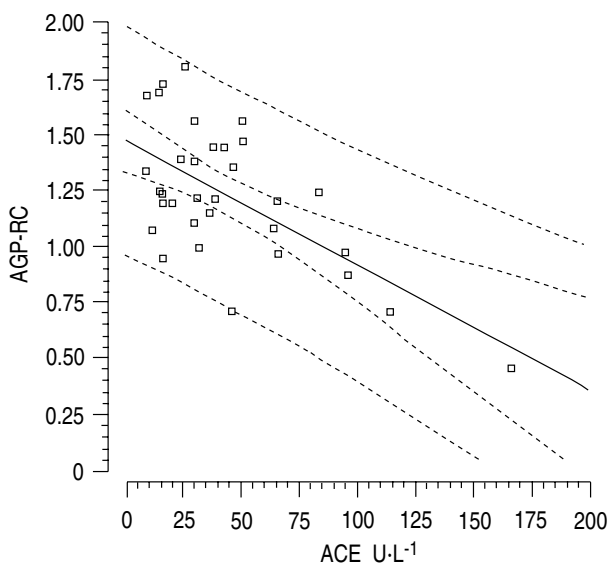
Fig. 4. – Regression curve showing the relationship between α_1-acid glycoprotein reactivity coefficient (AGP-RC) and serum angiotensin-converting enzyme (ACE) activity (linear model r= -0.6358, p<0.0001). Two pairs of dashed lines closest and farthest to the fitted line present the 95% confidence and prediction limits, respectively.
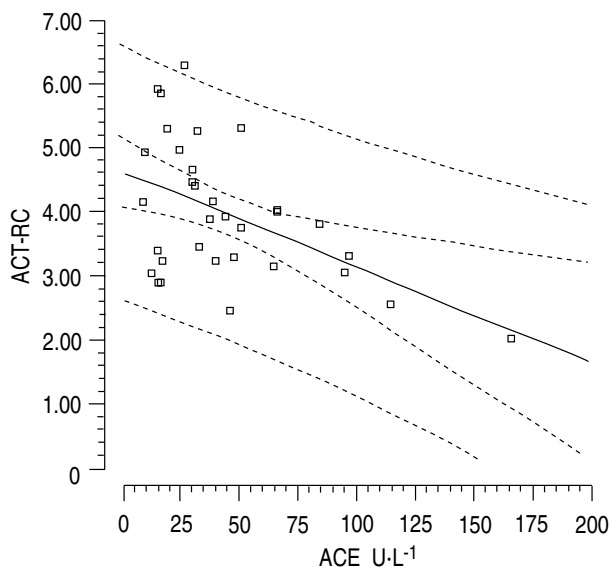
Fig. 5. – Regression curve showing the relationship between a$_1$-antichymotrypsin reactivity coefficient (ACT-RC) and serum angiotensin-converting enzyme (ACE) activity (linear model, r=-0.5019, p<0.005). Two pairs of dashed lines closest and farthest to the fitted line present the 95% confidence and prediction limits, respectively.

patients with active disease had decreased AGP-RC; whereas, normal proportions of various microheterogeneous forms of AGP were detected in those with nonactive disease. There was also a relationship between both AGP-RC and ACT-RC and serum ACE, which is a marker of disease activity in sarcoidosis [14, 16].

Cytokines have been shown to induce production of acute-phase proteins in the liver [18–21], and they also regulate their glycosylation [22–27]. Thus, both changes in the serum concentration and in the glycosylation profile of acute-phase proteins should be attributed to the action of cytokines.

From many cytokines studied, only interleukin-6 (IL-6) has been demonstrated to induce the synthesis of the full spectrum of human acute-phase proteins including CRP and serum amyloid A (SAA) [18, 19]. A study by DEVERGNE et al. [28] showed that cells expressing IL-1β genes predominated in sarcoid granulomas, whereas those producing interferon-γ (INF-γ), tumour necrosis factor-α (TNF-α) interleukin-lα (IL-1α), interleukin-2 (IL-2), and IL-6 were less frequent [28]. The low production of IL-6 in sarcoid lesions would eventually explain normal levels of serum CRP found in most cases.

Many reports confirm the role of IL-1β, TNF-α, and INF-γ in the pathogenesis of sarcoidosis. For instance, it has been demonstrated that alveolar macrophages obtained from patients with sarcoidosis spontaneously released IL-1β, TNF-α and INF-γ and that this ability correlated with the disease activity [29–33].

The action of IL-1β and/or TNF-α could explain lack of relationship between the synthesis and glycosylation of acute-phase proteins in patients with sarcoidosis; IL-1β and TNF-α have a moderate effect on the synthesis of some acute-phase proteins, including AGP and ACT although they fail to induce production of CRP or SAA [21]. The same cytokines may affect the glycosylation of acute-phase glycoprotein in hepatocytes [22, 27].

In the group studied, serum AGP but neither AGP-RC nor ACT-RC correlated with sIL-2R level. As macrophages present in sarcoid granulomas are a major source of sIL-2R in serum of patients with active sarcoidosis [34, 35], the implication is that alterations in the serum concentration of AGP and the changes in the glycosylation profile of this glycoprotein probably reflect different aspects of macrophage activation.

An interesting question is whether the changes in the glycosylation profile of acute-phase glycoproteins might affect their biological activity and/or function. It has been demonstrated by POS et al. [36] that microheterogeneity variants of AGP differ with regard to their immunomodulatory properties: the conA-nonreactive variant of AGP is more effective in modulation of lymphocyte proliferation than ConA-reactive AGP serum variants. Thus, the increase of serum conA-nonreactive AGP in patients with active sarcoidosis may play a part in the immunopathogenesis of the disease. Recently, it has been shown that AGP has an affinity for E-selectin and that this affinity can be changed by *in vitro* fucosylation of AGP [37]. As changes in the AGP fucosylation have been found in patients with chronic and acute

inflammatory diseases, this mechanism might represent a physiological feedback response on the selectin-mediated interaction between leucocytes and inflamed endothelium. As we focused on the structural changes in the heteroglycan side-chains of AGP, it is difficult to interpret our results in view of the latter data. To our knowledge, there are no reports suggesting that any changes in glycosylation of ACT might influence its function as antiprotease.

Determination of the glycosylation profile of acute-phase proteins, especially AGP, may provide a new interesting approach to assessment of the disease activity in sarcoidosis. However, the clinical applicability of this method regarding its sensitivity and specificity needs further evaluation.

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