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# PREVALENCE OF TRI- AND TETRAANTENNARY GLYCANS OF HUMAN $\alpha_1$-ACID GLYCOPROTEIN IN RELEASE OF MACROPHAGE INHIBITOR OF INTERLEUKIN-1 ACTIVITY

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*Abstract*—Based on the affinity for concanavalin A (Con A), human $\alpha_1$-acid glycoprotein (AGP) can be separated by chromatography on Con A–Sepharose gel into three variants: Con A unreactive AGP, Con A weakly reactive AGP, and Con A strongly reactive AGP. When exposed to native AGP or to its glycan variants, murine peritoneal macrophages released a factor that inhibited the interleukin-1 (IL-1) proliferative activity as measured in terms of the thymocyte comitogenic assay. Con A unreactive AGP, which contains tri- and tetraantennary glycans and no biantennae, proved to be more effective than Con A weakly and Con A strongly reactive variants, which contain one and two diantennary glycans, respectively. The inhibitory effect was not a function of the negative charge related to the sialyl residues and was not mediated by the mannosyl-fucosyl receptor.

## INTRODUCTION

The inflammatory reaction leads to immediate local responses and later systemic reactions. One of these events is the acute-phase response, during which alterations in the concentration of certain plasma proteins are observed. A common feature of many acute-phase proteins is their involvement in defense mechanisms against tissue damage and infections (i.e., $\alpha_1$-proteinase inhibitor, $\alpha_1$-antichymotrypsin, $\alpha_2$-macroglobulin, etc.). For some of them, however, including $\alpha_1$-acid glycoprotein (AGP), despite a large number of studies, the search continues for a single main identifiable function. The possible role of



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AGP in the modulation of the immune response supports one of the most promising hypotheses. Thus, AGP has been shown to suppress mitogen-induced lymphocyte proliferation, mixed lymphocyte reaction, antibody responses to sheep erythrocytes, capping of concanavalin A receptors and surface immunoglobulins on the lymphoid cell surface, and more recently, anti-CD3 stimulated lymphocyte proliferation (1–4). Some of these immunosuppressive properties were dependent on the structure of its carbohydrate moiety (2, 4).

Using concanavalin A (Con A), AGP can be separated into three variants: Con A unreactive AGP, Con A weakly reactive AGP, and Con A strongly reactive AGP. The Con A reactive variants are said to increase in response to acute inflammatory disorders (5), whereas an increase in the relative proportions of Con A unreactive AGP is observed during pregnancy (6) and in liver damage (cirrhosis, acute hepatitis) (7). The altered immune response associated to these physiopathological conditions led us to investigate interactions between macrophages, the primary source of IL-1, and glycan variants of AGP, which occur in the plasma in health or in disease states. In a preliminary report, we established the involvement of the multiantennary glycans of AGP in the secretion of an IL-1 inhibitor by macrophages (8). These results are here confirmed and the contribution of sialyl and fucosyl residues to the inhibitory effect of AGP is examined.

## MATERIALS AND METHODS

*Purification of Human AGP and its Con A Variants.* AGP was purified by means of immunoaffinity chromatography using an anti-AGP–Sepharose 4B column from various biologic fluids: pooled sera from healthy individuals ($AGP_H$), ascitic fluid from one cirrhotic patient ($AGP_C$), and serum from one eight-month-pregnant woman ($AGP_P$) (9). Native AGP was then passed through a Con A–Sepharose column (1.5 × 30 cm) (10) and separated into three peaks: Con A unreactive AGP (peak I), Con A weakly reactive AGP (peak II), and Con A strongly reactive AGP (peak III). In order to remove traces of Con A released from Con A–Sepharose gel, these different fractions were then chromatographed on an alpha-methylmannoside–agarose gel (Sigma) in 0.05 M Tris buffer, pH 7.6, containing 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Finally, a passage through a Detoxigel column (Pierce) (1 × 2 cm) was systematically performed in PBS buffer. The limulus test indicated less than 10 pg/mg of endotoxins in AGP preparations. The sample was extensively dialyzed and then lyophilyzed. Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed to check homogeneity and purity of the protein (11).

*Preparation of Con A Unreactive Asialo-AGP.* The terminal sialic acids of AGP were removed by incubating the glycoprotein and *Vibrio cholerae* neuraminidase (0.2 units/10 mg AGP) for 24 h at 37°C in 0.1 M acetate buffer, pH 5.5. Free sialic acid was eliminated by extensive dialysis against sodium acetate buffer and a second incubation was then performed. Asialo-AGP was chromatographed on an anti-AGP–Sepharose 4B column once more, in order to remove the neuraminidase. The samples were extensively dialyzed and then lyophilyzed.

*Carbohydrate Composition.* Quantitative carbohydrate analysis of purified AGP was performed by gas–liquid chromatography after methanolysis and trifluoroacetylation (12).

*Culture of Mouse Peritoneal Macrophages.* Thioglycolate-elicited peritoneal macrophages from DBA/2 mice were plated initially in T25 Falcon flasks at a concentration of 600,000 cells/ml (5 ml/flask) for 2 h. The nonadherent cells were removed by washing three times with Hanks' balanced salt solution–1% fetal calf serum (FCS). The adherent cells were cultured in RPMI 1640 medium with 25 mM HEPES buffer (5 ml/flask) in the presence or absence (macrophage control) of AGP (100 $\mu$g/ml). After a 24-h incubation, the cell-free supernatants were collected, centrifuged, and assayed for IL-1 inhibitory activity.

*Interleukin-1 Preparations.* Murine IL-1 was obtained by stimulating the culture of macrophages with *Escherichia coli* LPS (10 $\mu$g/ml) for 24 h at 37°C. Human recombinant IL-1$\beta$ was a kind gift from Dr. Boussaut (Rhone-Poulenc, France) and had a specificity of $5 \times 10^7$ units/mg.

*IL-1 Inhibitor Assay (13).* C3H/HeJ mouse thymocytes ($1.5 \times 10^6$) were cultured for 72 h in 0.2 ml of RPMI 1640 medium containing 25 mM HEPES and 5% FCS with serial twofold dilutions of macrophage supernatants, PHA (Wellcome) at a submitogenic concentration (1.5 $\mu$g/ml), and IL-1 [human rIL-1$\beta$ (50 pg/ml) or murine IL-1 (1 unit/ml); one unit per milliliter corresponds to the dilution that induces 50% maximal thymocyte proliferation]. The cultures were pulsed for the final 6 h with 0.5 $\mu$Ci of [$^3$H]TdR.

## RESULTS

*Con A Unreactive AGP Induces Release by Murine Macrophages of Inhibitor of Thymocyte Comitogenic Assay.* AGP isolated from healthy donors ($AGP_H$) contained Con A variants I, II, and III in a ratio of 49:38:13. Thioglycolate-elicited peritoneal macrophages from DBA/2 mice were treated with $AGP_H$ or its different Con A variants at 100 $\mu$g/ml for 24 h. The supernatants were then assayed for their effect on murine thymocytes in the presence of PHA (1.5 $\mu$g/ml) and murine IL-1. The macrophage supernatant obtained with Con A unreactive $AGP_H$ proved to be the most potent inhibitor of the comitogenic assay (Figure 1). The same result was observed with macrophages from C3H/HeJ mice that are known to be genetically unresponsive to LPS. Con A unreactive $AGP_H$ incubated in the culture medium without macrophages for 24 h at 37°C did not alter the comitogenic activity of human recombinant IL-1$\beta$ (Table 1). These results were confirmed with human $AGP_H$ preparations from various sources: two AGPs purchased from Sigma, AGP kindly supplied by Dr. Baudner (Behring), and those purified in our laboratory. When tested in the same assay, all Con A unreactive AGPs were effective to the same extent (Figure 2).

*Con A Unreactive AGP and Its Asialo Derivative had the Same Efficacy.* Con A unreactive asialo-AGP was prepared by a two-step incubation with *Vibrio cholerae* neuraminidase at 37°C for 24 h. This procedure removed 90–95% of the sialic acid residues of the glycoprotein as determined in terms of the thiobarbituric method of Warren after acid hydrolysis. Con A unreactive

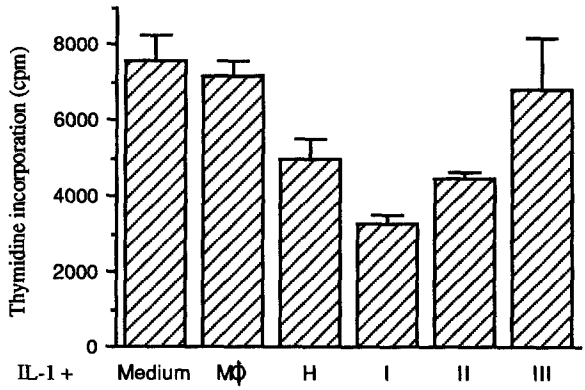


**Fig. 1.** Importance of the degree of antennarity of AGP glycans. Murine macrophages were incubated alone (Mϕ) or stimulated with AGP isolated from healthy subjects (H), Con A unreactive AGP (I), Con A weakly reactive AGP (II), or Con A strongly reactive AGP (III) at 100 μg/ml for 24 h. The different macrophage supernatants then were tested in the thymocyte comitogenic assay at $\frac{1}{8}$ final dilution, in the presence of PHA (1.5 μg/ml) and murine IL-1 (1 unit/ml).

$AGP_H$ and its desialylated form were added at different concentrations in macrophage cultures. The supernatants then were compared in the comitogenic assay: desialylation did not abolish the inhibitory effect on thymocyte proliferation since both AGPs were active at the same concentration (100 μg/ml) (Figure 3).

*Comparative Effect of Con A unreactive $AGP_H$, $AGP_C$, and $AGP_P$.* Con A unreactive $AGP_H$ is slightly more fucosylated than native $AGP_H$ and its Con

**Table 1.** Lack of Effect of AGP Incubated without Macrophages on Comitogenic Activity of Human Recombinant IL-1β[a]

| Thymocytes + PHA (2.0 μg/ml) + rhIL-1β (50 pg/ml) | [$^3$H]TdR uptake into thymocytes (cpm) in the presence of dilutions of different supernatants | | |
|---|---|---|---|
| | 1/8 | 1/16 | 1/32 |
| Medium | | 15 742 ± 3 343 | |
| Control Mϕ | 11 810 ± 424 | 14 795 ± 1 481 | 15 370 ± 1 958 |
| AGP | 12 689 ± 1 479 | 15 981 ± 321 | 15 548 ± 628 |
| AGP-Sup | 5 570 ± 596 | 5 851 ± 631 | 6 987 ± 851 |

[a] Thymocytes were cultured with PHA (2.0 μg/ml) and recombinant human IL-1β (50 pg/ml) in the presence of serial twofold dilutions of different supernatants: Control Mϕ and AGP-Sup were supernatants of macrophage cultures in the absence or presence of Con A unreactive $AGP_H$ at 100 μg/ml; AGP was a Con A unreactive $AGP_H$ solution at 100 μg/ml incubated in the same conditions as macrophage supernatants. (Con A unreactive $AGP_H$ was isolated from healthy subjects).

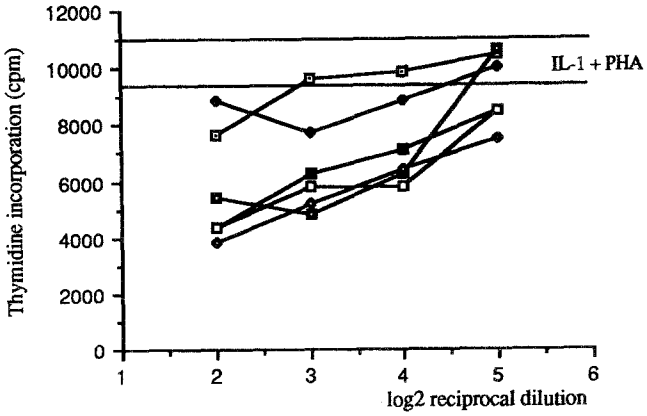


**Fig. 2.** Comparative effect of several Con A unreactive AGPs isolated from healthy subjects. Murine macrophages were cultured alone (control macrophage supernatant ⊡ ) or with Con A-strongly reactive AGP (♦), or different Con A unreactive AGPs at 100 $\mu$g/ml (◪: AGP given by Behring; ● and □: AGPs supplied from Sigma; ■: AGP purified in our laboratory). The activity of serial twofold dilutions of these macrophage supernatants was then assessed on thymocyte proliferation coinduced by PHA (1.5 $\mu$g/ml) and human recombinant IL-1$\beta$ (50 pg/ml).

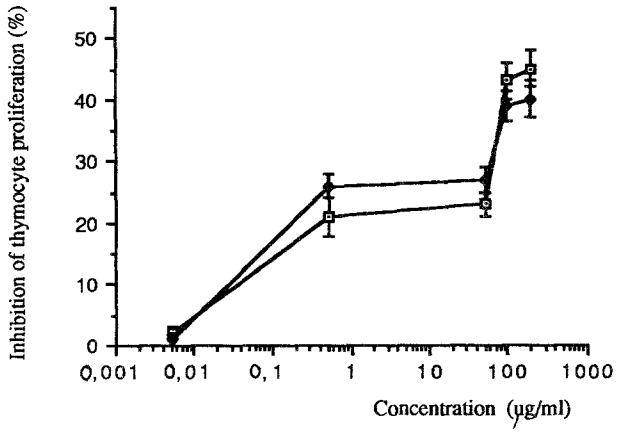


**Fig. 3.** Lack of effect of desialylation upon inhibitory activity of AGP. Con A unreactive AGP (⊡) and its asialo derivative (♦) were added at different concentrations in macrophage cultures. The supernatants were then assayed on thymocytes stimulated with PHA (1.5 $\mu$g/ml) and murine IL-1 (1 unit/ml). The percentage of inhibition was calculated using the formula: % inhibition = [1 − CPM [$^3$H]TdR (IL-1 + AGP-supernatant)/CPM [$^3$H]TdR (IL-1 + control macrophages)] × 100.

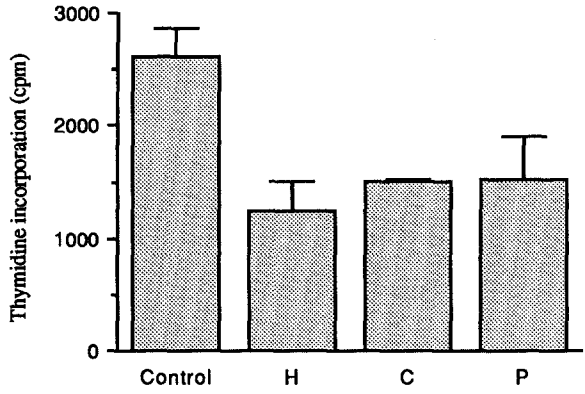


**Fig. 4.** Comparative effect of Con A unreactive AGPs isolated from healthy (H), cirrhotic (C), and pregnant (P) subjects. Macrophages were incubated alone (control macrophage supernatant) or with Con A unreactive AGP isolated from healthy donors (H), one cirrhotic patient (C), and one pregnant woman (P) (100 μg/ml). The anti-IL-1 activity of the supernatants was then measured by means of the thymocyte comitogenic assay.

A reactive variant (8), so we investigated whether the activity of Con A unreactive $AGP_H$ was related to its degree of fucosylation and/or to a higher proportion of multibranched glycans. We thus compared the effect of Con A unreactive AGPs isolated from healthy individuals ($AGP_H$), from one cirrhotic patient ($AGP_C$) and one eight-month-pregnant woman ($AGP_P$). $AGP_P$ was slightly underfucosylated whereas $AGP_C$ was remarkable by an increased content in fucosyl residues (Table 2).

DBA/2 mouse macrophages were treated with Con A unreactive variant of $AGP_H$, $AGP_C$, and $AGP_P$ at 100 μg/ml for 24 h. The anti-IL-1 activity of the macrophage supernatants was assessed on C3H/HeJ thymocytes costimulated with PHA (1.5 μg/ml) and murine IL-1 (1 unit/ml). The percentages of inhibition induced by the three AGPs were not significantly different.

**Table 2.** Carbohydrate Analysis of Con A Unreactive AGP Isolated from Healthy Subjects (H), Cirrhotic Patient (C) and Pregnant Woman (P)

| | Carbohydrate | | | | |
|---|---|---|---|---|---|
| | Fuc | Gal | Man[a] | GlcNAc | NeuAc |
| H | 0.48 | 3.78 | 3 | 5.55 | 3.55 |
| C | 1.19 | 3.53 | 3 | 6.08 | 3.59 |
| P | 0.28 | 3.54 | 3 | 6.21 | 3.61 |

[a]The molar ratio was calculated on the basis of three mannose per oligosaccharide.

## DISCUSSION

When exposed to AGP preparations, macrophages release a factor with inhibitory activity towards the proliferative effect of IL-1 on murine thymocytes. The effect of AGP would appear to be mediated by the macrophages, because AGP incubated at 37°C for 24 h in the culture medium in the absence of macrophages had no activity on thymocyte proliferation. Con A unreactive AGP, which contains only tri- and tetraantennary glycans and no biantennary glycans (14), proved to be the most effective preparation. Consequently, the inhibitory activity was at least partly dependent on a highly branched carbohydrate moiety. The fact that Con A weakly and Con A strongly reactive variants, which contain one and two diantennary glycans, respectively (14), were less effective than Con A unreactive AGP may be related to their binding to a receptor followed by their internalization into the macrophage and their lysosomal degradation, as shown by Pimpaneau et al. (15). Thus, these variants may induce a reaction that is different from the one now described here. These results were confirmed largely with Con A unreactive variants of AGPs isolated either by means of ion-exchange chromatography or immunoaffinity chromatography. The observed phenomenon therefore was not related to the purification procedure used.

AGP differs from other plasma glycoproteins by its high content of sialyl residues (11%), which contribute to its negative charge. The biological activities experimentally associated with AGP are varied and may or may not be due to charge-dependent interactions. We found that Con A unreactive AGP and its desialylated form were active at the same concentration. This result therefore suggests that desialylation of AGP did not alter the recognition by macrophages and that AGP did not act as a simple multivalent anion.

Con A unreactive AGP induced the release of an inhibitory factor by thioglycolate-elicited macrophages as well as resident macrophages (results not shown). Both macrophages express a mannosyl/fucosyl receptor on the cell surface (16). However, the mechanism of action of AGP would not appear to be mediated by this receptor, because Con A unreactive $AGP_C$, which is highly fucosylated, was not more effective than Con A unreactive $AGP_H$ and $AGP_P$.

Our data indicate that a high degree of glycosylation is an important feature with regard to the possible immunomodulatory role of AGP. Consistent with this is the observation that Con A unreactive AGP was shown to be more effective in the suppression of anti-CD3 stimulated lymphocyte proliferation (4) than Con A reactive forms, as was human $\alpha_1$-proteinase inhibitor in inhibition of natural killer cell activity (17). Another interesting model is illustrated by the IgE-binding factors, which share a common structural gene. The major difference is that the IgE-potentiating factor has affinity for lentil lectin and Con A,

whereas IgE-suppressive factor only has affinity for peanut agglutinin (18). Taken together, these findings further substantiate the considerable role of the carbohydrate moiety, which may determine the biologic function of certain glycoproteins.

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