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Immunomodulating activities of a natural α_1-acid glycoprotein and its carbohydrate chains attached to the protein–free polymer

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IMMUNOMODULATING effects of a neoglycoconjugate created on the basis of α_1-acid glycoprotein (AGP) carbohydrate chains and synthetic protein-free carrier have been investigated. It was demonstrated that this pseudo-AGP suppressed PHA- or anti-CD3 antibody-induced lymphocyte proliferation in a dose-dependent manner. Pseudo-AGP revealed a similar antiproliferative effect as the natural AGP samples. Stimulation of the LPS-induced proinflammatory cytokine production by mononuclear cells treated with both natural and pseudo-AGP has been also demonstrated. These data show that carbohydrate chains of AGP play a crucial role in the studied biological effect realization.

Key words: α_1-acid glycoprotein, Neoglycoconjugate, Antiproliferative effect, Proinflammatory cytokines, Interleukin-6, Tumour necrosis factor

Introduction

The inflammatory reaction leads to alterations in the concentration of certain plasma protein commonly referred to as acute-phase proteins. One of these proteins is α_1-acid glycoprotein (AGP) which has multiple immunomodulating effects. Thus, AGP exerts an inhibitory effect on the proliferation of lymphocytes stimulated with anti-CD3 antibodies[1], PHA[2] and in mixed lymphocyte culture.[3] AGP significantly enhances the production of inflammatory cytokines interleukin (IL)-1β, IL-6, and tumour necrosis factor-α (TNF-α).[4,5] In the same time monocytes/macrophages treated with AGP release interleukin-1 inhibitory factor.[6,7] These data confirm the role of AGP as an element of cytokine network. Immunomodulating properties of AGP depend on microheterogeneity of carbohydrate chains. In normal serum, three different glycoforms of AGP are present: conconavalin A (Con A) unreactive AGP (AGP-A), Con A weakly reactive AGP (AGP-B), and Con A strongly reactive AGP (AGP-C). These molecular forms contain carbohydrate chains with different number of antennae and neuraminic acid residues. Tri-and tetraantennary carbohydrate chains only are present on the AGP-A.[8–10] AGP-A is more effective in modulation of lymphocyte proliferation[1,6] and inhibition of interleukin-2 production by peripheral blood lymphocytes.[2] Nevertheless, there is no distinct answer to the question of what is the role of carbohydrate moiety and of the peptide core of AGP molecule. Thus, several investigations demonstrate the participation of AGP glycans in suppression of the mitogen-induced lymphocyte proliferation.[1,11] On the other hand, there is an opinion that the glycan moiety of AGP is not involved in the potentiation of cytokine secretion triggered by LPS.[4] In this work the discrimination between the mentioned possibilities has been carried out by means of quantitative translocation of carbohydrate chains from the protein onto an other polymer carrier.[12,13]

Material and Methods

Isolation of human AGP

AGP patterns were isolated from peripheral human blood. Blood serum was obtained from 20 normal individuals. AGP was isolated and purified by using the ammonium sulphate precipitation and chromatography on DEAE-cellulose and on Cibacron blue F3GA-Sepharose 4B (Pharmacia, Sweden). AGP-positive fractions were desalted by dialysis and lyophilized. Preparations of AGP were electrophoretically pure and tested for the presence of endotoxin in the Limulus amoebocyte lysate test (Atlas Bioscan, UK). Endotoxin content was below 500 pg/mg.

Pseudo-AGP preparation

Pseudo-AGP was obtained as described earlier.[12] Briefly, 9.3 mg of poly-4-nitrophenilakrilate, 3.2 mg of aminated AGP oligosaccharides and 100 µl diisopropilethylene in 1 ml of dimethylphormamide were mixed and kept at 37°C for 48 h. Then 200 µl of

aqueous NH_3 was added and the mixture was incubated for 16 h at 5°C. Pseudo-AGP has been purified by chromatography on Sepahadex LH-20 and lyophilized (yield was about 95%). Carbohydrate content in pseudo-AGP (including Neu5Ac) corresponds to parent AGP, as well as ratio of bi-, tri- and tetraantennary N-chains.

Cell cultures

Mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Ficoll-Verographin gradient centrifugation. The cells were washed twice and resuspended in RPMI-1640 medium (ICN, UK) supplemented with 10% heat inactivated donor horse serum, 2×10^{-3} M HEPES, 2 mM L-glutamine, 2.8×10^{-6} M 2-mercaptoethanol, and 20 μg/ml gentamycin. Cells (10^6 cells/ml) were cultivated for 16–18 h at 37°C with 3 μg/ml of *Neisseria meningitidis* lipopolysaccharide (LPS) and AGP at five different concentrations within the dose range 31.2–500 mg/ml in a humidified atmosphere containing 5% CO_2 in the wells (1.5 ml per well) of 24-well plates (Nunc, Denmark). The cells in the control wells were incubated with LPS only. The supernatants were collected and stored at –20°C until cytokine activity examination.

Inhibition of PHA- or anti-CD3 antibody-induced lymphocyte proliferation by AGP

Peripheral blood mononuclear cells (see above) were cultivated in flat-bottomed 96-well plates (Nunc, Denmark), and contained 5×10^4 cells in each well. The final concentration of PHA (Calbiochem, USA) was 5 μg/ml. In some experiments lymphocytes were stimulated with polyclonal anti-CD3 antibodies which were a generous gift from Dr M. Bliacher (Gabrichevsky Institute of Epidemiology and Microbiology, Moscow). Inhibition of lymphocyte proliferation by AGP was evaluated at six different concentrations within the dose range 31.2–1000 μg/ml. The control wells incubated without AGP contained a culture medium with mitogen or a culture medium only. The cells were incubated for 72 h at 37°C in a humidified atmosphere (95% air; 5% CO_2). Four hours before the end of cultivation, each well was pulsed with 40 kBq of [^{3}H]-thymidine (Isotope, Russia). The cells were harvested with a cell harvester and counted on a liquid scintillation counter. Four wells of each concentration were assayed and the counts per minute (counts/min) were averaged. Percentage inhibition was calculated by dividing the counts/min in each inhibited sample by the counts/min in the sample containing PHA subtracting only the background level (counts/min in the wells with culture medium only) from these values. The intensity of suppression was estimated by probit-analysis and expressed as ED_{50}: a dose of immunosuppressive agent at which lymphocyte proliferation was 50% of its maximum.

TNF activity assay

TNF activity was determined by the method of Ruff and Gifford[14] with some modifications. Briefly, L929 cells were seeded at a density of 3×10^4 cells per well in 96-well plates in 100 μl of medium 199 to which 10% heat-inactivated calf bovine serum and gentamycin had been added. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until the monolayer was formed. After the culture medium elimination, two-fold serial dilution of the samples (100 μl of each dilution) and 100 μl fresh culture medium with 20 μg/ml of actinomycin D (Serva, Germany) were added, and further incubated for 18 h at the same conditions. Supernatants were then removed and cells stained with 0.2% crystal violet (Sigma, USA). After washing and drying the plates were finally read at 540 nm on a Titertek Multiskan microElisa reader. Human recombinant TNF-α (Institute of Bioorganic Chemistry, Moscow, Russia) was used as the internal standard. For the comparison of an experimental and calibrating curves the probit-analysis method was used. TNF content in the samples was expressed in pg/ml.

IL-6 activity assay

IL-6 activity was determined using the IL-6-dependent hybridoma cell line D6C8.[15] Briefly, serial dilutions of culture supernatants and recombinant IL-6 (code 89/45, NIBSC, UK) as a standard were incubated in 96-well microplates with cells (5×10^4 cells/well), in a total volume of 200 μl at 37°C. The cells were cultivated for 48 h in RPMI-1640 medium supplemented with 5% human dialysed AB-serum. Four hours before the end of cultivation the cells were pulsed with 40 kBq per well of [^{3}H]-thymidine, harvested with a cell harvester and counted by using a liquid scintillation counter.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test and chi-square statistic.

Results

Inhibition of lymphocyte proliferation

Inhibition of PHA-induced lymphocyte proliferation in a dose-dependent manner by natural AGP (nAGP) has been shown. Both lot I and lot II markedly suppressed cell proliferation but the inhibitory effect of the latter was higher (Fig. 1). However, both curves

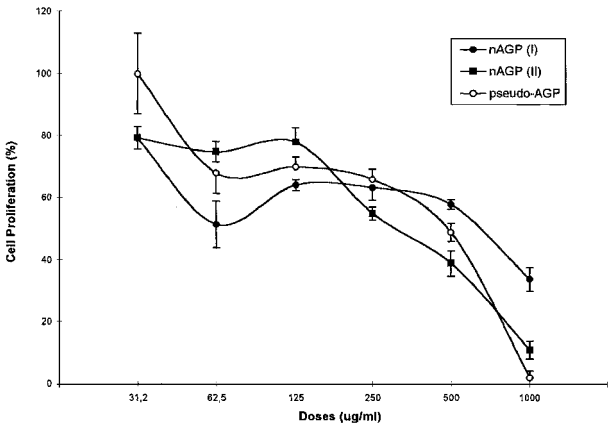

FIG. 1. Inhibition of PHA-induced lymphocyte proliferation with natural AGP (nAGP) and pseudo-AGP (see text). Peripheral blood mononuclear cells were stimulated with optimum PHA dose and incubated for 72 h in the presence of different concentrations of nAGP or pseudo-AGP.

had a profile of the same type. Protein-free neoglycoconjugate, so-called pseudo-AGP, revealed a similar antiproliferative effect. Also compared were AGP (lot II) with pseudo-AGP using lymphocyte stimulation with anti-CD3 antibodies. Nearly complete coincidence of the curves has been demonstrated (Fig. 2).

Table 1 summarizes ED_{50} values for nAGP and pseudo-AGP. For chi-squared calculation ED_{50} values for pseudo-AGP and nAGP were considered as observed and expected numbers respectively. These data show that no differences between pseudo-AGP and nAGP (lot II) have been observed.

Stimulation of cytokine production

TNF-α and IL-6 production by peripheral blood mononuclear cells, both were enhanced with nAGP (Fig. 3). Stimulation of TNF-α production by nAGP was observed within the dose range of 31–250 μg/ml. Pseudo-AGP showed the same stimulating properties. In the same time, the maximum levels of the cytokine

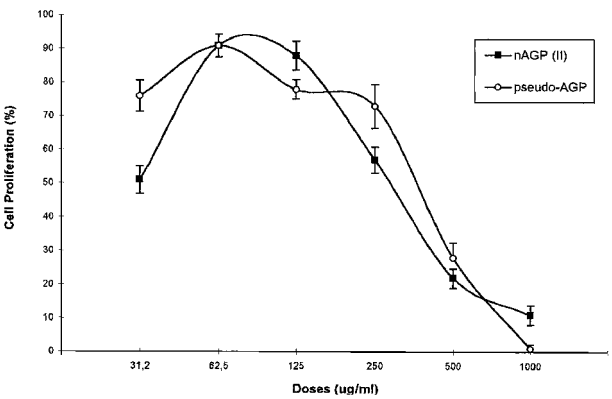

FIG. 2. Inhibition of anti-CD3 antibody-induced lymphocyte proliferation with nAGP and pseudo-AGP. For the experiment condition explanation see text and legend to Fig. 1.

Table 1. Comparison of antiproliferative effects of two AGP lots with a neoglycoconjugate (NGC)

Inhibition of cell proliferation by	Lymphocyte stimulation	ED_{50} values (μg/ml)*	χ^2	*P*
NGC	PHA	246 ± 25		
AGP (I)	PHA	306 ± 62	19.27	< 0.05
AGP (II)	PHA	233 ± 21	3.11	0.38
NGC	CD3ab	281 ± 16		
AGP (II)	CD3ab	279 ± 15	0.058	0.996

*Date are mean ± SEM.

production were higher than in the case of nAGP cell treatment and the curves describing nAGP and pseudo-AGP stimulation of LPS-induced TNF-α production were somewhat differ in profile (Fig. 3A). Stimulation of LPS-induced IL-6 production by nAGP and pseudo-AGP was observed too. In this case the curve profiles were similar (see Fig. 3B).

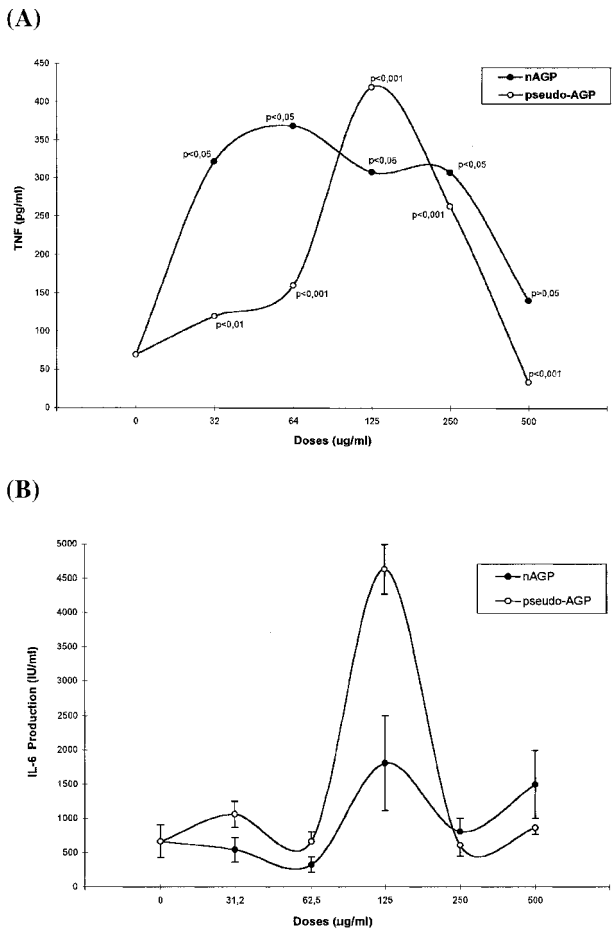

FIG. 3. The effect of natural AGP (nAGP) and pseudo-AGP on *in vitro* TNF (panel A) and IL-6 (panel B) production by peripheral blood mononuclear cells. The cells were incubated for 16–18 h with LPS in the presence of different concentrations of nAGP or pseudo-AGP. The supernatants were collected and TNF or IL-6 activity was quantified as described in Materials and Methods. On the panel A *P* values are presented in comparison with a control.

Discussion

Pseudo-AGP differs from natural AGP only in two items: (i) it has no protein part, (ii) carbohydrate chains are shuffled in it. As is shown above, pseudo-AGP completely retains the ability of nAGP to modulate antiproliferative response of lymphocyte to mitogens. Pseudo-AGP stimulates TNF and IL-6 production in a similar manner, its effect being more marked even than nAGP action. It was also shown earlier by flow cytometry analysis that pseudo-AGP bound selectively with blood cells similarly to nAGP.[16]

There is no serious contradictions between our data and results published by Boutten *et al.*[4] It is no doubt that native AGP, asialo-AGP and AGP glycoforms can potentiate the effect of LPS to the same degree, especially when a single dose of the preparations have been used. Earlier data showed that maximum immunosuppressive effect was obtained with asialo, agalacto derivative of AGP. Further enzymatic removal of mannose and *N*-acetyl glucosamine significantly reduced the inhibitory effects.[11] Our unpublished data confirm these results. Moreover, the full deglycosylation resulted in peptide core denaturation and glycoprotein became insoluble. Because of that the approach including quantitative translocation of carbohydrate chains from the protein onto protein free polymer carrier has been performed.

Thus, it seems that carbohydrate chains of AGP play a crucial role in the studied biological effect realization. It is also demonstrated that the localization of glycans on the carrier is not important for the effects displayed. In prospect, on the basis of these results, it is possible to create a family of artificial molecules with different properties by the various glycan translocation onto synthetic carrier.

References

1. Pos O, Oostendrop RAJ, van der Stelt ME, Scheper RJ, van Dijk W. Con A nonreactive human α_1-acid glycoprotein (AGP) is more effective in modulation of lymphocyte proliferation than Con A-reactive AGP serum variants. *Inflammation* 1990; **14**: 133–141.
2. Pukhalsky AL, Toptygina AP, Kalashnikova EA, Shyian SD, Nasonov VV, Bovin NV, Liutov AG, Bairushin FT. Immunomodulating effects of α_1-acid glycoprotein (orosomucoid) in cultivated human peripheral blood lymphocytes. *Bull Exp Biol Med* 1994; **118 (7)**: 71–73.
3. Shyian SD, Nasonov VV, Bovin NV, Medvedev A. Immunosuppressive activity of normal, embrionic and cancer α_1-acid glycoprotein and its molecular forms differing in glycosilation. *Exp Oncology* 1993; **15**: 46–50.
4. Boutten A, Dehoux M, Deschenes M, Rouzeau J-D, Bories FN, Durand G. α_1-Acid glycoprotein potentiates lipopolysaccharide-induced secretion of interleukin-1β, interleukin-6 and tumor nrcrosis factor-α by human monocytes and alveolar and peritoneal macrophages. *Eur J Immunol* 1992; **22**: 2687–2695.
5. Pukhalsky AL, Kalashnikova EA, Shiyan SD, Bovin NV. Effects of α_1-acid glycoprotein on inflammatory cytokin (TNF-α and IL-1β) production by peripheral blood mononuclear leukocytes *in vitro*. *Bull Exp Biol Med* 1998; **125**: 79–83.
6. Bories PN, Feger J, Benbernou N, Rouzeau J-D, Agneray J, Durand G. Prevalence of tri- and tetraantennary glycans of human α_1-acid glycoprotein in release of macrophag inhibitor of interleukin-1 activity. *Inflammation* 1990; **14**: 315–323.
7. Bories PN, Guenounou M, Feger J, Kodari E, Agneray J, Durand G. Human α_1-acid-glycoprotein-exposed macrophages release interleukin 1 inhibitory activity. *Biochem Biophys Res Commun* 1987; **147**: 710–714.
8. Bierhuizen MN, de Witt M, Govers C, Ferwerda W, Koeleman C, Pos O. Glycosilation of three molecular forms of human α_1-acid glycoprotein having different interactions with concanavalin A. *Eur J Biochem* 1988; **175**: 387–394.
9. Pimpaneau V, Midouz P, Durand G, de Baetselier P, Monsigny M, Roche AC. Endocytosis of α_1-acid glycoprotein variants and neoglycoproteins containing mannose derivatives by a mouse hybridoma cell-line ($2c_{11-12}$). Comparison with mouse peritoneal macrophages. *Glycoconjugate J* 1989; **6**: 561–574.
10. Shiyan SD, Nasonov VV, Bovin NV, Novikova LI, Aleshkin VA. Molecular forms of α_1-acid glycoprotein from human serum. Differences in the contents of bi-, tri- and tetraantennary N-chains. *Bioorg Khim* 1991; **17**: 663–670.
11. Bennet M, Schmid K. Immuno-suppression by human plasma α_1-acid glycoprotein: importance of the carbohydrate moiety. *Proc Natl Acad Sci USA* 1980; 77: 6109–6113.
12. Shiyan SD, Pukhalsky AL, Toptygina AP, Nasonov VV, Bovin NV. Conjugates of α_1-acid glycoprotein carbohydrate chains with polyacrylamide conserves immunomodulating activity of natural glycoprotein. *Bioorg Khim* 1994; **20**: 994–1000.
13. Bovin NV, Korchagina EY, Zemlynukhina TV, Byramova, Galanina OE, Zemlyakov AE, Ivanov AE, Zubov VP, Mochalova LV. Synthesis of polymer neoglycoconjugates based on N-substituted polyacrilamides. *Glycoconjugate J* 1993; **10**: 142–151.
14. Ruff MR, Gifford GE. Tumor necrosis factor. In: Pick E, ed. *Lymphokines*. New York: Academic Press, 1981; 235–241.
15. Kliushenkova EN, Malaitzev VV. Establishment of an interleukin-6(IL-6)-dependent hybridoma. *Bull Exp Biol Med* 1992; **114**: 179–181.
16. Shyian SD, Khaidukov SV, Pukhalsky AL, Toptygina AP. Interaction of peripheral blood leukocytes with α_1-acid glycoprotein, its carbohydrate chains and neoglycoconjugates. *Haematol Transfusiol* 1996; **41(2)**: 24–29.

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