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Interaction of an acute phase reactant, α_1-acid glycoprotein (orosomucoid), with the lymphoid cell surface: a model for non-specific immune suppression

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Summary. α_1-Acid glycoprotein (AG), a serum component elevated during acute inflammation, has been implicated in the suppression of various immunological responses. Pretreatment of lymphoid cells with AG at a concentration commonly found in patients with acute inflammation results in the inhibition of mitogen induced lymphoproliferation as well as capping of concanavalin A (Con A) receptors and surface immunoglobulin (sIg) on the lymphoid cell surface. In order to determine a potential interaction of AG with the lipid bilayer we examined the effects of purified AG on synthetic phosphatidyl choline vesicles. AG displaces 1-anilino-8-naphthalene sulphonate (ANS), an anionic surface probe from these vesicles yet is unable to perturb the binding of N-phenyl-1-naphthalamine (NPN), a hydrophobic probe of the membrane interior. The non-immunosuppressive asialo-derivative of AG is incapable of displacing ANS from the vesicles. The interaction of AG with the membrane may partially involve electrostatic forces mediated by sialic acid and/or steric hindrance of receptor mobility. The results suggest that AG has the capacity to perturb the lymphoid cell surface and interfere with events required for lymphocyte proliferation.

Abbreviations: AG, α_1-acid glycoprotein; Con A, concanavalin A; ANS, 1-anilino-8-naphthalene sulphonate; PC, 1,2-dimyristoyl phosphatidyl choline; NPN, N-phenyl-1-naphthalamine; LSM, lymphocyte separation medium; PHA-P, phytohaemagglutinin-P; PWM, pokeweed mitogen; c.p.m., counts per minute; $HBSS^-$, Hanks's balanced salt solution without Ca^{+2} and Mg^{+2}; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; ASAG, asialo AG; SA, sialic acid; IU, international unit.

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INTRODUCTION

α_1-Acid glycoprotein (AG) is a component of normal serum which becomes elevated during acute inflammation and is associated with numerous conditions including neoplasia, rheumatoid arthritis and severe burns (Jeanloz, 1966; Schmid, 1975). In normal individuals the serum level of AG ranges from 0·5 to 1·25 mg/ml whereas during the acute phase of inflammation this level has been shown to be elevated two- to five-fold (1·0–3·0 mg/ml; Jeanloz, 1966; Schmid, 1975; Cheresh *et al.*, 1982a). The function of AG has not been established, but recent findings suggest that when serum concentrations are elevated it may play an immunosuppressive role (Cheresh *et al.*, 1982a; Chiu *et al.*, 1977). Previous studies have shown that exogenous addition of purified AG to lymphoid cells in culture results in a dose-dependent inhibition of blast transformation, cytotoxic reactions, phagocytosis and antibody secretion (Chiu *et al.*, 1977; Bennett & Schmid, 1980; van Oss *et al.*, 1974; Cheresh *et al.*, 1982b). While liver is the primary site of AG synthesis and secretion, it has recently been demonstrated that it can be secreted by lymphocytes of the T cell lineage after activation in mixed lymphocyte cultures

(Gahmberg & Anderson, 1978). Moreover, indirect immunofluorescence has revealed that AG is present on the cell membrane of lymphocytes, monocytes and granulocytes.

In a recent study we reported that sera from metastatic breast cancer patients contain elevated levels of AG and that a positive correlation exists between AG concentration and the ability of serum to inhibit mitogen induced lymphoproliferation of normal lymphoid cells (Cheresh *et al.*, 1982a). Furthermore, we demonstrated that sera from breast cancer patients with elevated levels of this glycoprotein are capable of inhibiting concanavalin A (Con-A)-induced capping on the surface of normal lymphocytes. These studies have led us to explore the effects of exogenous addition of AG on immune responsiveness and to establish a model that may explain the interaction of AG with the lymphoid cell membrane that results in a generalized immunosuppressive effect.

In the present report we demonstrate that levels of AG commonly found in individuals with acute inflammation are inhibitory to mitogen-induced lymphoproliferation as well as ligand-induced capping on the surface of human mononuclear cells. Our results suggest that AG has the ability to interact with and perturb the polar head region of phospholipids, the most abundant lipid in the membrane of mammalian cells. Thus, AG through its interaction with the lipid bilayer may alter cell surface-associated events important in lymphoid cell activation.

MATERIALS AND METHODS

α_1-*Acid glycoprotein*

The AG used in this study was provided by Dr M. Wickerhauser from the American National Red Cross Blood Research Laboratory, Bethesda, MD. AG was isolated from normal plasma according to the method of Hao & Wickerhauser (1973), and was shown to be electrophoretically homogenous.

Collection of serum

Blood was obtained from normal individuals by venipuncture and allowed to clot for 2 hr at room temperature. The serum was separated from the clotted material by centrifugation at 1000 *g* for 15 min. The serum was allowed to stand at room temperature for 24 hr and then stored at 4° until use.

Isolation of mononuclear cells

Peripheral blood mononuclear cells were obtained from healthy individuals with type O blood. Blood was obtained by venipuncture using syringes which contained sufficient heparin to yield a final concentration of 10 units per ml and was diluted with an equal volume of sterile saline. Mononuclear cells were isolated by discontinuous density gradient centrifugation using lymphocyte separation medium (LSM, Litton Bionetics). Twenty millilitres of diluted blood were layered over 15 ml of LSM. The gradients were spun at 400 *g* for 40 min at 18–20°. Mononuclear cells were recovered from the interface and washed three times with medium, RPMI-1640 (Grand Island Biological Company, Gibco) supplemented with 100 units/ml of penicillin and 50 μg/ml of streptomycin (Associated Biomedic Systems). Washed cells were pelleted appropriately for each assay as described below.

Mitogen induced lymphoproliferation assay

Cultures of human mononuclear cells were set up in triplicate using microtitre plates (Falcon 3040). To each well was added 0·01 ml of Con A (Calbiochem Behring) at a concentration of 0·5 mg/ml, phytohaemagglutinin-P (PHA-P; isolated from *Phaseolus vulgaris*, Difco) at 0·1 mg/ml, or pokeweed mitogen (PWM; Grand Island Biological Company) at 0·5 mg/ml. Control cultures received 0·01 ml of growth medium instead of mitogen. Then $1{\cdot}0 \times 10^5$ mononuclear cells in 0·19 ml of growth medium supplemented with 10% normal human serum with or without the exogenous addition of AG (to give a final concentration of 2 mg/ml) were added to each well. Cultures were incubated for 72 hr at 37° in a 5% CO_2, humidified atmosphere. Eighteen hours before harvesting, cultures were pulsed with 0·01 ml of [^{3}H]-thymidine solution (50 μCi/ml). Harvesting was facilitated by a Skatron Cell Harvester (Flow Laboratories). Cells were collected on glass fibre filter paper (Flow Laboratories No. 78-105-5) and lysed with deionized water. Filter paper discs containing ^{3}H-DNA were placed in polyethylene vials with 5 ml of a scintillation cocktail of toluene containing 4 g/l of PPO and 50 mg/l of POPOP (Spectrafluor, Amersham). Vials were counted in a Packard Tri-Carb scintillation counter (Model No. 3330).

Data are presented as the counts per minute (c.p.m.) of experimental cultures with mitogen minus c.p.m. of control cultures without mitogen.

Capping of Con A receptors

Mononuclear cells (2×10^6 cells) from normal individuals were incubated for 1 hr at 37° in the presence of 0·2 ml of serum from a normal donor with or without the addition of AG (2 mg/ml). The cells were washed once with Hanks's balanced salt solution without Ca^{+2} and Mg^{+2} ($HBSS^-$). The cell pellet was resuspended in 0·2 ml fluorescein isothiocyanate (FITC)-Con A (Miles) (15 μg/ml) in $HBSS^-$. Incubation was carried out for various times in a 37° water bath after which 0·2 ml of a 4% paraformaldehyde solution was added in order to fix the cells. After fixation the cells were washed with phosphate-buffered saline (PBS) pH 7·4, containing 1 mg/ml bovine serum albumin. Cells were then placed on a slide and the number of cells with caps were determined using a Nikon Optiphot microscope equipped with epifluorescence. Each slide was examined blindly and a minimum of 200 cells were counted. A cell was considered capped when the fluorescence was distributed uniformly over 1/3 of the cell membrane or less.

Capping of surface immunoglobulin (sIg)

Mononuclear cells (2×10^6 cells) from normal individuals were preincubated as described above for Con A capping and subsequently washed with Media 199 (Gibco). Cell pellets were resuspended with 0·1 ml of a solution containing 100 μg/ml of FITC-labelled goat anti-human immunoglobulin (E. Y. Laboratories) and incubated at 4° for 1 hr. The cells were washed three times with Media 199 at 4° and allowed to incubate for 10 min at room temperature. We chose 10 min as the incubation time since previous determination of the kinetics of sIg capping under these assay conditions revealed that optimal capping time occurred very rapidly (approximately 5–10 min). The percentage of cells showing caps did not subside until after 20 min of incubation at room temperature. Capping was stopped by the addition of 0·2 ml of a 4% paraformaldehyde solution in PBS pH 7·4. After fixation the cells were washed twice with Media 199 containing 0·1% gelatin and mounted on slides. The number of caps was determined from a total of 200 fluorescent cells counted.

Interaction of AG with PC vesicles in the presence of ANS, an anionic surface probe

Synthetic vesicles were prepared by adding PC (Sigma) to PBS to reach a final concentration of 2 mg/ml. The mixture was sonicated (Model W 185 Heat Systems-Ultra Sonics) at 50 W for 15 min. This procedure has been shown to result in unilamillar vesicles of 250–500 A° diameter (Haynes & Staerk, 1974). ANS was added to 2 ml of vesicles in a 1 cm quartz curvette to reach a final concentration of 50 μg/ml. The mixture was allowed to stand at 37°C in a Spectrofluorimeter (Perkin Elmer MPF-3L) until the fluorescence intensity (excitation = 360 nm, emission = 480 nm) reached a plateau indicating that both the inside and outside vesicle surfaces were equilibrated with dye (Haynes, 1974). AG was added in 25 μl aliquots to reach a final concentration of 2 mg/ml. After each AG addition the fluorescent intensity was measured. In order to calculate the specific AG-induced displacement of ANS, a parallel experiment (as above) was performed in the absence of vesicles to determine the interaction between AG and ANS. The values obtained in the absence of vesicles were subtracted from the values obtained with vesicles. Control experiments were performed by carrying out the above procedures in the presence of asialo AG (ASAG, 2 mg/ml), sialic acid (SA, 0·54 mg/ml, the moler equivalent of AG), IgG (2 mg/ml) or PBS, instead of AG.

Interaction of AG with PC vesicles in the presence of NPN, a non-polar membrane interior probe

PC vesicles were prepared as described above, and mixed with 10 μl of NPN ($1{\cdot}4 \times 10^{-2}$ M solution in methanol). The vesicles were allowed to equilibrate with NPN (excitation = 340 nm, emission = 480 nm) until the fluorescent intensity reached a plateau. All subsequent steps were performed as previously described for the ANS experiments.

Preparation of ASAG

The ASAG derivative was prepared by incubating 40 mg of AG in 0·5 M sodium acetate buffer, pH 5·5 (containing 9 mM $CaCl_2$ and 154 mM NaCl) at 37° for 30 min in the presence of 1 international unit (IU) of neuraminidase (*Vibrio cholerae*, Calbiochem Behring). After 30 min another IU was added and the reaction was allowed to incubate an additional 30 min. The reaction mixture was passed over a G-25 Sephadex column and the protein peak was pooled and dialysed exhaustively against distilled H_2O and then lyophilized. The ASAG was shown to be 98% free of sialic acid by the thiobarbituric acid assay for sialic acid (Warren test; Warren, 1959).

Determination of the liquid to crystalline phase-transition temperature in the presence of AG

NPN was added to PC vesicles as previously de-

scribed. An aliquot of 225 μl of AG at a concentration of 20 mg/ml was added. The fluorescent intensity was measured at 480 nm and recorded as the vesicles in the cuvette were slowly cooled to 10°. The phase-transition temperature was determined by plotting the fluorescent intensity at 480 nm as a function of temperature. The plotted data result in two lines with different slopes that intersect at the point of phase-transition. Thus, the phase-transition temperature was determined from the plotted data.

Statistical analysis

Data were analysed by the Student's *t*-test for unpaired variates. A P value of $<0{\cdot}05$ was considered significant.

RESULTS

Mitogen-induced lymphoproliferation of human mononuclear cells in the presence of AG

The Con A-, PHA-P- and PWM-induced proliferative responses of lymphoid cells from human donors were determined in a replicate series of experiments with or without AG at a concentration found in serum during acute inflammation (2 mg/ml) (Fig. 1). Addition of AG consistently resulted in inhibition of lymphoproliferation. The data reveal that the glycoprotein causes a significant reduction in mitogen responsiveness at P values $<0{\cdot}01$, $0{\cdot}01$ and $0{\cdot}05$ for Con A, PHA-P and PWM, respectively. The reduced mitogenic activity was not due to AG-induced toxicity as measured by parallel cultures which showed $>95\%$ trypan blue exclusion.

Kinetic analysis of Con A receptor cap formation of lymphocytes pretreated with AG

Human mononuclear cells were incubated with or without exogenously added AG at a concentration of 2 mg/ml and then treated with FITC-labelled Con A. The number of cells showing capped fluorescence per 200 lymphocytes was recorded at various times during incubation at 37° (Fig. 2). The untreated cells showed a rapid increase in cap formation which reached a maximum at 15 min. When the cells were pretreated with AG (at a concentration found in serum during acute inflammation) there was only a minimal increase in the relative number of cells showing caps. There was a significant difference between treated and untreated cells at 10 and 15 min, $P<0{\cdot}05$, and $0{\cdot}01$, respectively. At incubation times greater than 20 min both groups demonstrated cells with intracellular fluorescence, presumably due to endocytosis.

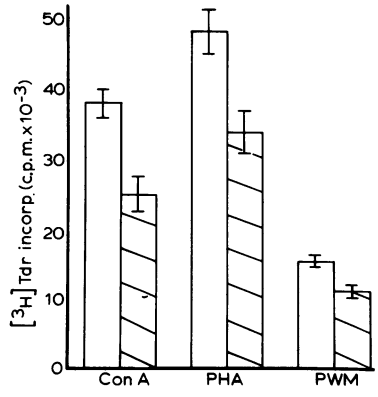

Figure 1. Effect of AG on mitogen-induced proliferation of human lymphocytes. *Open bars* represent the mean counts per minute ± SE of [^{3}H]TdR incorporated into the DNA of lymphoid cells from five individual donors. *Hashed bars* represent the mean counts per minute ± SE of [^{3}H]TdR incorporated into the DNA of lymphoid cells from the same donors in the presence of AG (2 mg/ml, final concentration). For additional experimental details see 'Materials and Methods'.

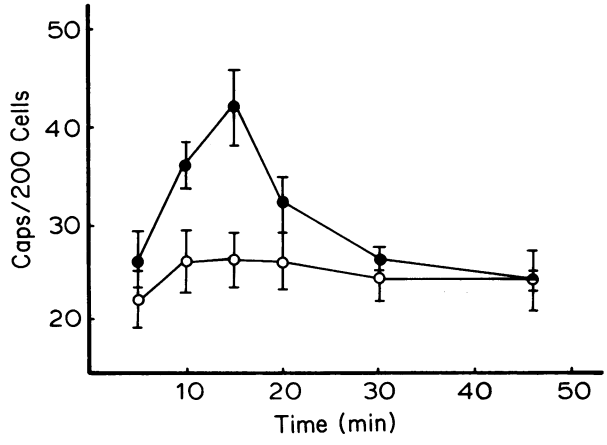

Figure 2. Effect of AG addition on the kinetics of Con A capping of normal lymphoid cells. (●) The mean number of caps/200 lymphocytes from six individual donors preincubated in human serum from six different donors. (○) The mean number of caps/200 lymphocytes from the same donors incubated in the same normal sera as above supplemented with AG (2 mg/ml). The *bars* represent the SE. For additional experimental details see 'Materials and Methods'.

Analysis of sIg receptor capping on lymphocytes pretreated with AG

Normal lymphoid cells were preincubated with or without exogenously added AG at a concentration of 2 mg/ml and then treated with FITC-labelled anti-sIg. The number of cells showing capped fluorescence per 200 fluorescently stained cells appears in Table 1. There was a significant reduction in caps on cells pretreated with AG ($P < 0.01$).

Table 1. SIg capping of lymphocytes pretreated with AG*

	Caps per 200 fluorescent cells
Untreated	57±4†
AG (2 mg/ml)	37±3

* For experimental details see 'Materials and Methods'.
† Mean±SE.

Interaction of AG with PC vesicles in the presence of ANS, an anionic surface probe, and NPN, a probe of the hydrophobic vesicle interior

The ability of AG to displace previously bound ANS, an anionic probe, from the surface of PC vesicles was determined. The percent reduction of ANS-associated vesicle fluorescence as a function of AG concentration appears in Fig. 3. The reduction of fluorescence is dose dependent reaching a maximum of 32% at levels of AG greater than 1·25 mg/ml. Thus, at AG levels commonly found in the serum during acute inflammation the molecule appears to saturate the PC vesicle. In a separate set of experiments we tested the ability of AG, ASAG, IgG, SA and PBS, to displace ANS from the PC membrane (Table 2). Duplicate experiments demonstrate that only AG is capable of significantly reducing ANS vesicle-associated fluorescence at concentrations found in serum during acute inflammation. All of the other compounds had minimal effects when tested at equivalently high concentrations. The ability of AG to displace previously bound NPN from the PC vesicles was also determined (Table 2). AG was incapable of displacing NPN from the vesicles, indicating a lack of interaction with the membrane interior.

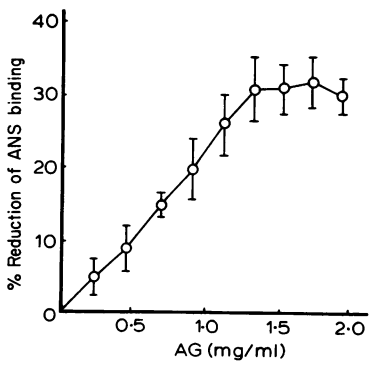

Figure 3. Dose-dependent effect of AG on the % reduction of ANS bound to PC vesicles. Each point represents the mean percent reduction ±SE ($n = 5$) of PC vesicle-associated ANS fluorescence measured at 430 nm. For additional experimental details see 'Materials and Methods'.

Table 2. Effect of AG (2 mg/ml) on the binding of ANS and NPN to PC vesicles*

	ANS (% reduction)		NPN (% reduction)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
AG	40	35	4	3
ASAG	10	5	ND[b]	ND
Sialic acid	5	4	ND	ND
IgG	0	5	6	4
PBS	3	5	4	7

* For experimental details see 'Materials and Methods'.
† Not determined.

Liquid to crystalline phase-transition temperature of PC vesicles in the presence of AG

Membranes composed of a single phospholipid have a particular liquid to crystalline phase-transition temperature dependent upon packing of the liquid molecules and the degree of saturation of the nonpolar tail (Alderson & Green, 1975). PC vesicles have been shown to have phase-transition temperature of 23° (Sackman & Trauble, 1972). The addition of 2 mg/ml of AG to PC vesicles does not change this phase-transition temperature, indicating that AG addition to the PC vesicles does not alter lipid packing or induce a structural change in the lipid bilayer (data not shown).

DISCUSSION

Many conditions associated with acute inflammation are characterized by a two- to five-fold elevation in serum AG levels (1–3 mg/ml) (Jeanloz, 1966; Schmid, 1975). Immune hyporesponsiveness of lymphocytes from cancer patients with increased serum levels of AG has been documented (Bradley *et al.*, 1977). We have recently reported that AG levels in sera from metastatic breast cancer patients correlates positively with the ability of these sera to inhibit mitogen induced proliferation of normal lymphoid cells in culture (Cheresh *et al.*, 1982a). Moreover, we demonstrated that sera from those patients with high levels of AG ($\geq 1{\cdot}5$ mg/ml) inhibits Con A capping on normal lymphocytes. In the present report we show that when AG (2 mg/ml) is added to cultures of normal lymphoid cells, it similarly inhibits proliferative responses induced by various mitogens. In parallel experiments we show that AG inhibits the redistribution (capping) of Con A receptors as well as sIg on the surface of normal lymphocytes. Capping, an energy-dependent receptor redistributional event, is not required for lymphocyte activation (Edelman, 1976). Inhibitors of capping, however, may affect other events involving receptor mobility which are necessary for triggering lymphocyte responses. Several other studies indicate that reduced receptor capping is associated with inhibition of lymphoid cell activation. Con A capping (Avertano *et al.*, 1980) and mitogen-induced blastogenesis of lymphocytes (Hallgren *et al.*, 1973; Gilman, Woda & Feldman, 1981) are both reduced with increasing age. Certain drugs which inhibit capping of lymphocyte receptors are also inhibitory to cellular proliferation. Cytochalasin B, known to perturb submembrane microfilaments, inhibits capping and cellular proliferation (Yoshinaga, Yoshinaga & Waksman, 1972). Local anaesthetics and tranquilizers which inhibit membrane depolarization are also inhibitory to mitogen induced blastogenesis and receptor redistribution on lymphoid cells (Cullen, Chretien & Leventhal, 1972; Ryan, Unanue & Karnovsky, 1974). The finding that elevated serum levels of AG (2 mg/ml) inhibits the lymphocyte capping process is a unique observation since AG is a naturally occurring biological substance.

Our results indicate that the interaction of AG with the lymphoid cell results in a non-specific inhibition. Exogenous addition of AG inhibits lymphocytes stimulated by any one of three different mitogens as well as capping of two distinct receptor–ligand complexes. Other investigators have described the numerous suppressive effects of AG (Chiu *et al.*, 1977; Bennett & Schmid, 1980; van Oss *et al.*, 1974). AG does appear to have some direct interaction with the mitogen PHA (Chiu *et al.*, 1977). When added simultaneously with PHA it blocks agglutination of sheep erythrocytes. When cells are pretreated with AG, however, agglutination proceeds as usual. In these same studies it was shown that AG is not cytotoxic to mononuclear cells from human peripheral blood since inhibition by AG of PHA-induced blastogenesis (at optimal concentrations of mitogen) can be reversed by further addition of PHA. AG does have a direct effect on lymphoid cells. When human peripheral blood mononuclear cells were pretreated with AG, washed, and then challenged with an optimum dose of PHA, blastogenesis was inhibited. The response was suppressed almost to the same degree as that of cells incubated continuously in the presence of AG. Our results confirm that AG is capable of interacting directly with lymphocytes. When mononuclear cells were preincubated with AG and washed, they were inhibited in their ability to support surface redistribution of receptors (Fig. 2 and Table 1). AG does not prevent ligand binding. We have examined the effects of AG pretreatment on the capacity of lymphocytes to bind Con A and anti-sIg. Using flow cytometric analysis we observed no change in the number of cells binding either ligand and detected no change in the amount of ligand bound per cell (unpublished results).

Since the effect of AG does not seem to involve masking of specific ligand receptors, we examined the interaction of this glycoprotein with phospholipid vesicles in order to characterize its effect on the lipid portion of the membrane. We used synthetic vesicles composed of PC because it is the most abundant phospholipid on the outermost surface of mammalian cells (Bretscher & Raff, 1975). Similar to other phospholipids, PC contains a zwitterion polar head group and a hydrophobic tail. With the use of two fluorescent probes that normally bind to these regions we have demonstrated that AG is capable of displacing an anionic probe which binds at the polar head region, but not a non-polar probe which binds at the membrane interior. Earlier published experiments indicate that negative charge interaction at the site of the polar head region is capable of displacing the anionic probe, ANS (Haynes, 1974; Flanagan & Hesketh, 1973). When we remove the highly electronegative sialic acid moiety from the AG molecule the remaining glycoprotein is incapable of displacing ANS. This supports the

concept that the interaction of AG with the polar head region may be in part electrostatic in nature.

Further support of the surface-restricted interaction of AG was shown by its inability to alter the liquid–crystalline phase-transition temperature of the PC vesicle. This was shown for all concentrations of AG tested (0–2 mg/ml). Molecules like cholesterol that interact with the hydrophobic portion of phospholipid cause a change in the phase-transition temperature which may be due to an alteration of lipid packing or a change in membrane fluidity (Bretscher & Raff, 1975).

Binding of AG to cell membranes may result in a marked increase in cell surface associated sialic acid and hence net negative surface charge. Such an increase in negative surface charge could alter antigenic recognition, cellular cooperation, and/or specific membrane-associated events leading to lymphoid cell activation. Nagura *et al.* (1973) demonstrated that a highly negative surface charge was associated with a reduction in phagocytic activity of rat peritoneal macrophages. Partial neutralization of the negative electric charge density by addition of protamine sulphate resulted in restoration of phagocytosis. Similarly, neuraminidase treatment of lymphocytes resulting in decreased negative surface charge was accompanied by an increased blastogenic response (Han, 1975). Preliminary experiments in our laboratory using polycationized ferritin (Danon *et al.*, 1972; Valet *et al.*, 1979) indicate that pretreatment of normal lymphoid cells with AG does significantly increase the net negative surface charge. Resulting electrostatic repulsion between surface receptors could explain the ability of AG to inhibit ligand-mediated cross-linking and capping. Alternatively, AG could bind to the exposed lipid between available surface receptors and sterically hinder receptor migration after ligand cross-linking.

The model presented here for the interaction of AG with the surface of lymphoid cells may explain the ability of this glycoprotein to inhibit numerous immunological responses. The biological role of AG may reside in its ability to affect the lipid bilayer of lymphoid cells, thus altering cell surface-associated events required for lymphoid cell activation.

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