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Interactions of alpha$_1$-acid glycoprotein with the immune system

I. PURIFICATION AND EFFECTS UPON LYMPHOCYTE RESPONSIVENESS

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Summary. Alpha$_1$-acid glycoprotein (AAG) is a constituent of normal serum which is elevated in concentration in the acute phase of inflammation; its physical and chemical properties have been defined but its biological function is uncertain. In the present study, the effect of AAG on the proliferative response of lymphocytes to several different stimuli was determined. For this purpose AAG was prepared by precipitation of human ascites fluid with sulphosalicylic acid and passage of the supernate through SP-Sephadex; the eluted protein migrated as a single band during immunoelectrophoresis, polyacrylamide gel electrophoresis and chromatography on Bio-Gel A-1·5 m in 6 M guanidine HCl. This AAG was found to markedly inhibit the proliferative response of human peripheral blood lymphocytes to PHA; it also inhibited blastogenesis induced by Con A and PWM, but to a lesser extent. AAG was not cytotoxic to lymphocytes, and its inhibitory effects were reversed at higher mitogen concentrations. Lymphocytes preincubated with AAG remained less reactive to PHA indicating that, although AAG appeared to react with PHA, its effect was directed predominantly to the cell. Further, AAG markedly inhibited the mixed lymphocyte response, and this effect was directed to the responder cells. Thus, AAG is another acute phase reactant with the ability to modulate lymphocyte responsiveness.

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INTRODUCTION

One feature of the inflammatory reaction is the 'acute phase response', during which increases in the concentration of certain serum proteins are seen. The physical and chemical properties of many of the acute phase proteins have been established, but the biological advantage of these changes remain poorly understood. This laboratory recently has undertaken investigations to characterize the interactions of acute phase reactants with the immune system. The present studies are concerned with the effects of alpha$_1$-acid glycoprotein (AAG) upon the lymphoid system.

Many physical and chemical properties of AAG are known, and these have recently been reviewed (Jeanloz, 1972). AAG was first observed in 1882 as a reddish-brown residue that remained in solution

after serum was boiled; these properties led to its early designation as the 'orosomucoid'. The mol. wt of AAG has been estimated to be 41,000; it is approximately 42% carbohydrate. It has a high (10–12%) sialic acid content and a low (pI 1·82) isoelectric point. Electrophoretic variants have been demonstrated in desialized AAG (pI 5·0), which have been attributed to amino acid substitutions near the N terminus. Curiously, one CNBr-cleaved fragment has sequence homology to immunoglobulin (Emura, Ikenaka, Collins & Schmid, 1971).

The biological properties of AAG are less well characterized. It can bind vitamin B_{12} (Miller & Sullivan, 1962) and progesterone (Westphal, Ashley & Selden, 1961), and inhibit thromboplastin (Nilsson & Yamashina, 1958). Its immunogenicity is increased upon desialization (Athineos, Thornton & Winzler, 1962). In fact, it has been suggested that the antigenicity of cells generally may be controlled by serum sialoproteins, which in this view would bind to and protect bystander cells from damage during the inflammatory process (Apffel & Peters, 1969).

We report here that AAG inhibits expression of certain lymphocyte responses, including blast transformation induced both by mitogens and the mixed lymphocyte reaction. Several alphaglobulins previously were found to regulate lymphocyte and/or immune responsiveness, and these include immunoregulatory alpha globulin (IRA) (Menzoian, Glasgow, Nimberg, Cooperband, Schmid, Saparoschetz & Mannick, 1974), alphafoetoprotein (Murgita & Tomasi, 1975a, b; Yachnin, 1975, 1976) and a crude preparation of alpha-glycoproteins (Chase, 1972). The acute phase reactant C-reactive protein also has been shown to have this capacity (Mortensen, Osmand & Gewurz, 1975; Mortensen & Gewurz, 1976), as have low density serum lipoproteins (Curtiss & Edgington, 1976). AAG is distinct from these factors, and thus can be considered yet another alphaglobulin and acute phase reactant that interacts with lymphocytes to modulate their responsiveness.

MATERIALS AND METHODS

Antisera

Rabbit anti-human serum and -AAG, and radial immunodiffusion plates for quantification of AAG, were obtained from Behring Diagnostics, Somerville, New Jersey.

Purification of AAG

AAG was purified from pooled human ascites fluids stored at 4° after the addition of sodium azide (final concentration 0·1%); subsequent purification steps also were performed at 4°. The fluids were clarified by filtration through Celite (Hyflo Super-Cel, Johns-Manville Products Corporation, Lompoc, California), mixed with an equal volume of a 6% solution of sulphosalicylic acid (Fisher Scientific Company, Fair Lawn, New Jersey) following the method of Rudman, Treadwell, Vogler, Howard & Hollins (1972), and centrifuged at 5000 *g* for 30 min. The supernatants were brought to pH 7·5 with 1 M NaOH, concentrated on an ultrafiltration apparatus (Amicon Corporation, Lexington, Massachusetts) using a UM-10 filter, and passed through a sulphopropyl-Sephadex column (2·5 × 50 cm) in 0·08 M phosphate buffer at pH 5·0. The eluate, which contained the AAG, was dialysed sequentially against distilled water and phosphate-buffered saline, pH 7·5 (PBS). The total protein was estimated by the Lowry assay using bovine serum albumin as the standard. The purity of the AAG preparation was established by polyacrylamide gel electrophoresis at pH 8·28 (staining with Coomassie blue and scanning with a Beckman Acta CV spectrophotometer), and by immunoelectrophoresis in agarose gels using B-2 buffer (Beckman Instruments, Fullerton, California). To further determine its purity, AAG (10 mg) was also treated with 6 M guanidine HCl and applied to a 1·5 × 50 cm Bio-Gel A-1·5 m gel filtration column (Bio-Rad Laboratories, Richmond, California) equilibrated in 6 M guanidine HCl; 2·0 ml fractions were collected, and the absorbance at 230 nm was compared to the elution profiles of marker proteins.

Lymphocyte cultures

Human lymphocytes were separated from peripheral blood of healthy donors using density sedimentation on Ficoll–Hypaque. The cells were washed three times in Hanks's balanced salt solution (HBSS) and suspended in RPMI-1640 supplemented with 10% foetal calf serum (FCS). Cell suspensions (10^5 cells per well) were cultured in microplates (Microtest II, Falcon Plastics, Division of BioQuest, Oxnard, California) with varying concentrations of the mitogens phytohaemagglutinin (PHA; Difco Laboratories, Detroit, Michigan), concanavalin A (Con A; Nutritional Biochemical Co., Cleveland, Ohio) or pokeweed mitogen (PWM; Calbiochem, San Diego, California); AAG (50 μl) diluted in HBSS was added

to bring the final volume to 0·2 ml/culture. The suspensions were incubated at 37° for 96 h, pulsed with 0·5 μCi/well of ^{3}H-labelled thymidine (1·9 ci/mM; Schwartz-Mann Division, Becton Dickinson & Co, Orangeburg, New Jersey) and collected after 18 h using a multiple automatic sample harvester as described by Hartzman, Segall, M. Bach & F. Bach (1971).

Unidirectional mixed lymphocyte reactions (MLR) also were performed according to the method of Hartzman *et al.* (1971). An equal number (10^5) of mitomycin C (Calbiochem)-inactivated stimulator cells and untreated responder cells were incubated in the presence of varying concentrations of AAG in HBSS (total volume, 0·2 ml per culture). The suspensions were reacted for 5 days, pulsed with ^{3}H-labelled thymidine and harvested as described above.

Formation of rosettes

Lymphocytes (2×10^6/ml) were incubated overnight with varying amounts of AAG. E rosettes were formed according to the method of Jondal, Holm & Wigzell (1972). EAC rosettes were formed according to Bianco, Patrick & Nussenzweig (1970) using sheep erythrocytes sensitized with haemolysin (Hyland Laboratories, Costa Mesa, California) and reacted with human serum (1:50) at 37° for 30 min in gelatin-Veronal-buffered saline. Cells considered positive had three or more clearly attached sheep erythrocytes.

Viability

Lymphocytes were incubated with AAG for varying intervals, 0·1% trypan blue was added and, after 15 min, cells were scored for viability on the basis of dye exclusion.

PHA-induced haemagglutination

PHA was serially diluted in PBS in microtitre plates (Cooke Engineering, Alexandria, Virginia), AAG was added to each well, and sheep erythrocytes were added to a final concentration of 1%. The mixtures

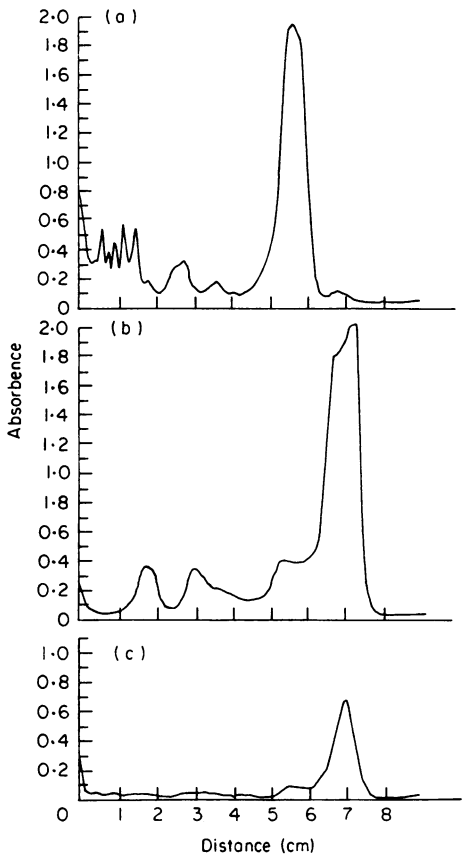

Figure 1. Polyacrylamide gel electrophoresis of (a) human ascites fluid; (b) the supernatant of ascites fluid after precipitation with 6% sulphosalicylic acid; and (c) purified AAG after sulphopropyl-Sephadex C-50 chromatography at pH 5·0 in 0·08 M phosphate buffer. The gels were stained by Coomassie blue and scanned at 600 nm.

Table 1. Purification of AAG from human ascites fluid

Step	Protein* (mg)	AAG† (mg)	Purification factor	Recovery (%)
Ascites-fluid‡	38,750	600	1	100
1. Sulphosalicylic acid	715	459	43	76
2. Sulphopropyl-Sephadex	405	375	62	63

* Determined by the assay of Lowry *et al.* using bovine serum albumin as the standard.
† Determined by radial immunodiffusion.
‡ Ascites fluid (500 ml starting volume) was clarified and treated exactly as described.

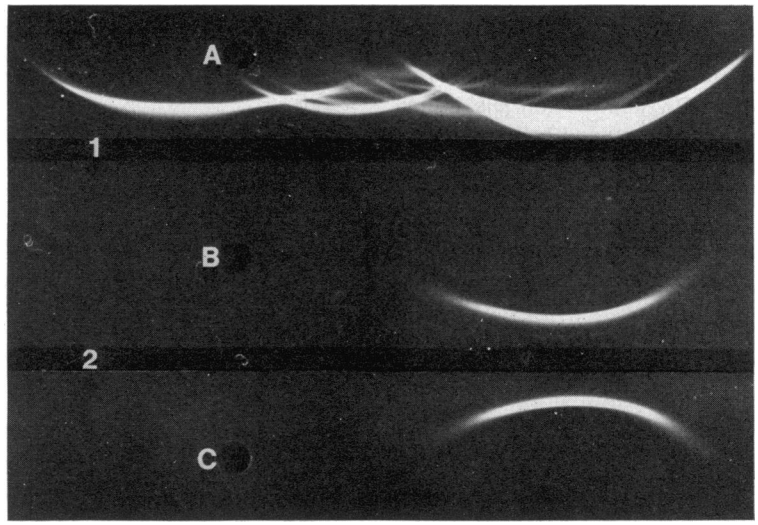

Figure 2. Immunoelectrophoresis of whole human ascites fluid (wells A and C) and purified AAG (well B) using anti-whole human serum (trough 1) and monospecific anti-AAG (trough 2).

were incubated overnight at room temperature, and agglutination was scored by inspection.

Capillary precipitation assays

AAG (1000 μg/ml) was placed in interfacial contact with varying concentrations of PHA (0·12–3·9 μl/ml) in capillary tubes and incubated overnight at room temperature; precipitation was scored by inspection.

RESULTS

Isolation and purification of AAG

Human ascites fluid, chosen as the starting material because of its large content of AAG, was clarified with Celite and treated with an equal volume of 6% sulphosalicylic acid. This served to precipitate most of the proteins while AAG remained in the supernatant with a 76% recovery and a 43-fold purification. This supernatant was passed through sulphopropyl-Sephadex (pH 5·0), from which AAG eluted with a final recovery of 63% and purification of 62-fold (maximum possible purification, 64-fold). Data from a representative purification procedure are summarized in Table 1 and Fig. 1.

The final preparation migrated in polyacrylamide gel electrophoresis as a single band forward of albumin, displaying a small trailing shoulder which may have represented aggregated or degraded AAG (Fig. 1). Upon immunoelectrophoresis no reaction was observed with polyvalent antisera to normal human serum although a strong reaction was observed with antiserum to human AAG (Fig. 2). It also migrated as a single molecular species during chromatography on Bio-Gel A-1·5 m in 6 M guanidine hydrochloride (Fig. 3), emphasizing the absence of lower mol. wt impurities such as IRA (see below), or subunits of C-reactive protein (Osmand, Mortensen, Siegel & Gewurz, 1975). Radioimmunoassay of the final preparation (kindly performed by Dr

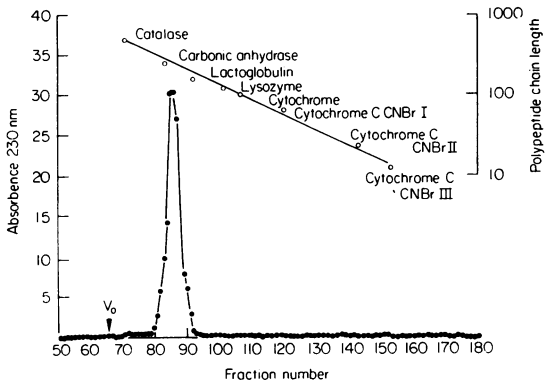

Figure 3. Chromatography of the purified AAG preparation on Bio-Gel A-1·5 m in guanidine hydrochloride monitored at 230 nm.

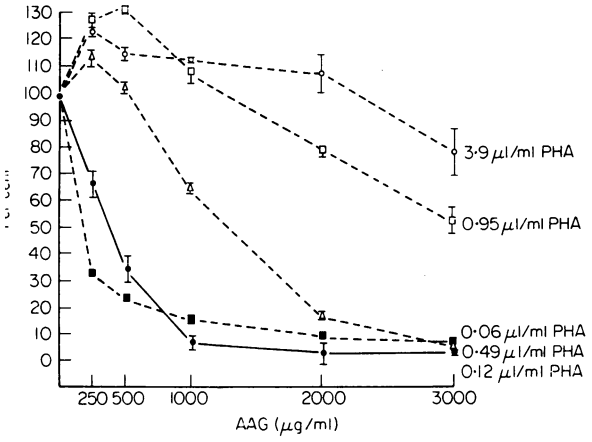

Figure 4. The effect of AAG upon PHA-induced uptake of ^{3}H-labelled thymidine by human peripheral blood lymphocytes performed in triplicate, showing the mean ± 1 s.d. The solid line indicates optimum dose of PHA at 0·12 μl/ml which gave 35,045 ± 1336 c.p.m., while the dotted lines indicate doses below and above the optimum where 0·06 μl/ml gave 27,114 ± 2125 c.p.m.; 0·49 gave 34,888 ± 1336 c.p.m.; 0·95 μl/ml gave 21,873 ± 2560 c.p.m.; and 3·9 μl/ml gave 13,885 ± 3078 c.p.m.

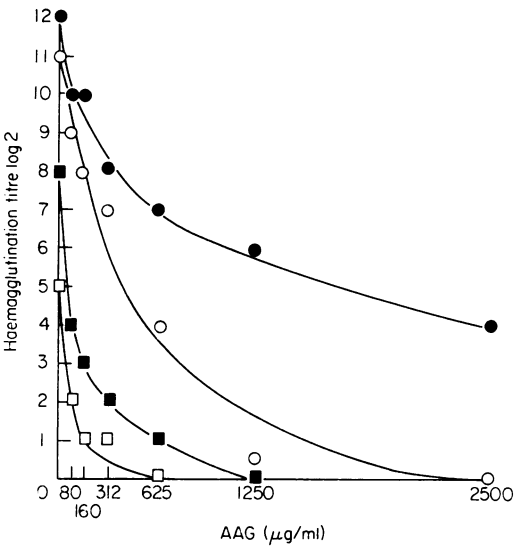

Figure 5. Inhibition of the PHA-induced agglutination of sheep erythrocytes by varying amounts of AAG: 0·16 μl (□), 0·63 μl (■), 1·25 μl (●), and 5·0 μl (●) PHA/ml, which yielded agglutination titres of 32, 256, 2048 and 4096, respectively.

Thomas Waldman of the National Institutes of Health) showed the virtually complete absence (1 part/1730,000 parts AAG) of alpha-foetoprotein.

The effect of AAG upon lymphocyte responsiveness to PHA

The effect of AAG upon lymphocyte responsiveness to PHA was tested in the first group of experiments. The ability of human peripheral blood lymphocytes to respond to the amount of PHA (0·12 μl/ml) which induced maximal blastogenesis was completely inhibited by amounts of AAG as low as 1000 μg/ml (Fig. 4). Larger amounts of PHA overcame this inhibition, while larger amounts of AAG restored the inhibitory effect. This emphasized that the degree of inhibition was a function of both the amount of stimulation and the quantity of inhibitory AAG present, and that the inhibitory effect of AAG did not involve cytotoxicity.

Experiments were designed to test the site(s) of action of AAG. When AAG was placed in interfacial contact in capillary tubes with concentrations of PHA used in the previous experiments, no precipitate was observed. However, AAG was found to markedly inhibit the ability of PHA to agglutinate sheep erythrocytes (Fig. 5); such inhibition was not seen with cells prereacted with AAG. To determine whether AAG could act at the level of the cells as well, lymphocytes were pre-incubated with AAG for 18 h (37° and 5% CO_2), washed, and challenged with the optimum dose of PHA. Cells pretreated with AAG in this way indeed were hyporesponsive to PHA as shown in Table 2. Thus, AAG appeared to influence the proliferative response to PHA both at the level of the lymphocyte and at the level of the mitogen.

The effect of AAG upon lymphocyte stimulation by Con A and PWM

The effects of AAG upon blastogenesis induced by Con A and PWM also were assessed. In the presence of 500 μg/ml AAG, the response to an optimum dose of Con A (8 μg/ml) was reduced by nearly 50%, and remained so when amounts as great as 3000 μg/ml AAG were used. By contrast, AAG even in amounts up to 3000 μg/ml had no effect on the blastogenesis induced by the amount of PWM (1:200) optimum for blastogenesis; at lower dilutions of PWM (e.g.

Table 2. Effect of preincubation with AAG upon the blastogenic response of peripheral blood lymphocytes to PHA*

AAG (μg/ml)	^{3}H-labelled thymidine incorporation (per cent control ± s.d.)		Per cent viable cells ± s.d.†
	AAG present	AAG preincubated	
0	100 ± 5·6	100 ± 4·2	85 ± 2
1000	64 ± 1·8	88 ± 1·8	84 ± 5
2000	17 ± 1·4	67 ± 4·5	86 ± 2
3000	5 ± 0·2	26 ± 0·5	88 ± 1

* Lymphocytes (10^6/ml) were incubated 18 h (37° in 5% CO_2) with varying amounts of AAG, washed and placed into cultures with the amounts of PHA (0·12 μl/ml) shown to induce the maximal blastogenic response under these conditions; ^{3}H-labelled thymidine was added on day 4 and the cells were harvested and analysed as described on day 5.

† The viability of cells incubated with AAG alone was measured by trypan blue dye (0·1%) exclusion on days 1–4. Similar results were obtained on each day and viabilities on day 4 are shown.

1:800), inhibition of blastogenesis up to 75% was observed when 3000 μg/ml AAG was used. Thus, AAG inhibited the lymphocyte response to Con A and PWM, but this was less intense than was its inhibition of PHA-induced blastogenesis.

The effect of AAG upon the mixed lymphocyte reaction (MLR)

The effect of AAG also was tested in the MLR. As shown in Fig. 6, the unidirectional MLR was significantly inhibited with as little as 7 μg/ml AAG, during which ^{3}H-labelled thymidine was incorporated at a level of only 60% that of the control. With 31 μg/ml AAG, the MLR was 50% as intense as in the untreated control, and amounts of AAG as high as 3000 μg/ml did not reduce the response beyond this level. These results further emphasize that the inhibitory action of AAG is directed at least in part to the lymphocyte. It may be noted that this inhibition was accomplished with approximately ten-fold lower concentrations of AAG than those needed to inhibit the lymphocyte response to PHA, perhaps because of the known smaller size of the responding cell population (Oppenheim & Rosenstreich, 1976).

To determine whether the inhibition in the MLR was induced via the responding or stimulating lymphocytes, or both, cells were preincubated for 18 h (37° and 5% CO_2) with AAG, washed and placed in cultures in appropriate combinations. The results are shown in Table 3. The responder cells preincubated with 1000 μg/ml AAG were distinctly inhibited, while stimulator cells incubated with identical amounts of AAG were not. Larger amounts of AAG were required for this inhibition than were

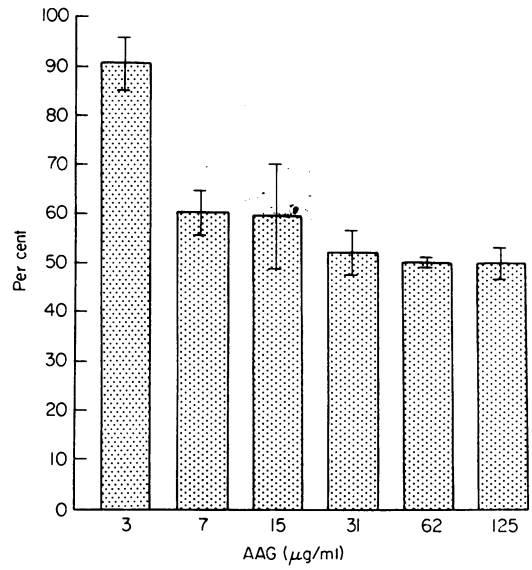

Figure 6. Inhibition of unidirectional mixed lymphocyte culture reactions performed in triplicate, showing the mean ± 1 s.d. Cells incubated without AAG had an uptake of ^{3}H-labelled thymidine at 18,727 ± 2138 c.p.m.

Table 3. Effect of preincubating with AAG upon lymphocyte responsiveness in the unidirectional MLR

Cell combination*	AAG (μg/ml)	^{3}H-labelled thymidine uptake (c.p.m. ± s.d.)	Per cent control (± s.d.)
A	0	3999 ± 2116	
B	0	383 ± 137	
AB	0	24706 ± 1075	—
A_aB	100	20765 ± 1500	84 ± 6
A_aB	1000	9111 ± 1208	37 ± 5
AB_a	100	21826 ± 1622	88 ± 7
AB_a	1000	18540 ± 1827	75 ± 7

* A represents lymphocytes from donor A; B represents mitomycin C-inactivated lymphocytes from donor B; A_a represents lymphocytes from donor A preincubated with AAG for 18 h (37° in 5% CO_2); and B_a represents lymphocytes from donor B pretreated with AAG in the same fashion. All groups of lymphocytes were adjusted to 10^5 cells/0·2 ml culture, and reacted as described in the text.

needed when this protein was present throughout the incubation. Again, these results emphasize that AAG can inhibit by direct interaction with the lymphocyte.

Effect of AAG upon rosette formation with E and EAC

The ability of AAG to inhibit blastogenesis induced by optimal concentrations of PHA and Con A, but not by PWM, suggested that it might react preferentially with T lymphocytes. To further explore this possibility, the effect of AAG upon the ability of lymphocytes to form spontaneous E and EAC rosettes, respectively, was evaluated. As shown in Table 4, lymphocytes treated with 3000 μg/ml AAG showed a slight but significantly decreased ability to form E rosettes, but no inhibition of EAC rosette formation was observed.

Table 4. Effect of AAG on formation of T and B rosettes

AAG (μg/ml)	Per cent E rosettes ± s.d.	Per cent EAC rosettes ± s.d.
0	52 ± 1	18 ± 2
500	51 ± 4	16 ± 3
1000	42 ± 6	18 ± 2
2000	43 ± 1	16 ± 4
3000	35 ± 1	17 ± 2

* Lymphocytes incubated overnight with varying AAG for 18 h (37° in 5% CO_2) and assessed for their ability to form T and B rosettes as described in the text.

DISCUSSION

The elevated serum concentration of AAG during the acute phase of inflammation led us to explore whether this acute phase reactant could influence lymphocyte responsiveness. Several procedures for isolation of AAG have been presented, including separation by ammonium sulphate precipitation (Weimer, Mehl & Winzler, 1950) with further purification by DEAE (Krotoski & Weimer, 1966) and CM (Bezkorovainy & Winzler, 1962) cellulose chromatography. In an alternative approach, Rudman *et al.* (1972) resolved AAG into a single band on polyacrylamide gel electrophoresis by precipitation of most of the serum proteins with sulphosalicylic acid, and passage of the supernatant through Sephadex G-75 and Dowex-1. In the present study, the latter procedure was modified by using only SP-Sephadex (pH 5·0) for the chromatographic step, and AAG was thus readily prepared in large quantities. The final product was >95% pure as determined by polyacrylamide gel electrophoresis, immunoelectrophoresis and chromatography on Bio-Gel A-1·5 m in 6 M guanidine HCl. Hydrolysis of sialic acid residues and denaturation of the protein was minimized by performing all procedures at 4°, and as demonstrated by others (Ganuly & Westphal, 1968), these preparative procedures were unlikely to have caused substantial degradation of this protein.

The present study demonstrated that AAG inhibited both blastogenesis of lymphocytes in response to mitogens and allogeneic cells and their ability to form E rosettes. The inhibition of blastogenesis in response to optimal doses of PHA and Con A but not PWM, the inhibition of T- but not B-cell rosette formation, and the inhibition of the responding but not the stimulating cells in the MLR, all suggested that the inhibitory effect of AAG was directed predominantly to T cells. Since as little as 7 μg/ml AAG resulted in a 40% inhibition of the MLR, and increasing the amount of AAG to levels as high as 3000 μg/ml did not further reduce the inhibition below about 50%, AAG appeared to have its effect upon a subset of T cells. The mechanism underlying these inhibitions is not yet clear. While AAG seemed to react with the mitogen PHA, it had an additional effect at the level of the cell, as shown by both preincubation experiments and by its inhibition of the MLR. Thus, AAG may combine with and/or otherwise alter the lymphocyte surface, or modulate intracellular factors or pathways which are known to be associated with the proliferative responses of these cells. Analysis of the mechanism(s) of inhibition awaits further elaboration.

We carefully considered the possibility that other serum immunosuppressive factors, such as IRA or alpha-foetoprotein, might be present in the preparation of AAG. IRA had previously been demonstrated to inhibit blastogenesis induced by mitogens and allogeneic cells, as well as to inhibit formation of E rosettes and cells secreting specific antibody

(Menzoian *et al.*, 1974) it has been identified as a polypeptide of mol. wt 8000, and apparently is bound noncovalently by serum alphaglobulins (Occhino, Glasgow, Cooperband, Mannick & Schmid, 1973). The presence of this material was excluded by chromatography on Bio-Gel A-1·5 m in guanidine HCl, by which no material of mol. wt lower than 40,000 was detected. Alpha-foetoprotein also has been demonstrated to inhibit lymphocyte responses *in vitro* (Murgita & Tomasi, 1975, a, b; Yachnin, 1975, 1976), but analysis by radioimmunoassay showed it to be absent from the AAG preparations. C-reactive protein and low density lipoproteins, which have completely different physicochemical properties than has AAG (Curtiss & Edgington, 1976; Osmand *et al.*, 1975), also were shown to be absent from the final AAG preparation.

In a survey of normal human serum, only certain proteins were found to inhibit the MLR, and these were characterized by low isoelectric points, mol. wt between 8–25000 and 30–60000 and precipitation in 1–2 M ammonium sulphate (Miller, 1976). These physical parameters are compatible with the known properties of AAG and suggest that it is one of the of serum macromolecules capable of suppressing lymphocyte blastogenesis. Indeed, the concentrations of AAG used in the present experiments are well within the range found in normal (400–1000 μg/ml) and acute phase (1000–2500 μg/ml (Winzler, 1971) sera. The amounts of AAG required for inhibition of mitogen-induced responses (~500 μg/ml) are well within the range of concentrations found in normal sera, and that (30 μg/ml) which inhibited the MLR compares favourably with amounts of CRP (40 μg/ml) (Mortensen, Osmand & Gewurz, 1975; Mortensen & Gewurz, 1976), low density lipoprotein (~50 μg/ml) (Curtiss & Edgington, 1976), murine (100 μg/ml) (Murgita & Tomasi, 1975b) and human (~250 μg/ml) (Yachnin, 1976) alpha-foetoprotein, IRA (250 μg/ml) (Cooperband, Badger, Davis, Schmid & Mannick, 1972) and foetuin (~1250 μg/ml) (Yachnin, 1975) required to inhibit the MLR by 50%. Further, the inability of high concentrations of AAG either to decrease lymphocyte responsiveness to optimum amounts to PWM or to inhibit the formation of EAC rosettes argues against the proposition that AAG has nonspecific effects directed against lymphocytes generally. Therefore, AAG must be considered as an additional serum protein which has the ability to influence lymphocyte responsiveness. Since another acute phase reactant, C-reactive protein, has been shown to have pronounced ability to modulate lymphocyte and platelet responsiveness (Mortensen *et al.*, 1975; Mortensen & Gewurz, 1976; Fiedel & Gewurz, 1976), the present results support the hypothesis that the acute phase response may represent in part a coordinated effort to limit, modulate, and/or direct host immune responses during the period of intense inflammation and tissue destruction.

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