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Inhibition of Plaque Formation, Rosette Formation and Phagocytosis by Alpha Globulin

M. GLASER, I. OFEK* AND D. NELKEN

Lil and Ben Stein Transplantation Laboratory, Department of Immunology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Summary. The immunosuppressive effect of an alpha globulin fraction isolated from normal human plasma is described.

The administration of this material to rats suppressed both the primary and secondary antibody responses against sheep erythrocytes. Alpha globulin also depressed the ability of mice spleen cells to confer immunity to irradiated isologous recipients.

The *in vitro* addition of alpha globulin to mouse peritoneal leucocytes (MPL) and whole human blood caused a significant reduction of their phagocytic activity against bacteria.

The mode of action of alpha globulin in relation to its immunosuppressive activity is discussed.

INTRODUCTION

A number of reports have indicated that α-globulin rich fractions derived from normal mammalian sera are effective inhibitors of the immune responses *in vivo* (Kamrin, 1959; Mowbray, 1963a; Mowbray, 1963b; Mannick and Schmid, 1967; Glasgow, Cooperband, Schmid, Parker, Occhino and Mannick, 1971). By *in vitro* studies it was found that these fractions inhibit the proliferation of lymphocytes (Cooperband, Bondevik, Schmid and Mannick, 1968; Cooperband, Davis, Schmid and Mannick, 1969; Milton, 1971), and interfere with antigen induced macrophage immobilization (Davis, Cooperband and Mannick, 1971). It was thought, therefore, that α-globulin acts by antagonizing the antigen recognition mechanism of lymphoid cells.

In a previous communication we described the *in vitro* inhibitory effect of α-globulin on plaque and rosette formation (Glaser, Cohen and Nelken, 1972). The present study deals with the *in vivo* and *in vitro* activity of this material on the immune response. Plaque and rosette formation were studied in greater detail and the effects of α-globulin on haemagglutinin formation and on phagocytosis were also examined.

MATERIAL AND METHODS

Animals

White male 'Sabra' rats of a strain developed at the Hebrew University, weighing 120–150 g, were obtained from the breeding farm and raised in the animal quarters till

* Present address: Streptococcal Reference Laboratory, Government Central Laboratories, Ministry of Health, Jerusalem, Israel.

reaching sexual maturity. They were used in the haemagglutination, the haemolytic plaque and the rosette assays.

Adult female BALB/c mice were used in the transfer experiments. Humans and BALB/c mice were used as a source of leucocytes in the phagocytosis experiments.

Preparation of α-globulin fraction

This was prepared as previously described (Mannick and Schmid, 1967).

Immunization and treatment

Blood from a single adult sheep was collected periodically in Alsever's solution and stored at 4°.

White rats were injected intraperitoneally with suspensions of $2{\cdot}5 \times 10^8$ sterile washed sheep red blood cells (SRBC) in 0·5 ml saline. The animals were divided into three groups: The first group received intravenously 20 mg of α-globulin in 1·0 ml of saline 0–30 minutes prior to SRBC injection; the second group was treated as above and 10 days later was challenged with an identical dose of SRBC; the third group was first immunized with SRBC and 10 days later was injected with α-globulin followed by a second dose of SRBC. Control animals were treated with saline or with 20 mg of human serum albumin (HSA-Magen David Adom, Blood Services, Tel Aviv-Jaffa) at the same time their counterparts received α-globulin. The animals were bled 6 days after each immunization, their spleens removed and spleen cell suspensions prepared.

Haemagglutination test

Haemagglutination titrations were done as described elsewhere (Nelken, Gurevitch and Neuman, 1957). To test for mercaptoethanol sensitivity, duplicate aliquots of serum were prepared; to one an equal volume of saline was added, to the other an equal volume of 0·2 M 2-mercaptoethanol (2-ME) in saline, and these were incubated at 37° for 30 minutes. The sera were then titrated.

Assay for plaque-forming cells (PFC)

The direct haemolytic plaque assay was performed as described by Jerne, Nordin and Henry (1963) modified as follows: suspensions of $0{\cdot}1 \times 10^6$ spleen cells in 0·1 ml Veronal buffer pH 7·4 were plated in duplicate, incubated at 37° in a humidified incubator with 5 per cent CO_2—95 per cent air for 75 minutes, flooded with 1·0 ml of 10 per cent fresh frozen guinea-pig serum (complement) and incubated for an additional 45 minutes.

The indirect (facilitated) plaque test was carried out according to the method described by Sterzl and Riha (1965) and Dresser and Wortis (1965), by adding 1·0 ml of 10 per cent complement containing 1:50 dilution of rabbit anti-rat IgG serum. After a further 45 minutes incubation the newly developed plaques were counted.

Rabbit anti-rat IgG serum

This antiserum was raised in rabbits by five weekly intracutaneous injections of 5 mg of rat IgG in 0·5 ml of saline isolated from whole rat serum on DEAE Sephadex according to the method described by Perper, Okimoto, Cochrum, Ramsey and Najarian (1967). Ten days after the last injection the rabbits were bled and the serum was inactivated at 56° for 30 minutes and absorbed twice with an equal volume of packed SRBC.

The specificity of the antiserum was determined by immunoelectrophoresis according to Scheidegger (1955).

Rosette formation test

The test described by Zaalberg (1964) and by Nota, Liacopoulos-Briot, Stiffel and Biozzi (1964) was used modified as follows: suspensions of 5×10^6 spleen cells in 0·25 ml of Veronal buffer were incubated with suspensions of 20×10^6 SRBC in 0·25 ml of Veronal buffer in a 37° water bath for 2 hours. The rosette-forming cells (RFC) were counted per 2000 lymphocytes.

Transfer experiments

These experiments were performed on four groups of BALB/c mice. The first group received intravenously a 50×10^6 spleen cell suspension in 0·5 ml of Veronal buffer preincubated with α-globulin (2 mg/10^6 cells) in a 37° water bath for 30 minutes. The cells were derived from BALB/c mice 5 days after intraperitoneal injection of 10^8 SRBC in 0·5 ml of saline (immunized group). The second group was treated as above with the exception that the spleen cells were derived from normal BALB/c mice (unimmunized group). The third and the fourth group were used as controls and received spleen cell suspensions as in the first and second groups respectively, except that the cells were pretreated with saline or HSA in the same concentration as the α-globulin preparation.

Mice were exposed in lucite containers to total body X-irradiation of 550 r using a Vanguard Picker X-ray machine (280 kV, 20 mA). They were irradiated at a target distance of 50 cm (dose rate 110 r/min) with 0·8 mm Cu filters. The irradiation was performed 2 hours before injections of spleen cells, and 1 day later the animals were injected intravenously with suspensions of 4×10^8 SRBC in 0·1 ml of saline. After further 7 days, the animals were bled and their spleens removed to test for haemagglutination, plaque and rosette formation.

Rabbit anti-mouse IgG serum (kindly supplied by Dr D. Sulitzeanu from our department) for the detection of indirect plaques was used in a 1:200 dilution.

Bacterial strains

Staphylococcus albus (from the stock culture collection of the Department of Bacteriology, the Hebrew University-Hadassah Medical School, Jerusalem) was subcultured in Bacto-nutrient broth containing 0·2 per cent glucose, for 18 hours at 37°. The bacteria were washed twice with saline buffered with 0·05 M phosphate pH 7·4 (PBS) and adjusted turbidometrically to 30 optical density (Klett spectrophotometer at 420 mμ). This density was equal approximately to 10^7 cocci per 1 ml.

β-Haemolytic *Streptococcus pyogenes* group A type 12, strain 512 (kindly supplied by Dr S. Rabinowitz from the Streptococcus Reference Laboratory, Ministry of Health, Jerusalem) was cultivated in Todd-Hewit Broth (Difco). The bacterial cells were diluted from the logarithmic phase of growth.

Phagocytosis

The procedure for phagocytosis with MPL was performed as described previously (Bergner-Rabinowitz, Beck, Ofek and Davies, 1969) with a slight modification. A leucocytic monolayer (10^6 cells) on a cover slip in a small Petri dish was prepared and 1·0 ml of α-globulin or HSA at varying concentrations were added. After 1 hour incubation at 37°, 1·0 ml of the bacterial suspension (*Staphylococcus albus*) was added. The reaction mixture was further incubated for 30 minutes and the cover slips were prepared for phagocytosis estimation as described (Bergner-Rabinowitz *et al.*, 1969). In other experi-

ments leucocytes or bacteria were preincubated with α-globulin or HSA and then washed before being used in the phagocytosis experiments. The percentage of leucocytes containing cocci was determined by counting 200 cells in each test.

Bactericidal method

The test was performed as described previously (Lancefield, 1957) with the following modifications: 0·1 ml of α-globulin or HSA at varying concentrations were added to 0·3 ml of fresh normal heparinized human blood. After different times of incubation on a shaker apparatus at 37°, 0·1 ml of a bacterial inoculum (*Streptococcus pyogenes*) containing 10^4 cocci was added. The survival of organisms was estimated by quantitative colony counts on blood agar pour plates made with 0·1-ml aliquots from the reaction mixtures after 3 hours incubation.

Intracellular killing

The test was performed with *Streptococcus pyogenes* and MPL as described elsewhere (Beck, Bergner-Rabinowitz and Ofek, 1969) with the following modifications:

α-Globulin or HSA (6 mg/ml) were added to the leucocytes which had been allowed to phagocytose the bacteria for a 10-minute period at 37° in the absence of the protein and had subsequently been washed free from all but engulfed or associated organisms. After a 30-minute incubation period at 37°, samples (0·2 ml) were withdrawn into 4 ml of 3 per cent saponin (T. T. Backer Chemical Co., Phillisburg, N.J.) solution (w/v) causing total lysis of the leucocytes and release of the ingested bacteria. The number of living intracellular microorganisms was estimated by quantitative colony counts.

In other experiments the intracellular killing test was performed with whole human blood. In this test the blood (0·8 ml) was incubated with suitable concentration of *Streptococcus pyogenes* (0·2 ml) for 10 minutes at 37° on a shaker apparatus. The reaction mixture was centrifuged for 4 minutes at 1000 rev/min and washed twice with saline to remove all but engulfed bacteria. 0·4 ml of plasma from the same donor was added and the mixture was further incubated with 0·2 ml of α-globulin or HSA (6 mg/ml) for 30 minutes at 37° on a shaker apparatus. The survival of organisms was estimated by colony counts after addition of 3 per cent saponin solution.

Viability test

Duplicate samples of 0·1 ml from the leucocyte suspensions were mixed with an equal volume of 1 per cent trypan blue (National Aniline Division, N.Y., N.Y.) in saline and left for 2 minutes. The percentage of viable leucocytes which did not take up the dye in each sample was then microscopically estimated by counting 200 leucocytes.

RESULTS

The effect of α-globulin rich fraction on the primary and secondary immune responses of rats to SRBC is shown in Tables 1, 2 and 3.

In the primary immune response (α-globulin was injected shortly prior to SRBC immunization and the animals were killed 7 days later) the direct and indirect plaque formation, the rosette formation, as well as the 2-ME sensitive and resistant haemagglutinin production were all suppressed (Table 1).

TABLE 1

EFFECT OF TREATMENT WITH α-GLOBULIN ON THE PRIMARY RESPONSE OF RATS TO SRBC

	Haemagglutinin titre (reciprocal)						PFC/10^6 spleen cells						RFC/10^6 spleen cells		
	Without 2-ME			With 2-ME			Direct			Facilitated					
Treatment	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
α-Globulin-treated group	4·2	2·20	0·69	0	0	0	90	17·79	5·62	0	0	0	1000	264	83
*Control group	121·60	80·95	25·60	4·20	2·20	0·69	550	60	18·97	210	46·66	14·75	4000	585	185

Eight animals in each group.
P values, differences between experimental and control groups, in all tests were less than 0·001.
* HSA or saline treated group.

TABLE 2

EFFECT OF TREATMENT WITH α-GLOBULIN BEFORE PRIMARY SRBC IMMUNIZATION ON THE SECONDARY RESPONSE OF RATS TO SRBC

	Haemagglutinin titre (reciprocal)						PFC/10^6 spleen cells						RFC/10^6 spleen cells		
	Without 2-ME			With 2-ME			Direct			Facilitated					
Treatment	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
α-Globulin-treated group	15·40	9·98	3·15	4·20	2·20	0·69	200	42·81	13·54	150	28·08	8·88	4500	346	109
* Control group	131·20	74·59	23·58	14·00	7·83	2·47	430	49·44	15·63	1500	228·52	72·26	10000	1290	408

Eight animals in each group.
P values, differences between experimental and control groups, in all tests were less than 0·001.
* HSA or saline treated group.

TABLE 3

EFFECT OF TREATMENT WITH α-GLOBULIN AFTER PRIMARY SRBC IMMUNIZATION ON THE SECONDARY RESPONSE OF RATS TO SRBC

	Haemagglutinin titre (reciprocal)						PFC/10^6 spleen cells						RFC/10^6 spleen cells		
	Without 2-ME			With 2-ME			Direct			Facilitated					
Treatment	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
α-Globulin-treated group	8·0	4·89	1·54	2·4	0·84	0·26	100	15·98	5·05	80	17·79	5·62	3000	246	77
*Control group	118·4	60·43	19·11	12·4	4·78	1·51	420	33·74	10·67	1350	129·09	40·82	9800	163	50

Eight animals in each group.
P values, differences between experimental and control groups, in all tests were less than 0·001.
* HSA or saline treated group.

TABLE 4

EFFECT OF α-GLOBULIN ON THE CAPACITY OF MICE SPLEEN CELLS TO PRODUCE ANTIBODIES AGAINST SRBC IN SUBLETHALLY IRRADIATED ISOLOGOUS RECIPIENT

	Haemagglutinin titre (reciprocal)						PFC/10^6 spleen cells						RFC/10^6 spleen cells		
	Without 2-ME			With 2-ME			Direct			Facilitated					
Treatment	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
α-Globulin-treated normal cells	8·00	4·89	2·19	4·40	2·19	0·97	50·0	12·74	5·70	20	4·74	2·12	800	111·80	50·00
HSA-treated normal cells	121·60	85·86	38·40	16·80	9·95	4·45	260	19·03	8·51	110	12·74	5·70	5000	158·11	70·71
α-Globulin-treated immunized cells	16·0	9·79	4·38	16	9·79	4·38	30	7·90	3·53	70	12·74	5·70	2000	223·60	100
HSA-treated immunized cells	128·00	78·3	35·05	64·0	39·19	17·52	140	19·03	8·51	300	33·35	14·91	9000	790·56	353·55

Five animals in each group.
P values, differences between experimental and control groups, in all tests were less than 0·01.

In the secondary immune response (α-globulin was injected as in the primary response and the animals were killed 7 days after the second SRBC immunization) the same effect was observed (Table 2).

The suppression of the secondary immune response was even greater when the α-globulin was injected after the first and shortly prior to the second SRBC immunization (Table 3).

In other experiments it was found that sensitized or normal mice spleen cells incubated with HSA or Veronal buffer produced antibodies in irradiated isologous recipients. The same spleen cells pretreated with α-globulin failed to induce similar levels of antibodies (Table 4).

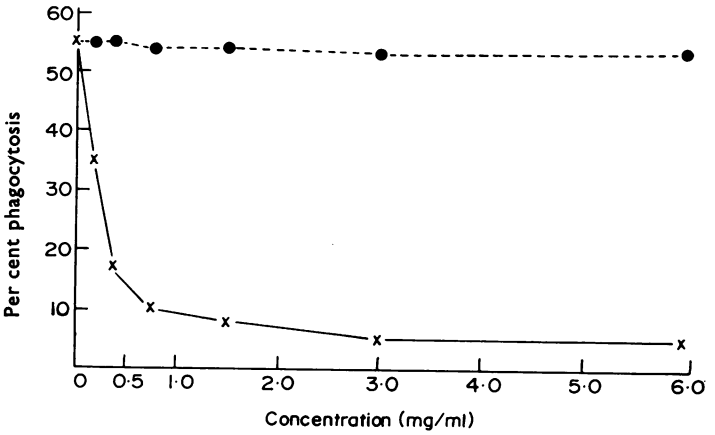

FIG. 1. Effect of α-globulin on phagocytosis of *Staphylococcus albus* by MPL. All values are averages of six experiments. x—x, α-globulin; ●—●, HSA.

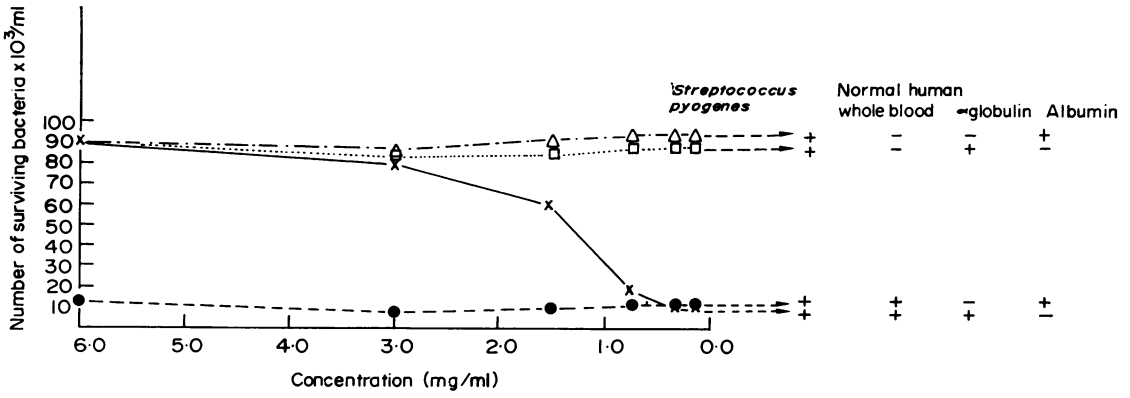

FIG. 2. Effect of α-globulin on the bactericidal activity of normal human whole blood on *Streptococcus pyogenes*. All values are averages of six experiments.

The effect of α-globulin on the phagocytic properties of MPL is shown in Fig. 1. The material was able to inhibit the phagocytosis of Staphylococcus albus by leucocytes as revealed by light microscopy, and this effect was related to its concentration.

The minimal effective concentration (found to reduce the phagocytosis by at least 75%) was approximately 0·4 mg/10^6 cells. This concentration was not found to be toxic to the MPL as judged by trypan blue dye uptake. No such inhibition was obtained when

the MPL or bacteria were pretreated with α-globulin (3 mg/ml) and washed before using them in the phagocytosis experiments.

Marked suppression in the bactericidal capacity of whole human blood exposed to α-globulin was observed (Fig. 2). The time of preincubation of the leucocytes with the material before addition of bacteria did not affect the degree of suppression even when the α-globulin was added ten minutes after the addition of bacteria, however, when phagocytosis was permitted to occur for 30 minutes before the α-globulin was added, no inhibition of bactericidal activity occurred. Moreover, in other experiments when α-globulin was added to MPL or whole human blood, which had been allowed to phagocytose bacteria in the absence of the material and had subsequently been washed free from all but engulfed organisms, no reduction in intracellular killing was noted.

No inhibition of phagocytosis and of bactericidal activity was observed when equal concentrations of HSA were used.

DISCUSSION

Recent reports indicate that naturally occurring α-globulins suppress immune responses both *in vivo* (Kamrin, 1959; Mowbray, 1963a; Mowbray, 1963b; Mannick and Schmid, 1967; Glasgow *et al.*, 1971), and *in vitro* (Cooperband *et al.*, 1968; Cooperband *et al.*, 1969; Milton, 1971).

It has been suggested that α-globulin acts directly on lymphocytes and/or other cells which are involved in the immune response (Milton, 1971).

In the present investigation it was found that the α-globulin rich fraction isolated from normal human plasma can suppress both the primary and secondary response of rats and mice to SRBC. This was found to be the case both by serum antibody titrations and by lymphocyte tests. Our results indicate that treatment of rats with 20 mg of α-globulin intravenously depressed both the 19S and 7S antibody response. The fact that rats which were challenged with SRBC 10 days after they were injected with α-globulin and SRBC, had a markedly diminished response to this antigen (a time when all immunosuppressive activity of the initial α-globulin treatment should have dissipated as was shown by Glasgow *et al.*, 1971) could perhaps be explained by the ability of the α-globulin preparation to confer partial tolerance to this antigen. On the other hand, it seems to us that as the primary response was markedly reduced by the injection of the α-globulin fraction, the number of antibody-producing cells, at 10 days, when the booster injection of SRBC was given, was relatively small and no significant secondary response could even be expected.

More evidence on the immunosuppression of the primary and secondary responses by α-globulin was gained by the transfer experiments. In these experiments spleen cells previously incubated with α-globulin were unable to restore the immune response in irradiated mice.

It could be argued that α-globulin may act by way of antigenic competition and thus cause suppression of the immune response. However, the control experiments, in which no reduction of the immune response was detected when similar amounts of homologous serum albumin were used instead of the α-globulin preparation, seem to rule out such a possibility.

Recent studies have shown that besides lymphocytes, α-globulin may affect other cells involved in the immune response (Davis *et al.*, 1971). Using the phagocytosis experiments, our studies suggest that α-globulin inhibits both uptake of *Staphylococcus albus* by MPL

and bactericidal activity against *Streptococcus pyogenes* by normal human whole blood. As has been shown for lymphocytes (Cooperband *et al.*, 1968), the viability of α-globulin treated macrophages was indistinguishable from that of cells treated with HSA as measured by the trypan blue method. It seems, therefore, that the differences in the percentage of phagocytosis cannot be attributed to a lethal effect of α-globulin on the cells. Alpha-globulin still exerts its antiphagocytic activity when applied 10 minutes after the addition of bacteria, but not when the material was added after 15–30 minutes, when most of the bacteria were already engulfed. Since α-globulin did not inhibit the intracellular killing when added after the phagocytosis was permitted to occur, its possible site of action is probably on the plasma membrane of the leucocyte. Furthermore, it is not firmly bound to these cells since washing of the treated cells before addition of bacteria removed all the inhibitory effects of the α-globulin on the phagocytic activity. Similar phenomena were reported for lymphocytes (Cooperband, Badger, Davis, Schmid and Mannick, 1972).

On the basis of our experiments, the normal values of plasma α-globulin should not effect phagocytosis. To attain a blood level equal to our minimal effective *in vitro* concentration, the value would have to be twenty times its normal concentration. Such values have been reported in various diseases (McFarlane and Oppenheim, 1969; Riggio, Schwartz, Stenzel and Rubin, 1968).

These results raise the possibility that α-globulin may directly affect the primary cellular defence mechanism. Further experiments on the susceptibility to infection of α-globulin treated animals and people with high α-globulin serum levels would shed more light on this problem.

α-Globulin was active in reducing the immune response after the interaction of antigen and lymphocytes and it was also active when given to phagocytic cells before or shortly after their interaction with the antigen. Therefore, this material may have more than one effect on the immune response.

At present, experiments are being performed in order to isolate the immunosuppressive substance from the α-globulin rich fraction.

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