Transformation of Human Lymphocytes: Inhibition by Homologous Alpha Globulin

Abstract. *An alpha globulin fraction prepared from normal human plasma by column chromatography prevents homologous lymphocyte transformation and the stimulation of DNA, and also protein synthesis induced by phytohemagglutinin and specific antigens. These observations support the concept of a normal circulating immunosuppressant factor which prevents lymphoid cell proliferation.*

There is evidence that the immune response may be to some extent under humoral regulation. Antibody itself, notably immunoglobulin G, can apparently operate as a very effective feedback mechanism to limit and even to terminate further antibody synthesis (*1*). Humoral factors of thymic origin appear able to stimulate immune responsiveness (*2*) and increase proliferation of lymphoid cells and synthesis of DNA and protein (*3*). The most recently described and least understood of these regulating factors is an immunosuppressive α-globulin (or α-globulins) present in normal plasma or serum (*4*). Allograft rejection and antibody production can be suppressed in animals injected with this α-globulin (*5*). The mechanism by which a normal plasma protein fraction exerts immunosuppression is still unknown.

In studies with this material, heterologous serum has usually been used in intact animals. Thus the immunosuppression could have resulted from antigenic competition by the injected foreign protein. We have sought to eliminate this possibility by determining whether the suppressive α-globulin factor from human plasma would influence reactivity of human lymphoid cells under the controlled conditions available in vitro. We now report that the α-globulin is a potent inhibitor of lymphocyte transformation and can prevent the stimulatory effects of both phytohemagglutinin (PHA) and specific antigen.

Human blood lymphocytes from normal donors were prepared as described (*6*). Heparinized blood (20 unit/ml) in a 50-ml syringe was held at a 45° angle for 2 hours, and the upper portion of plasma was separated after sedimentation of the red blood cells. The cells in this plasma were centrifuged at 300*g* and washed three times with culture medium 199 containing 200 units of penicillin, 200 μg of streptomycin, and 20 percent homologous AB serum. In the final leukocyte culture, 60 to 90 percent of the cells were lymphocytes, 10 to 40 percent were polymorphonuclear leukocytes, and up to 3 percent were monocytes. Cultures (0.5 ml) were established with 0.5×10^6 lymphocytes in conical glass tubes (1 by 12 cm) with loosely applied screw caps: they were maintained at 36°C in an atmosphere of water-saturated CO_2 in an incubator for 6 days at a 75° slant. Duplicate or quadruplicate cultures for each experiment were established, and all the data were tabulated as a mean.

Synthesis of DNA or protein in these cultures was determined by the incorporation of ^{32}P-labeled phosphate and ^{14}C-L-leucine, respectively, into these moieties, which were then isolated, and the radioactivity was counted (*7*). The morphology was examined, the cells were counted, and the viability was determined (*6*) when the growing period was terminated. Some of the lymphocyte cultures were stimulated with an amount of phytohemagglutinin M (0.03 ml per milliliter of culture), giving maximum stimulation of DNA synthesis. Phytohemagglutinin (Difco) lot No. 267303 was generally used for these studies; and lot No. 497034, later. This latter batch produced only 30 percent as much stimulation as that previously used. Immune stimulation of the lymphocytes was effected with diphtheria or tetanus toxoids in amounts of 10 to 50 flocculating units per milliliter, and purified tuberculin in amounts of 0.2 to 20 μg/ml. The toxoids were specially prepared and known to be free of cytotoxicity (*6*). The dose of antigen which provided maximum stimulation for the individual donor was used to assay the immunosuppressive α-globulin.

For the isolation of α-globulin (*5*), plasma protein was first precipitated at *p*H 5. The supernatant was applied to a DEAE-cellulose column, and the column was eluted with acetate buffer (*p*H 5) of increasing ionic strength. The fraction eluted between 0.1 and 0.2*M* acetate was used for our studies. This fraction was brought to neutral *p*H with glycine buffer, exhaustively dialyzed against saline, concentrated by diaflo-ultrafiltration, and sterilized by filtration through Millipore HA filters. It was used in the dose range of 0.01 to 10 mg per milliliter of tissue culture. Four batches of the α-globulin were prepared from different pools of human plasma at different times. Each batch was active, although there was some variability in activity when related to the number of milligrams of isolated protein. When examined by immunoelectrophoresis, the most active preparation contained one α_1-globulin and two α_2-globulins in addition to small amounts of albumin. The variable activity probably resulted from differences in the concentration of the active protein.

Controls for the α-globulin effect upon lymphocytes included buffers eluted from DEAE-cellulose columns without protein. After concentration of this "control buffer," purified bovine serum albumin (BSA) was added to a final concentration similar to that of the active isolated α-globulin. In some experiments, we used the incubation medium with or without BSA in appropriate concentration as the control.

The absence of cytotoxicity in the α-globulin or control buffer was determined by (i) counting the viable leukocytes in the cultures, trypan blue being the indicator (*8*); and (ii) counting the surviving cells in primary cultures of rabbit-kidney cells by the method of Rosenau and Moon (*9*). For the latter, the number of cells attached to the culture vessel after 2 days was determined in duplicate cultures that had been established with either the α-globulin, the control buffer with BSA, or the standard culture medium. The data are reported as the percentage of surviving cells, calculated in the following manner: number of recovered cells with α-globulin (or control buffer) times 100, divided by number of recovered cells in standard culture medium.

Sixteen cultures established with incubation medium and assayed with trypan blue demonstrated 67 ± 6 percent viable cells after 6 days. Ten cultures established with control buffer plus BSA demonstrated 72 ± 3 percent survival, and sixteen cultures with the α-globulin had 65 ± 8 percent nonstaining cells after 6 days. In two experiments in which the Rosenau and Moon method was used, the standard cultures were established with a standard deviation of 8 percent. Identical cells incubated with control buffer plus BSA demonstrated 108 ± 18 percent surviving cells, and cells incubated with the α-globulin showed 135 ± 15 percent cells surviving. Neither procedure disclosed any

Table 1. Suppression by human alpha globulin of PHA-induced proliferation in homologous lymphocyte cultures.

Additions	$^{32}PO_4$ incorporated into DNA over 6 days (count/min)	Transformed cells after 6 days in culture (%)
Unstimulated		
None	55	3
Controll buffer*	43	2
α-Globulin†	25	1
PHA stimulated		
None	1113	61
Control buffer*	1307	58
α-Globulin†	20	1

* Containing 2 mg of bovine serum albumin per milliliter of total culture. † α-Globulin concentration was 2 mg per milliliter of total culture.

Table 2. Suppression of stimulated protein synthesis in human lymphocyte cultures by human alpha globulin.

Donor	^{14}C-L-Leucine incorporated into protein after 6 days in culture (count/min)		
	Stimulation	With α-globulin	With BSA
A.B.	None	214	203
A.B.	PHA	195	2569
S.C.	None	462	558
S.C.	PHA	462	4131
S.C.+A.B.	Histoincompatability	254	2185

Table 3. Concentration-dependent suppression of stimulated DNA synthesis by alpha globulin. Radioactivity was measured on the 6th day.

α-Globulin* (mg/ml)	^{32}P in DNA (count/min)	BSA* in control buffer (mg/ml)	^{32}P in DNA (count/min)
Unstimulated cultures			
0.001	22	0.001	18
.01	20	.01	27
.1	9	.1	24
1.0	12	1.0	9
2.0	16	2.0	36
Immune stimulated cultures (diphtheria toxoid)			
0.001	67	0.001	84
.01	52	.01	112
.1	12	.1	91
1.0	9	1.0	42
2.0	14	2.0	119
PHA stimulated cultures			
0.001		0.001	728
1.0	567	.01	957
.01	654	.1	572
.1	29	1.0	627
2.0	37	2.0	719

* Final concentration.

overt signs of toxicity in the preparations.

The isolated α-globulin, in protein concentrations similar to those effective in suppressing graft rejection in mice (*5*) (that is, 2 to 10 mg/ml), or control buffer was added to lymphocyte cultures stimulated by PHA (Table 1). The control buffer with added albumin did not influence either the stimulated or unstimulated cultures. The addition of the α-globulin to unstimulated cultures may have produced a slight inhibition of DNA synthesis; however, the cell morphology was unchanged. Cell transformation and DNA synthesis induced by phytohemagglutinin were markedly inhibited by the addition of α-globulin.

Moreover, the α-globulin also suppressed immune-induced transformation and DNA synthesis. In a typical experiment with cultured cells from a donor who demonstrated skin sensitivity to tuberculin and to diphtheria and tetanus toxoid, unstimulated cultures incorporated 24 count/min of ^{32}P into DNA and contained 3 percent transformed cells after 6 days in culture. When these cells were exposed to diphtheria toxoid we found 96 count/min attributable to ^{32}P in DNA and 8 percent transformed cells; with tetanus toxoid, 80 count/min due to ^{32}P-DNA, and 7 percent blastoid cells; with tuberculin, 65 count/min due to ^{32}P-DNA, and 7 percent transformed cells. In duplicate cultures containing identical materials plus 2 mg of the α-globulin per milliliter, we found in the unstimulated cultures 20 count/min due to ^{32}P-DNA, and 1 percent transformed cells; with diphtheria toxoid 10 count/min due to ^{32}P-DNA, and 1 percent blastoid cells; with tetanus toxoid 10 count/min ^{32}P-DNA, and 1 percent transformed cells; with tuberculin and 27 count/min ^{32}P-DNA, and 1 percent transformed lymphocytes. The effect of the α-globulin on protein synthesis by lymphocytes in culture is seen in Table 2. The α-globulin prevented the stimulatory effect of PHA and allogeneic lymphocytes, but had a minimum effect on unstimulated cells.

Information was obtained on the approximate amounts of α-globulin needed to inhibit DNA synthesis in PHA and immune stimulated lymphocytes (Table 3). The lowest doses employed had no effect on cellular response to either stimulant; beyond this, the range of doses yielding graded suppression was relatively narrow. With the most active preparation in concentrations of 1 mg/ml and greater, inhibition was complete. Similar concentrations of BSA in control buffer did not influence DNA synthesis.

Inhibition of the proliferation normally evoked by PHA and immune reactions did not involve cytotoxicity, at least as assayed by increased cell permeability. The quantity of protein added constituted only a very small portion of the total protein present in the tissue culture medium (20 percent serum or approximately 14 mg per milliliter of culture). Addition of an equal amount of purified albumin had no discernible effect. Consequently, inhibition of lymphocyte transformation by the isolated α-globulin fraction appears to be specific. That small quantities of an homologous serum protein can inhibit specific immune cellular reactions is interpreted as indicating that this effect involves more than antigenic competition. It suports the concept that there is a normal circulating humoral factor which functions in the regulation of immune reactivity and prevents lymphoid cell proliferation. The specific cellular mechanism of this effect remains to be determined.

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