Inhibition of the Mixed Lymphocyte Reaction with Fab Fragments from Anti-Human ALG[1] (36579)

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The degree of disparity between the HL-A antigens of a donor and host indicates the likelihood of graft acceptance or rejection. Grafts in donor–host combinations having all of the HL-A antigens in common are more likely to survive than those in which a number of HL-A antigenic differences exist. Thus the degree of allograft reactivity can be estimated by histocompatibility typing of donor and host lymphocytes (1–3).

The mixed lymphocyte culture (MLC) technique is currently being used in conjunction with histocompatibility typing techniques to predict allograft reactivity in various donor–host combinations. A correlation has been demonstrated between the extent of the MLC response and the degree of HL-A antigenic disparity (4, 5). Thus, agents which suppress the MLC response may be expected to have an immunosuppressive effect on allograft reactivity *in vivo*.

Antilymphocyte globulin (ALG) is a potent immunosuppressive agent (6). Nevertheless, the use of ALG may be precluded in certain situations, such as bone marrow transplantation, because its lymphocytotoxic effect may destroy the cells of the graft.

This report is concerned with rendering ALG noncytotoxic without eliminating its *in vitro* immunosuppressive activity. This is accomplished by digesting ALG with papain to produce Fab fragments which are not cytotoxic yet retain the antibody specificity of the intact globulin (7). Both ALG and Fab fragments derived from ALG were tested for their ability to inhibit one way mixed lymphocyte reactions of cells of known HL-A types.

Materials and Methods. Freshly drawn heparinized human blood was allowed to sediment in plastic syringes for 1–3 hr. The plasma was centrifuged for 20 min at 200*g* to obtain leukocytes which were resuspended in RPMI-1640 medium (100 units/ml penicillin, 1 mg/ml streptomycin added). Stimulating cells were incubated at a concentration of 1×10^6 cells/ml for 30 min at 37° with Mitomycin C (27 μg/10^6 cells), washed once, and resuspended to the original volume in medium. One milliliter of stimulating and 1 ml of responding cells (2×10^6 cells/ml) were mixed and incubated in 16 × 125 mm glass culture tubes at 37° in a humidified 5% CO_2 atmosphere. After 6 days incubation, 0.1 μCi of ^{14}C-thymidine (Beckman, sp act 54 μCi/μmole) was added to each culture tube; 18–24 hr later, the cultures were harvested by the following procedure. The cells were washed twice with Hanks BSS, extracted for 1 hr with 4 ml of cold 5% trichloroacetic acid (TCA), washed once with 5% TCA and dissolved in 0.2 ml of 0.1 *N* NaOH for 10 min at room temperature. Ten milliliters of toluene base scintillation cocktail were added to each tube, the contents of which were transferred to glass liquid scintillation counting vials. Thymidine uptake is expressed in cpm as an average value of triplicate cultures. The stimulation index was calculated by dividing the average counts per minute in mixed cultures by the average counts per minute in control cultures consisting of responding and stimulating cells from the same individual.

Equine anti-human lymphocyte globulin (ALG) was obtained from Kallestad Laboratories (Lot No. 500C070). Fab fragments

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TABLE I. Cytotoxic Effect of ALG and ALG Derived Fab on Peripheral Blood Leukocytes.[a]

Expt.			Reciprocal ALG or Fab dilutions									Saline control
			4	8	16	32	64	128	256	512	1024	
1	Standard cell suspension	ALG	+	+	+	+	+	+	+	—	—	—
		Fab	—	—	—	—	—	—	—	—	—	—
2	Standard cell suspension	ALG	+	+	+	+	+	+	+	+	—	—
		Fab	—	—	—	—	—	—	—	—	—	—
3	Cells presensitized with Fab (1:2)	ALG	—	—	—	—	—	—	—	—	—	—

[a] The percentage of cells stained with trypan blue was determined after incubation of the ALG or Fab preparations with peripheral blood leukocytes at 37° for 30 min. An end point of 50% stained cells was taken as the cytotoxic titer. Dilutions were made from stock solutions containing 10 mg/ml of protein.

were obtained by digesting a portion of the ALG preparation with papain (8). The fragments were separated from undigested ALG by passage through a column of Sephadex G 200. By ultracentrifugal analysis the purified fragments migrated as a homogeneous population with an average sedimentation coefficient of 3.1 S.

In the cytotoxic tests 0.1 ml of dilutions of the ALG or Fab prepared from stock solutions of 10 mg/ml, were incubated for 30 min at 37° with 0.1 ml of peripheral blood leukocytes (5×10^6 cells/ml) in the presence of guinea pig complement. Trypan blue was added to each dilution and the percentage of cells stained was determined by counting at least 100 leukocytes in a hemacytometer. An end point of 50% stained cells was taken as the cytotoxic titer.

Results. The cytotoxic effect of ALG on standard leukocyte suspensions is illustrated in Table I by parallel Expts. 1 and 2. In contrast to the marked cytotoxic effect exhibited by the ALG preparation, little or no cytotoxicity for peripheral blood leukocytes was demonstrated by the Fab fragments.

A solution of Fab fragments (5 mg/ml) was mixed with an equal volume of leukocyte suspension (5×10^6 cells/ml). Following incubation, the cells were washed once and used in a cytotoxic test with undigested ALG. The results in Expt. 3 of Table I show that presensitization of cells with the fragments blocked the cytotoxic activity of the ALG preparation.

Table II includes data from six experiments illustrating inhibition of the one way MLC reaction by the ALG and ALG derived Fab preparations. There was no increase in the amount of radioactivity incorporated in control cultures treated with ALG or Fab preparations. Thus, neither the ALG nor ALG derived Fab fragments, in the amounts used in these experiments, stimulated a nonspecific lymphocyte proliferation. Also, the one way MLC is neither inhibited nor stimulated by IgG or Fab preparations from normal equine serum (Table II, Expts. 7 and 8).

The stimulation indices reported in Table II show that lymphocytes from HL-A nonidentical individuals responded vigorously in one way MLC. The addition of ALG to such cultures consistently inhibited the lymphocyte response, decreasing the stimulation indices to control values. The Fab preparations exerted a similar inhibitory effect on the lymphocyte response, as the stimulation indices again approached control values.

Inhibition of the MLC response by preparations of either ALG or ALG derived Fab was not dependent on the HL-A antigenic patterns of the particular lymphocytes being tested.

Discussion. The data presented show that preparations of ALG and Fab fragments derived from ALG can effectively inhibit the one way MLC reaction. The mechanism of such inhibition remains to be determined. Although the effect of ALG may be due merely

TABLE II. Inhibition of the One Way MLC Reaction by ALG and ALG Derived Fab Fragments.

Expt.	HL-A antigens of: Responders	HL-A antigens of: Stimulators	Treatment of cell cultures[a]	Av cpm	Stimulation index
1	2, 10	2, 10	—	347	—
	2, 10	2, 10	ALG	300	0.86
	2, 10	2, 10	Fab	292	0.84
	2, 10	2, 9, 12	—	6148	17.71
	2, 10	2, 9, 12	ALG	434	1.25
	2, 10	2, 9, 12	Fab	312	0.90
2	2, 9, 12	2, 9, 12	—	479	—
	2, 9, 12	2, 9, 12	ALG	197	0.41
	2, 9, 12	2, 9, 12	Fab	268	0.56
	2, 9, 12	2, 10	—	2504	5.23
	2, 9, 12	2, 10	ALG	160	0.33
	2, 9, 12	2, 10	Fab	529	1.25
3	1, 2, 8	1, 2, 8	—	322	—
	1, 2, 8	2, 9, 12	—	8062	25.01
	1, 2, 8	2, 9, 12	ALG	154	0.48
	1, 2, 8	2, 9, 12	Fab	201	0.62
4	2, 9, 12	2, 9, 12	—	240	—
	2, 9, 12	1, 2, 8	—	4218	17.61
	2, 9, 12	1, 2, 8	ALG	204	0.85
	2, 9, 12	1, 2, 8	Fab	155	0.64
5	1, 5	1, 5	—	972	—
	1, 5	2, 10	—	10,952	11.31
	1, 5	2, 10	ALG	612	0.63
	1, 5	2, 10	Fab	1219	1.25
6	2, 9, 12	2, 9, 12	—	218	—
	2, 9, 12	1, 10, 8	—	2341	10.71
	2, 9, 12	1, 10, 8	ALG	164	0.75
	2, 9, 12	1, 10, 8	Fab	221	1.01
7	2, 10	2, 10	—	341	—
	2, 10	2, 10	IgG (normal)	302	0.88
	2, 10	2, 9, 12	—	4321	13.51
	2, 10	2, 9, 12	IgG (normal)	4081	12.71
8	2, 10	2, 10	—	281	—
	2, 10	2, 10	Fab (normal)	301	1.07
	2, 10	2, 9, 12	—	2728	9.70
	2, 10	2, 9, 12	Fab (normal)	2565	9.12

[a] 0.5 mg of IgG or Fab preparations were added to appropriate cultures. Other experimental conditions are given in the text.

to a lymphocytotoxic effect the noncytotoxic Fab fragments were as effective as intact ALG in inhibiting the MLC reaction. Possibly the ALG and Fab preparations react with surface antigens of the responding lymphocytes, thereby preventing the blastogenic response to recognized allogeneic antigens, even in the absence of a cytotoxic effect. Alternatively, or in addition, the surface antigens on stimulating lymphocytes may be blocked by combination with ALG or Fab fragments, preventing recognition by the responding lymphocytes.

The ability of the Fab fragments to block

the lymphocytotoxic effect of ALG (Table I) indicates that these fragments retain the antibody specificity associated with the intact ALG preparation. However, the mechanism(s) by which the ALG molecules and the Fab fragments inhibit the MLC reaction may or may not be similar.

This investigation has shown that Fab fragments of ALG are capable of suppressing the mixed lymphocyte response *in vitro*. Since the MLC is used to measure potential allograft reactivity (9, 10), it has been suggested that agents which inhibit the MLC reaction may have a similar suppressive action on allograft reactivity *in vivo*. Studies of the *in vivo* immunosuppressive action of Fab fragments derived from ALG are in progress.

Summary. The human mixed lymphocyte culture reaction (MLCR) as measured by incorporation of ^{14}C-thymidine is inhibited in the presence of an appropriate concentration of equine anti-human lymphocyte globulin (ALG). Fab fragments prepared from ALG also inhibit the MLC reaction. However, unlike the intact ALG molecules, the Fab fragments are noncytotoxic for human lymphocytes. ALG derived Fab fragments could be potentially useful in suppressing allograft reactivity *in vivo*.

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