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The Relationship of the Major Murine Histocompatibility Region Associated IA Antigens to Mitogen Responses[1]

JOHN E. NIEDERHUBER, M.D.,[2,3] JEFFREY A. FRELINGER, Ph.D.,[4] ELIZABETH DUGAN, Ph.D.,[4] AND DONALD C. SHREFFLER, Ph.D.[3]

Departments of Surgery, Microbiology, and Human Genetics, The University of Michigan Medical Center, Ann Arbor, Michigan 48104

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The mouse and other mammals have a large number of histocompatibility (H) gene loci (perhaps several hundred) controlling cell-membrane alloantigens. Only one locus, however, is a strong H locus and is termed the major histocompatibility complex (MHC). A difference between donor and recipient at the MHC will prevent the growth of nearly all tumor allotransplants and result in rapid rejection of skin allografts. The murine MHC, (*H-2*), is located in the *IX*th linkage group of the 17th chromosome and is composed of five regions denoted as *K, I, S, G, D* [1]. The *H-2* gene complex has served as a model for MHC of other mammals and the similarity of *H-2* and the *HL-A* gene complex of man is striking.

In addition to the classical *H-2* histocompatibility antigens of the *K* and *D* regions, the *I* region, originally defined by immune response (*Ir-1*) genes [2], contains genes coding for a system of lymphocyte alloantigens. These antigens have been designated Ia (*I* region associated) [3] and to date 21 specificities have been identified (for review, Ref. [4]). The Ia antigens have a restricted distribution with expression on the majority of B lymphocytes and a subpopulation of T lymphocytes [5–11].

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[2]Present address: Department of Microbiology, The University of Michigan Medical Center, Ann Arbor, Mich. 48104.

[3]Recipient of U.S. Public Health Service Research Career Development Award.

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The purpose of this study was to determine if specific mitogen reactive cells expressed the Ia surface molecules and if there was any association between the Ia determinant and specific mitogen receptors.

MATERIALS AND METHODS

Mice. B10.BR ($H\text{-}2^k$) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. All other strains used in experiments or for antisera production were maintained in Dr. Niederhuber's or Dr. Shreffler's colony at The University of Michigan. In each experiment, mice were matched for age and sex.

Antisera. Antisera specific for Ia antigens were prepared by reciprocal immunizations of A.TH ($H\text{-}2^{t2}$) and A.TL ($H\text{-}2^{t1}$) mice with lymphoid tissues. A.TH anti-A.TL (Ia^k) serum and A.TL anti-A.TH (Ia^s) serum have been extensively characterized by microcytotoxic and absorption studies [4, 10]. These sera are consistently cytotoxic for 70% of lymph node cells and 50% of spleen cells. Previous studies have demonstrated that the majority of B lymphocytes are Ia positive while only a subpopulation of T cells carry the Ia determinants [10]. The IgG fraction of A.TH anti-A.TL serum was prepared by Pevicon block electrophoresis, eluted with 0.1 *M* phosphate buffer, and dialyzed against tissue culture media [12].

Anti-Thy-1.2 serum was prepared in A.AKR ($H\text{-}2^{a1}$, $Thy\text{-}1^a$) mice against A.AL ($H\text{-}2^{a1}$, $Thy\text{-}1^b$) thymocytes. Anti-H-2 serum specific for the $H\text{-}2K^s$ determinant (H-2.19) was prepared in $(A \times A.AL)_{F1}$ ($H\text{-}2^a/H\text{-}2^{a1}$) mice against A.TL ($H\text{-}2^{t1}$) cells and anti-

serum specific for *H-2K*^{k} antigens (H-2.11 and H-2.23) was prepared in A.TL (*H-2*) mice by immunizing with A.AL (*H-2*^{a1}) cells. All normal sera and antisera are clarified by centrifugation, heat inactivated, and sterilized by passage through a 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass.). Sera are stored at −70°C in undiluted aliquots.

Culture conditions. Spleen cells were dispersed by teasing in serum-free RPMI 1640 media (Microbiological Associates, Bethesda, Md.) supplemented with 3 ml of Hepes (1 *M*) and 50 μg/ml gentamycin/100 ml media. Quadruplicate cultures of 5×10^5 viable cells were incubated in multi-well Linbro plates (Linbro Chemical Co., New Haven, Conn.). Cultures were stimulated with a dose range of T-cell and B-cell specific mitogens. The B-cell mitogen LPS was derived from Serratia marcesens (Difco Laboratories, Detroit, Mich.) and further purified according to the method of Nowotny *et al.* [13]. Con-A was purchased from Calbiochem (Elk Grove Village, Ill.), PHA-M was obtained from Difco Laboratories, and Leucoagglutinin, a highly purified form of PHA was from the Pharmacia Fine Chemicals, Uppsala, Sweden.

Culture plates of lymphoid cells stimulated with LPS were incubated for 48 hr at 37° C in an atmosphere of 5% CO_2. Con-A and PHA stimulated cultures were incubated for 72 hr. During the last 12–18 hr of culture, 0.2 μCi of ^{3}H-thymidine (2 Ci/m*M*) was present in the cultures. Cultures were harvested with a multiple sample harvester (Otto Hiller Co., Madison, Wis.), collected on glass fiber filters and counted in a liquid scintillation counter.

Thymus-dependent lymphocytes were isolated from whole spleen using the nylon-wool (LP-1 leuko-pak leukocyte filter, Fenwal Laboratories, Morton Grove, Ill.) method described by Julius *et al.* [14]. These cells were 80–90% sensitive to anti-Thy-1 serum plus complement and had no significant response to LPS.

B cells were prepared from whole spleen by twice treating the mixed cell population with anti-Thy-1.2 serum and complement. The resistant cell population was 95% B cells and demonstrated no T-cell functions.

Antiserum treatment of cultured cells. For blocking experiments, spleen cells or purified T- and B-cell subpopulations were incubated for 30 min at 37° C with anti-Ia serum, control serum, or normal serum without complement. These cells were then washed extensively by multiple centrifugations in media before culturing. In experiments where cells bearing the Ia determinants were actually eliminated from the cultured cell population, the cells were first incubated with antisera in a dilution of 1:5 or 1:10 at 37° C for 20–30 min. The cells were then pelleted by slow centrifugation, the antiserum was decanted, and the cells were resuspended in agarose absorbed rabbit complement, incubated for 30 min at 37° C, and then washed by centrifugation in media. Lysed and damaged cells were removed by centrifugation on a gradient of Lymphoprep (density 1.077 g/ml) (Nyegaard and Co. A/S, Oslo, Norway). The cells were then counted in a hemacytometer and adjusted to 5×10^6 viable cells/ml. Each culture has 5×10^5 cells and the final volume in each culture well was 0.3 ml.

RESULTS

Anti-Ia serum blocking of LPS stimulation. Spleen cells were incubated with appropriate anti-Ia serum without complement for 30 min at 37° C. The cells were then washed by centrifugation in media 3–4 times. Equal numbers of viable cells exposed to normal serum, anti-H-2 serum, or anti-Ia serum were cultured with a dose curve of 0.1 to 100 μg/ml LPS. The results of five experiments show a persistent partial inhibition (35.6% ± 11.8) ($P < 0.005$) of the response to 50 μg/ml of LPS (Table 1). The peak response in control cultures occurred with LPS concentration of 50 μg/ml. No significant inhibition was observed with nonappropriate anti-Ia^s serum (3.4% ± 13.7) or with anti-H-2K^s serum in Expts 2 and 3. In Expt 3 (Table 1),

TABLE 1
Inhibition of the LPS Mitogenic Response by Incubating Lymphocytes with Anti-Ia Serum before Culturing with LPS

Expt	Strain	I region	Antiserum blocking	cpm ± SD[a]	% Suppression
1	A.TL	k	None	10461 ± 1944[b]	—
			Anti-Ia^s	11830 ± 3752	−13
			Anti-Ia^k	8088 ± 914	23
2	A.TL	k	None	10704 ± 2198	—
			Anti-Ia^s	8110 ± 1922	24
			Anti-H-2K^s	9741 ± 3876	9
			Anti-Ia^k	5114 ± 1190	52
3	A.TL	k	None	4870 ± 900	—
			Anti-Ia^s	4959 ± 1964	− 2
			Anti-H-2K^s	4374 ± 1764	10
			Anti-Ia^k	2837 ± 708	42
4	B10.BR	k	None	21569 ± 792	—
			Anti-Ia^s	19852 ± 1314	8
			Anti-Ia^k	15902 ± 854	26
5	A.TL-B[c]	k	None	12239 ± 597	—
			Anti-Ia^s	14059 ± 1043	−15
			Anti-Ia^k	7969 ± 950	35
			Mean suppression		35.6 ± 11.8
			Control		0.4 ± 16.1
			P		<0.005

[a]Expressed as counts per minute ^{3}H-thymidine incorporation for quadruplicate cultures.
[b]Cultures were stimulated with 50 μg/ml LPS.
[c]A.TL-B = splenic B cells prepared by anti-Thy-1.2 serum and complement treatment of whole spleen.

the spleen cells were incubated with antisera for 3 hr, then washed, and cultured with LPS without any greater degree of suppression. Incubation with more concentrated serum dilutions of 1:2 also failed to increase suppression, and if incubations with sera were prolonged beyond 3 hr, nonspecific suppression occurred.

To eliminate the possibility that the persistent decrease in responsiveness was the effect of anti-Ia serum on T cells, the whole spleen cell suspension was treated with anti-Thy-1.2 serum and rabbit complement. The anti-Thy-1.2 resistent splenic B cells were then incubated with A.TH anti-A.TL (Ia^k) serum for 30 min, washed, and cultured with LPS (Expt 5, Table 1). The same partial suppression was observed. Therefore, a Thy-1 positive, Ia positive auxillary T cell was not involved in the suppression.

The inhibitory effect observed with anti-Ia serum was not found when cells were pretreated with antisera specific for H-2K transplantation antigens, nor with anti-Ia serum pretreatment of an irrelevant target cell. This indicates that there is no association between the H-2K determinants on the cell membrane and the receptors(s) for LPS. It also demonstrates that simply attaching an antibody to the cell surface will not interfere with the mitogen proliferative response.

Nonspecific inhibition of *in vitro* responses by even low concentrations of whole mouse serum in the culture media makes it virtually impossible to have the desired antibodies present throughout the culture period. In order to eliminate this problem, the IgG fraction of A.TH anti-A.TL (α Ia^k) serum was isolated by agarose block electrophoresis. This diluted antiserum retained a cytotoxic titer of 1:256 and could be present throughout the culture period without caus-

TABLE 2
Inhibition of the LPS Mitogenic Response when the IgG Fraction of A.TH Anti-A.TL Serum Was Added to Cultures

Expt	Strain	I region	cpm ± SD[a]		% Suppression
			Media	Anti-Ia (IgG)[b]	
1	B10.BR	k	9438 ± 2650[c]	6973 ± 1862	26
	B10.S	s	9552 ± 1578	8529 ± 780	11
2	B10.BR	k	8209 ± 506	6181 ± 750	25
	B10.S	s	5093 ± 1536	5414 ± 670	−6
3	A.TL	k	3316 ± 664	1944 ± 346	41
	B10.S	s	4236 ± 1416	4443 ± 675	−5
4	B10.BR	k	2246 ± 538	1738 ± 208	23
	B10.S	s	2817 ± 348	3272 ± 340	−16
5	B10.BR	k	1742 ± 611	995 ± 345	43
	B10.S	s	3795 ± 337	2842 ± 294	25
6	B10.BR	k	2626 ± 889	1393 ± 358	53
	B10.S	s	4163 ± 868	3732 ± 1234	10
			Mean suppression		35.2 ± 12.2
			Control		3.2 ± 14.9
			P		<0.005

[a] Expressed as counts per minute ^{3}H-thymidine incorporation for quadruplicate cultures. There was no mitogen effect of anti-Ia^k (IgG) when it was added to spleen cell cultures.
[b] Pool of inhibitory γ-fractions from agarose block electrophoesis.
[c] Cultures were stimulated with 50 μg/ml LPS. Responses were 2.7–5.0 × control with no LPS.

ing nonspecific suppression or mitogenic response. The results of six experiments are summarized in Table 2. The mean suppression was 35.2% ± 12.2 ($P < 0.005$) which was no greater than the suppression observed when cells were incubated briefly with anti-Ia antibodies before culturing with LPS. This consistent partial suppression of the LPS response suggests an association between the LPS receptor and the Ia molecule(s).

Anti-Ia serum blocking of Con-A and PHA stimulation. In order to test for a similar inhibition of T-cell mitogen stimulation, spleen cells were incubated for 30 min with normal serum, anti-H-2K serum or anti-Ia serum without complement, washed by centrifugation, and cultured for 72 hr with mitogen. The anti-Ia serum was A.TH anti-A.TL (α Ia^k) serum which is known to have anti-T-cell cytotoxic activity. In four experiments, there was no difference in the Con-A or PHA response of serum pretreated cells when compared to cells exposed only to culture media. Three additional experiments were performed using the T-cell mitogen leucoagglutinin again without any effect on the response. These experiments were always performed over the complete dose range of each mitogen to rule out a change in response kinetics in anti-Ia serum blocked cells.

LPS response of splenic B cells resistant to anti-Ia serum and complement. B10.BR (*H-2^k*) spleen cells were incubated with normal A.TH serum or A.TH anti-A.TL serum (anti-Ia^k) diluted 1:2 followed by an incubation with rabbit complement. The cells were centrifuged through a Ficoll–Hypaque gradient, adjusted to equal numbers of viable cells, and cultured with a dose range of LPS. The spleen cells resistant to anti-Ia antibodies and complement did not respond to LPS.

To eliminate the possible involvement of T cells, spleen cells were first treated with anti-Thy-1.2 serum and rabbit complement. After washing the cells by centrifugation in media, the anti-Thy-1.2 serum and complement incubation was repeated. The cells resistant to

anti-Thy-1.2 serum are greater than 95% B cells as determined by surface markers and functional assays. The splenic B cells were then treated with normal serum and rabbit complement or A.TH anti-A.TL (Ia^k) serum and complement. The remaining viable cells after anti-Ia^k serum treatment constituted approximately 8% of the original spleen cell population and equal numbers of these cells were cultured with 50 μg/ml LPS. The B cells resistant to complement dependent anti-Ia serum lysis did not respond to LPS (Fig. 1), indicating that the LPS reactive spleen cells are Ia positive B cells.

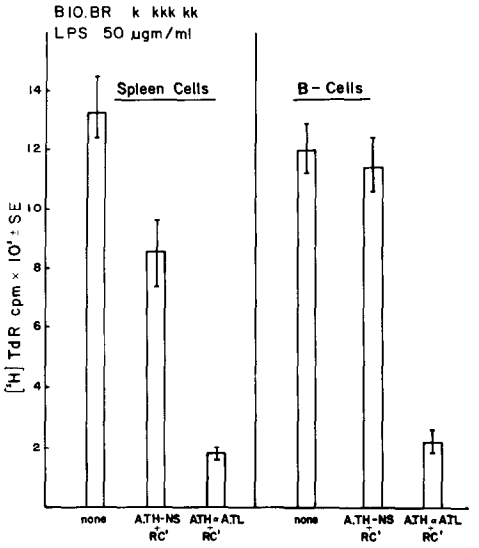

FIG. 1. Proliferative response of B10.BR ($H\text{-}2^k$) spleen cells and splenic B cells to 50 μg/ml LPS. Spleen cells or anti-Thy-1.2 serum and complement resistant B cells were treated either with A.TH normal serum and rabbit complement or with A.TH anti-A.TL (αIa^k) serum and complement. Equal numbers of surviving viable cells (5×10^5) were cultured for 48 hr with LPS. Each bar represents the mean of four cultures ± SE.

The differential expression of the Ia marker on PHA and Con-A reactive T cells. Spleen cells from B10.S ($H\text{-}2^s$) mice were subjected to lysis by A.TL anti-A.TH (anti-Ia^s) serum and rabbit complement, killing 50–60% of the lymphocytes. Equal numbers of viable Ia^s negative cells were cultured with Con-A or leucoagglutinin. These cells

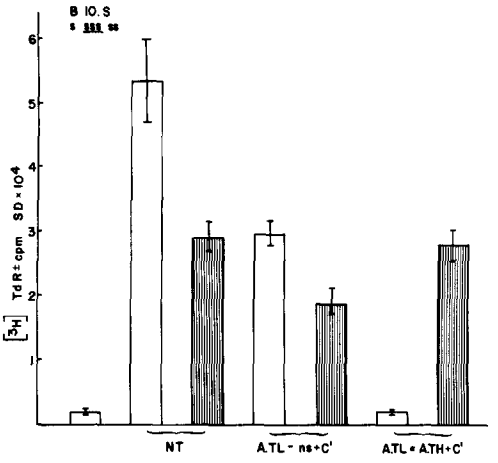

FIG. 2. Con-A and leucoagglutinin proliferative response of A.TL anti A.TH (αIa^s) serum and rabbit complement treated B10.S ($H\text{-}2^s$) spleen cells. NT is the response obtained when cells were exposed only to culture media. A.TL-ns + C' represents the response of cells treated with normal serum and rabbit complement. Equal numbers of remaining viable cells (5×10^5) were cultured for 72 hr with either Con-A, 1 μg/ml (open bars) or leucoagglutinin, 1 μg/ml (shaded bars). Each bar represents the mean response of four cultures expressed as cpm ± SD. The open bar at the left of the graph is the background response in unstimulated cultures.

responded normally to leucoagglutinin but did not respond to Con-A (Fig. 2). Four experiments were performed including two with B10.BR ($H\text{-}2^k$) spleen cells and A.TH anti-A.TL (Ia^k) serum with identical results.

The experiments were repeated using splenic T cells isolated by incubating the spleen cell mixture on nylon–wool columns. The first cells washed off the column by the addition of 15 ml culture media were greater than 90% Thy-1.2 positive T cells. The nylon–wool T cells were incubated with anti-Ia serum and complement. Equal numbers of surviving T cells were cultured with Con-A, PHA, or leucoagglutinin. These cells failed to respond to Con-A (Fig. 3). Although not shown in Fig. 3, these cells gave a normal response to PHA and leucoagglutinin.

The Con-A sensitive lymphocyte, therefore, is a Thy-1 positive, Ia positive T cell distinct from the PHA or leucoagglutinin reactive Thy-1 positive, Ia negative T cell.

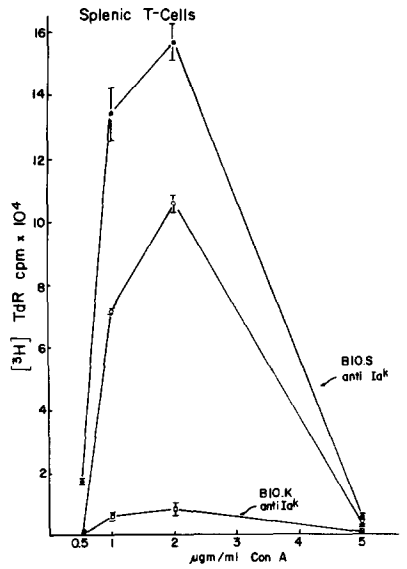

FIG. 3. Con-A proliferative response of nylon-wool purified splenic T-cells resistant to treatment with A.TH anti A.TL (αIa^k) serum and rabbit complement. Equal numbers of surviving viable cells (5×10^5) were cultured for 72 hr with mitogen. (○—) Nontreated splenic T cells from B10.K ($H\text{-}2^k$) mice; (●—) anti-Ia^k serum plus complement treated B10.S ($H\text{-}2^s$) cells; and (□—) anti-Ia^k serum plus complement treated B10.K cells. Each point represents the mean response of four cultures expressed as counts per minute ± SD.

DISCUSSION

These experiments have defined a subpopulation of Con-A reactive T lymphocytes which is Ia and Thy-1 positive and distinct from the subset of T cells which is PHA or leucoagglutinin reactive. The latter subpopulation is Thy-1 positive but is not susceptible to lysis by anti-Ia serum and complement. The demonstration of a functional class of Ia reactive T cells supports the findings of our previous cytotoxic and absorption studies indicating the existence of an Ia positive T cell population [10]. Demonstration of Ia positive thymocytes by other investigators using the fluorescence-activated cell sorter [18] and of Ia sensitive Con-A activated thymocyte and splenic blasts [16, 17] give additional support to these observations.

The fact that distinct subsets of T lymphocytes are specific for Con-A and PHA mitogens and that they also have differing Ia expression is consistent with a number of recent findings. For example, Cantor and Boyse [18, 19] and Kisielow *et al.* [20] have demonstrated that "helper" T cells are distinct from "killer" T cells and that "helper" cells express the Ly-1 antigen while "killer" cells express Ly-2,3. Graft-vs-host cells comprise yet another subpopulation and express Ly-1 and Ly-2 antigens.

Cytotoxic and absorption studies have indicated that the Ia antigens are expressed on the membranes of most B lymphoid cells and macrophages. It was, therefore, not surprising that the removal of the majority of the splenic B cells by anti-Ia serum and complement eliminated the response to LPS. More significant were the experiments designed to test if the simple binding of specific anti-Ia antibodies to the cell membrane would interfere with mitogen stimulation. These experiments were important for two reasons. First, it was recently shown that LPS is a potent polyclonal B cell activator nonspecifically inducing immunocompetent B cells to proliferate and/or secrete antibody [21, 22]. This cell triggering occurs without the involvement of the variable part of the Ig receptor and is not dependent upon accessory cells (for review, Ref. [23]). Second, we observed that covering of the Ia surface molecule(s) of spleen cells with specific anti-Ia antibodies inhibited the *in vitro* primary and secondary humoral response to heterologous erythrocytes [24]. This blocking of the humoral response was specific for anti-Ia serum with no inhibition occurring with anti-H-2K serum. These experiments implicate the involvement of the Ia determinant in cell triggering and/or cell collaboration in the immune response. The finding, therefore, that simple pretreatment of splenic B cells with anti-Ia serum without complement also suppressed the LPS proliferative response suggests the involvement of the Ia molecule in the events of B cell triggering. The fact that this suppression was only partial (~30%) necessitated the performance of a number of experiments to insure that this was a consistent observation.

It is unlikely that the observed blocking

was mediated by the Fc portion of the anti-Ia antibody molecule since antibodies specific for H-2K and H-2D determinants had no detectable effect. For the observed blocking with anti-Ia whole serum or with the IgG fraction of anti-Ia serum to occur via the Fc portion of the antibody, one must assume that the anti-Ia heavy chains have a unique constant region. This would be highly unlikely.

The inhibition was clearly a B cell specific event since it was not affected by elimination of splenic T cells. The most obvious interpretation is that the Ia molecule(s) and LPS receptors(s) are either the same molecule, or they are in the same supramolecular complex on the cell membrane. The fact that inhibition was only partial suggests, rather, that if LPS triggering is a simple switch event, then some cells have Ia and LPS receptors in close association on their membranes and are inhibited while other cells have non-Ia associated LPS receptors and are triggered. This could also imply the existence of at least two classes of LPS receptor molecules.

Andersson *et al.* [25] have observed that antibodies directed against the Ig receptors on B cells had no effect on the LPS proliferative response but did inhibit the LPS induction of polyclonal IgM synthesis. Furthermore, "capping" of surface Ig on B cells did not interfere with their proliferative response to LPS [26]. Therefore, it also will be important to test the ability of anti-Ia serum to inhibit polyclonal responses to LPS and other B cell mitogens.

The failure to block Con-A stimulation by the pretreatment of spleen cells with anti-Ia serum may simply reflect the fact that the receptors for this mitogen are quite distinct from surface Ia determinants. The absence of mannose, the membrane binding sugar for Con-A, in the Ia antigen structure also may be significant.

There is no question that the Ia molecules are distinct from classical transplantation antigens. They are found on molecules with a molecular weight of 25,000 to 33,000 daltons [11], are glycoproteins [27], and cap independently of the H-2K and H-2D transplantation antigens [28]. Recently it has been shown that anti-Ia sera react directly with both cell-bound and soluble molecules involved in a number of immune phenomena [29]. This surface marker and the antisera directed against the multiple specificities of this region, therefore, will be important tools in the further study of the events of antigen recognition, cell triggering, and cellular interaction in the immune response.

SUMMARY

Genes located in the *I* region of the H-2 complex control a system of lymphocyte alloantigens (Ia) which are expressed on subpopulations of T and B cells. Specific anti-Ia serum plus rabbit complement removed the B-lymphocyte population responsive to the mitogen LPS and the subpopulation of T cells responsive to Con-A. Lymphocytes sensitive to PHA or leucoagglutinin were not removed by anti-Ia serum and complement. Significant inhibition of the proliferative response to LPS was also obtained by brief periods of cell pretreatment with anti-Ia antibodies without complement. This inhibition was specific with the appropriate anti-Ia serum and did not occur with anti-H-2K sera or when cells of a different *I* region were pretreated.

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