

## EFFECTS OF ANTI-Ia SERA ON MITOGENIC RESPONSES

### II. Differential Expression of the Ia Marker on Phytohemagglutinin- and Concanavalin A-Reactive T Cells\*

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The major histocompatibility complex (H-2) of the mouse codes for two major classes of cell surface structures, the classical transplantation antigen of the *K* and *D* regions and the Ia antigens, which are products of genes mapping in the *I* (immune response) region (for review, reference 1). In contrast to the wide tissue distribution of the H-2K and H-2D major histocompatibility antigens, the Ia antigens have a restricted distribution with principal expression on subpopulations of T and B lymphocytes (2–8). The mapping of such important immune functions as mixed lymphocyte reaction, graft versus host reactivities, immune response control (*Ir* genes), and cell-cell interaction determinants to the *I* region has stimulated efforts to identify possible relationships between Ia determinants and these immune functions (9–14).

In recent experiments, we have been able to show inhibition of the primary and secondary *in vitro* responses to heterologous erythrocytes by a brief incubation of the spleen cells with anti-Ia sera in the absence of complement before culturing the cells with antigen (15). This inhibition was not observed with antisera against *H-2K* or *H-2D* determinants. These anti-Ia sera were also capable of significantly inhibiting the proliferative response of B lymphocytes to the mitogen lipopolysaccharide (LPS)<sup>1</sup> (16). Similar inhibition was not observed with anti-H-2K antibodies. Pretreatment of the B-lymphocyte population with anti-Ia serum and complement eliminated the LPS-responsive cells (16). In this report we have used appropriate anti-Ia sera to determine whether Concanavalin A (Con-A)- and Phytohemagglutinin (PHA)-sensitive T cells were contained in the subpopulation of Ia positive T cells.

#### Material and Methods

*Mice.* B10.Br (*H-2<sup>k</sup>*) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. All other mice were maintained in Dr. Niederhuber's and/or Dr. Shreffler's colony at The University of Michigan.

\* Supported by Damon Runyon DRG 1260, Michigan Kidney Foundation, U. S. Public Health Service Program Project 2-PO1-GM-15419-7, and National Institutes of Health Grants RO1-AI-11962-01 and RO1-AI-12153-01.

† Recipient of U. S. Public Health Service Research Career Development Award.

§ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

<sup>1</sup> *Abbreviations used in this paper:* Con-A, concanavalin A; LPS, lipopolysaccharide; PHA, phytohemagglutinin.

**Antisera.** Anti-Ia sera were prepared, as previously described, by reciprocal immunization of A.TH ( $H-2^a$ ) and A.TL ( $H-2^l$ ) mice (2). A.TH anti-A.TL ( $Ia^k$ ) and A.TL anti-A.TH ( $Ia^l$ ) sera have been extensively characterized by cytotoxic and absorption tests and are specific for *I*-region determinants (1). Batches of sera comprising several bleedings from a single series of immunizations consistently give a biphasic titration curve in the dye exclusion microcytotoxic test. The high plateau of 50–60% spleen cell lysis breaks to a lower plateau of 20–30% at a dilution of 1:160 to 1:320. The lower plateau persists to a titer of 1:1,280 to 1:2,560 (7). Anti-H-2 sera, specific for the H-2K<sup>b</sup> determinant, (H-2.19) were prepared in (A × A.AL)<sub>F1</sub> ( $H-2^a/H-2^l$ , Thy 1.2) mice against A.TL ( $H-2^l$ ) cells, and serum specific for H-2K<sup>b</sup> antigens (H-2.11 and H-2.23) was prepared in A.TL ( $H-2^l$ ) mice against A.AL ( $H-2^{al}$ ) cells. Anti-Thy 1.2 serum was prepared by immunizing mice of congenic strain A.AKR ( $H-2^{ak}$ , *Thy-1<sup>a</sup>*) with A.AL ( $H-2^{al}$ , *Thy-1<sup>b</sup>*) thymocytes.

**Culture Conditions.** Dispersed spleen cell suspensions were prepared in serum-free RPMI 1640 media (Microbiological Associates, Bethesda, Maryland) supplemented with 3 ml of HEPES (1 M) and 50 µg/ml of gentamycin per 100 ml. Con-A was purchased from Calbiochem, San Diego, Calif., and PHA-M was obtained from Difco Laboratories, Detroit, Mich. In some experiments, Leucoagglutinin, a highly purified form of PHA (Pharmacia Fine Chemicals, Uppsala, Sweden) was used to stimulate cultured cells.

Quadruplicate cultures of  $5 \times 10^5$  viable lymphoid cells/well were incubated in multiwell Linbro plates (Linbro Chemical Co., New Haven, Conn.) for 72 h. During the last 18 h of culture, 0.2 µCi of [<sup>3</sup>H]thymidine (2 Ci/mM) was present in the cultures. The 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-derived lymphocytes were prepared from whole spleen using nylon wool (LP-1 leukopak leukocyte filter, Fenwal Laboratories Inc., Morton Grove, Ill.), as described by Julius et al. (17). Such cells are 80–90% Thy-1 positive.

**Antiserum Treatment of Cells.** For blocking experiments, spleen cells were incubated with anti-Ia serum without complement, washed by centrifugation in media, and then cultured. In lysis experiments, the cells were incubated with appropriate antisera in a dilution of 1:5 or 1:10 at 37°C for 20 min, centrifuged, and resuspended in agarose and spleen cell-adsorbed EDTA-treated rabbit complement, diluted in RPMI 1640, and incubated for 30 min at 37°C. The cells were then washed by centrifugation in media, counted in a hemacytometer, and adjusted to  $5 \times 10^6$  viable cells/ml for distribution at a concentration of  $5 \times 10^5$  cells per culture. The final vol in each culture was 0.3 ml.

## Results

**Response of Spleen Cells Resistant to Treatment with Anti-Ia Serum and Complement.** When spleen cells from B10.BR ( $Ia^k$ ) mice were incubated with A.TH anti-A.TL (anti- $Ia^k$ ) serum and rabbit complement, approximately 60% of the cells were killed, as determined by trypan blue dye exclusion. Anti-Ia resistant spleen cells, at a concentration of cells equal to control cultures, responded normally to PHA-M and Leucoagglutinin, but the Con-A response was only 0–40% of control. The Con-A response was entirely absent in anti-Thy-1.2 resistant spleen cells (Table I, Fig. 1). In several experiments, the Con-A response was not completely eliminated, even though there was essentially no response by cells treated with anti-Thy-1.2 serum plus complement. This appeared to be the result of insufficient antibodies, since treating the cells a second time with anti-Ia serum and complement before culturing eliminated the population sensitive to Con-A.

Target cells of both  $H-2^k$  and  $H-2^s$  haplotypes were tested with the appropriate anti-Ia serum and rabbit complement with identical results (Table I, Fig. 2). In some experiments an inappropriate target cell was used as a control.

**Response of Nylon-Wool Purified Splenic T Cells Resistant to Anti-Ia Serum and Complement.** When splenic T cells were purified on nylon-wool columns,

TABLE I  
*Response after Treatment with Anti-Ia Serum and Complement*

Exp.	Mitogen	Target cells	Antiserum and complement	[ <sup>3</sup> H]TdR	Reduction
1.	None	B10.BR ( <i>H-2<sup>k</sup></i> ) Spleen cells	None	<i>cpm ± SD</i> 1,652 ± 231	%
	Con A	"	A.TH-normal serum	51,262 ± 5,630	66
	"	"	A.TH α A.TL ( <i>Ia<sup>k</sup></i> ) Anti-Thy 1.2	17,460 ± 2,230 2,940 ± 558	
2.	None	B10.BR ( <i>H-2<sup>k</sup></i> ) Spleen cells	None	718 ± 53	78
	Con A	"	A.TH-normal serum A.TH α A.TL ( <i>Ia<sup>k</sup></i> )	46,626 ± 2,164 10,328 ± 1,350	
3.	None	B10.S ( <i>H-2<sup>s</sup></i> ) Spleen cells	None	1,968 ± 243	93
	Con A	"	None	53,470 ± 6,400	
	"	"	A.TL-normal serum	30,126 ± 2,010	
	"	"	A.TL α A.TH ( <i>Ia<sup>s</sup></i> )	1,953 ± 65	
	Leucoagglutinin	"	None	29,129 ± 2,600	
4.	None	B10.S ( <i>H-2<sup>s</sup></i> ) Spleen cells	None	2,206 ± 759	90
	Con A	"	A.TL-normal serum A.TL α A.TH ( <i>Ia<sup>s</sup></i> )	15,151 ± 1,960 1,488 ± 146	
	Leucoagglutinin	"	A.TL α A.TH ( <i>Ia<sup>s</sup></i> )	28,025 ± 2,520	
	Leucoagglutinin	"	A.TL α A.TH ( <i>Ia<sup>s</sup></i> )	28,025 ± 2,520	
5.	None	B10.K ( <i>H-2<sup>k</sup></i> ) Nylon T cells	None	224 ± 192	92
	Con A	"	None	106,259 ± 4,490	
	"	"	A.TH α A.TL ( <i>Ia<sup>k</sup></i> )	8,662 ± 3,358	
	"	B10.S ( <i>H-2<sup>s</sup></i> ) Nylon T cells	A.TH α A.TL ( <i>Ia<sup>k</sup></i> )	156,572 ± 10,926	
	Leucoagglutinin	B10.K ( <i>H-2<sup>k</sup></i> ) Spleen	None	12,048 ± 1,564	
	"	B10.K ( <i>H-2<sup>k</sup></i> ) Nylon T	None	13,759 ± 2,566	
"	"	A.TH α A.TL ( <i>Ia<sup>k</sup></i> )	22,732 ± 2,562	0	

enrichment of Thy-1 positive cells from 30–40% to 80–90% was obtained. The nylon-wool purified T cells eluted with the first 15 ml of media were responsive to Con-A, PHA, and Leucoagglutinin. No LPS response was detected, indicating a low B-cell contamination in the purified cell suspensions.

Purified T cells from B10.K (*H-2<sup>k</sup>*) and B10.S (*H-2<sup>s</sup>*) mice were treated with A.TH anti-A.TL (anti-*Ia<sup>k</sup>*) serum and rabbit complement. The surviving, anti-*Ia* resistant cells were cultured for 72 h with Con-A and PHA. The anti-*Ia* resistant cell response to Con-A was decreased 10-fold compared to controls treated with normal A.TH serum (Fig. 3). No reduction of the Con-A response was observed in the control T-cell (B10.S) cultures (Table I). As previously described for the treatment of intact spleen cells, the response of the T cells to PHA was not

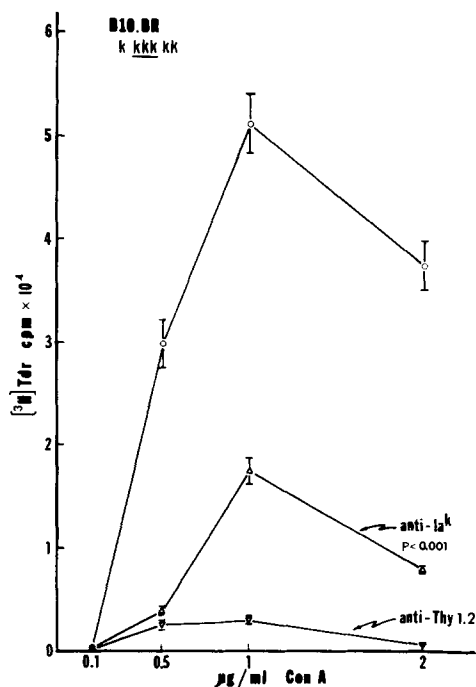


FIG. 1. Con-A-stimulated proliferative response of B10.BR ( $H-2^k$ ) spleen cells. The spleen cells were treated with either normal A.TH serum, A.TH anti-A.TL serum ( $Ia^k$ ), or anti-Thy-1.2 serum and rabbit complement. Equal numbers of viable cells ( $5 \times 10^5$ ) were cultured for 72 h with mitogen. The symbols represent: (○—○) normal A.TH serum, (△—△) A.TH anti-A.TL ( $Ia^k$ ) serum, and (▽—▽) anti-Thy-1.2 serum. Each point is the mean counts per minute of four cultures  $\pm$  SD.

altered. These experiments were also performed over a dose range of Con-A of 0.5–5  $\mu\text{g/ml}$  to exclude a shift in the kinetics of the response to Con-A in the  $Ia$ -resistant subpopulation.

These experiments have shown that the Con-A-reactive T-cell population is  $Ia$  positive and is a separate subpopulation from the PHA-responsive population, which is  $Ia$  negative. Target cells of both  $H-2^k$  and  $H-2^s$  gave identical results with appropriate anti- $Ia$  serum and complement.

**Pretreatment with Anti- $Ia$  Serum.** It was previously reported that pretreatment of splenic lymphoid cells with anti- $Ia$  serum without complement inhibited the in vitro humoral response to heterologous erythrocytes and the proliferative response to LPS (15, 16). Similar pretreatment of spleen cells with anti- $Ia$  serum without complement before stimulation with T-cell mitogens, Con-A, and PHA had no effect on their response (Fig. 4 a and b). The anti- $Ia$  serum used in these experiments was the A.TH anti-A.TL used above, which is known to have anti-T-cell cytotoxic activity (7). Such experiments were always performed with anti-H-2K and/or anti-H-2D sera, with normal serum, and with untreated cells as controls. This experiment was repeated four times with identical results. In addition to the PHA-M, Leucoagglutinin, a highly purified form of PHA, was tested with identical results.

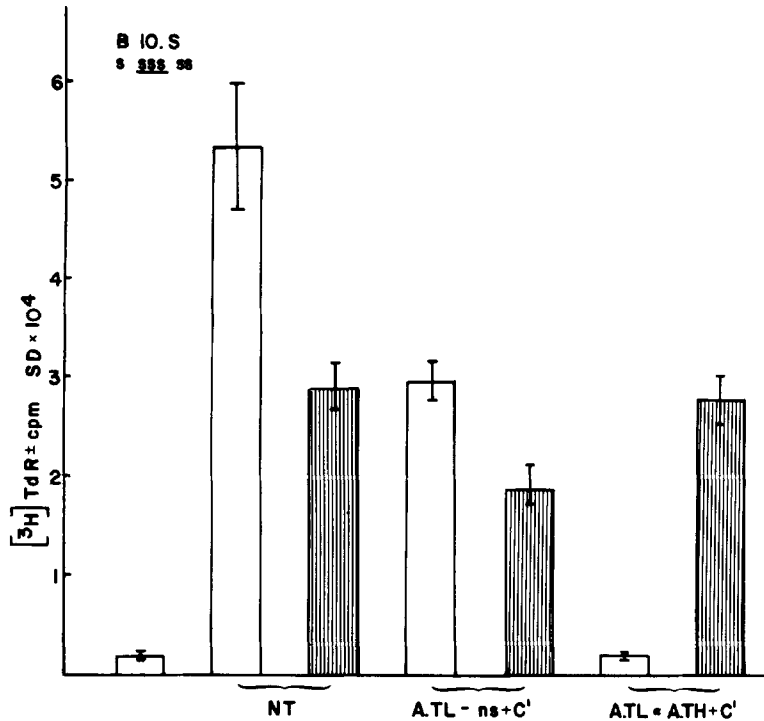


FIG. 2. Con-A and PHA (Leucoagglutinin) proliferative response of anti-Ia<sup>s</sup> resistant B10.S (H-2<sup>s</sup>) spleen cells. Spleen cells were either not treated with serum and complement indicated by NT, were treated with A.TL normal serum and complement indicated by A.TL-ns, or treated with A.TL α A.TH serum and complement indicated by A.TL α A.TH. Equal numbers of viable cells ( $5 \times 10^6$ ) were cultured for 72 h with either Con-A  $1 \mu\text{g/ml}$ , open bars, or Leucoagglutinin  $1 \mu\text{g/ml}$ , shaded bars. Each bar represents the mean response of four cultures expressed as counts per minute  $\pm$  SD. The open bar at the left of the graph is the background response in unstimulated cultures.

### Discussion

Although it has been accepted that Ia antigens are present on the membrane of most B-lymphoid cells, controversy has existed regarding their expression on T cells. We have previously reported the absorption of anti-Ia activity by thymocytes, direct cytotoxic reactivity with cortisone-resistant thymocytes, and levels of cytotoxicity with spleen and lymph node cells requiring lysis of at least a portion of T cells (7). Recently other investigators have reported evidence supporting the existence of an Ia-positive subpopulation of T cells. Most significant have been the demonstrations of Ia-positive thymocytes using the fluorescence-activated cell sorter (18), of Ia sensitive Con-A activated thymocyte and splenic blasts (19, 20), and of a characteristic Ia peak of 30,000 mol wt in the immunoprecipitation assay with thymus cells (B. Schwartz, personal communication).

With anti-Ia antibodies and complement it has been possible to eliminate the Con-A response of spleen cells and nylon-wood purified T cells. However, the ability to respond to another T-cell mitogen PHA and its more purified form, Leucoagglutinin, was not affected, indicating that PHA-sensitive and Con-A-

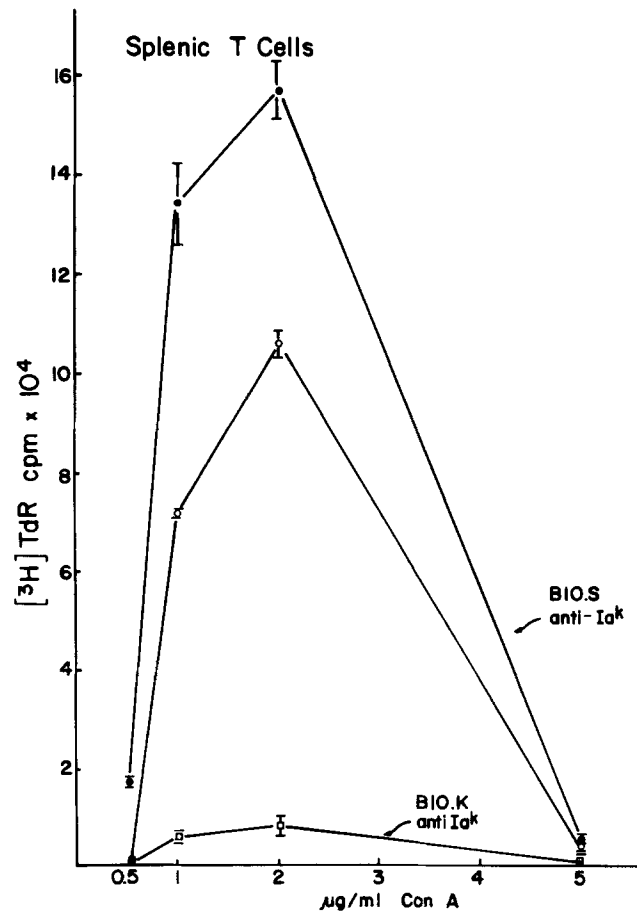


FIG. 3. Con-A-stimulated proliferative response of nylon-wool purified splenic T-cells. Splenic T-cells from B10.S ( $H-2^g$ ) or B10.K ( $H-2^k$ ) mice were treated with A.TH anti-A.TL serum ( $Ia^k$ ) and complement. Equal numbers of viable cells ( $5 \times 10^5$ ) were cultured for 72 h with mitogen. The symbols represent: (○—○) nontreated splenic T-cells, (●—●) anti- $Ia^k$  serum treated B10.S cells, and (□—□) anti- $Ia^k$  serum treated B10.K cells. Each point is the mean counts per minute of four cultures  $\pm$  SD.

sensitive T cells are independent subpopulations with differential expression of Ia antigens. The removal of Con-A-sensitive cells by anti-Ia serum and complement was more efficient when nylon-wool-purified T cells were used. The need to increase the antibody concentration or to treat spleen cells a second time with anti-Ia serum and complement may reflect a lower density of the Ia antigens relative to other surface markers such as H-2 and Thy-1 on the T-cell membrane.

The failure to block Con-A or PHA stimulation by simply incubating the spleen cells with anti-Ia antibodies is in contrast to the partial but significant inhibition of the proliferative response to several B-cell mitogens including LPS (16). The absence of mannose, the membrane binding sugar for Con-A, in the Ia antigen molecule may be significant to this observation. We interpret this lack of blocking to indicate that the receptors for these mitogens are distinct from Ia determinants on the cells membrane.

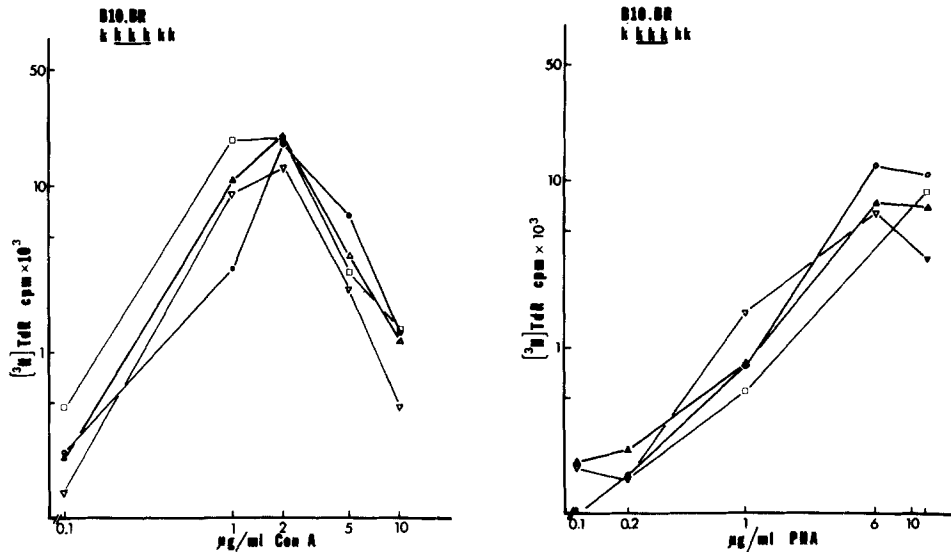


FIG. 4. (a) Con-A proliferative response of B10.BR spleen cells at concentrations of 0.1–10  $\mu\text{g/ml}$ . The cells were pretreated with anti-Ia serum for 30 min, washed  $\times 2$ , and cultured for 72 h with Con-A. The symbols represent the antiserum pretreatment: (O—O) no serum, (□—□) anti-Ia<sup>k</sup>, (∇—∇) anti-Ia<sup>s</sup>, and (Δ—Δ) anti-D<sup>d</sup>. Each point is the mean of four cultures. (b) PHA proliferative response of B10.BR spleen cells at concentrations of 0.1–12  $\mu\text{g/ml}$ . The cells were pretreated with anti-Ia serum for 30 min, washed  $\times 2$ , and cultured for 72 h with PHA. The symbols represent the antiserum pretreatment: (O—O) no serum, (□—□) anti-Ia<sup>k</sup>, (∇—∇) anti-Ia<sup>s</sup>, and (Δ—Δ) anti-D<sup>d</sup>. Each point is the mean of four cultures.

The removal of a subset of T cells which are sensitive to Con-A and distinct from PHA reactive Ia-negative cells is consistent with the findings of other investigators concerning the existence of discrete T-cell subsets and the association of Ia determinants and/or Ia-positive T cells with certain T-cell functions. For example, Cantor and Boyse (21, 22) and Kisielow et al. (23) have demonstrated that "helper" T cells express LY-1, but not LY-23, which is expressed on "killer" T cells. Graft vs. host reactive cells, however, carry both LY-1 and LY-2 surface markers, suggesting the existence of at least three T-cell subpopulations. There is also good evidence that the Ia antigens stimulate in mixed lymphocyte reaction, and that the stimulating cell, but not the responding cell, can be blocked by anti-Ia sera (24). Lonai has been able to remove this stimulating T cell by treatment with anti-Ia serum and complement (25). Of interest, too, is the finding that the activity of an antigen-specific helper cell replacing factor and an antigen-specific suppressor factor prepared from primed thymocytes can be removed by an Ia immunoabsorbent column (26–28).

Recently we have found that we can inhibit the generation of Con-A-induced nonspecific suppressor T cells by first treating spleen cells with anti-Ia and complement before culturing with Con-A (Niederhuber, unpublished data). Once generated, the suppressor cells, however, are not sensitive to anti-Ia and complement treatment. This is consistent with the data presented here and

suggests that T cells may express Ia only at certain times in their particular course of differentiation. David et al. have examined the Ia sensitivity of Con-A-activated thymocytes (19) and find these cells to be easily lysed with anti-Ia serum and complement when examined in dye exclusion and  $^{51}\text{Cr}$  cytotoxic tests. In contrast, Hauptfeld et al. have found only marginal anti-Ia serum cytotoxicity of Con-A-activated splenic lymphocytes, suggesting a difference in the expression of Ia by thymus cells and peripheral T cells (20).

We have previously observed that when nylon-wool-purified splenic T cells were subjected to treatment with anti-Ia serum and complement in a dye exclusion microcytotoxic assay, negligible killing was observed (7). The ability to treat large numbers of such T cells with anti-Ia serum and complement to eliminate a functional property, such as the response to Con-A, would seem to be in direct conflict to these earlier cytotoxic observations. It is possible that although the cells are not staining as dead cells in the cytotoxic assay, the cell membranes may be significantly damaged so that once the cells are cultured, they cannot respond normally and so are functionally killed. The second possibility is that while antibody alone is not sufficient to block the Con-A binding site, antibody-complement complexes on the membrane might be sufficient blockers. Alternatively, it is possible that only a small number of cells are actually activated by Con-A, and these cells recruit a large number of bystanding T cells to join in blast transformation and proliferation. Thus, a small number of sensitive cells could easily be killed by the anti-Ia serum plus complement treatment and not be detected, and in the absence of these Con-A sensitive cells, the cells normally recruited would not be induced to proliferate. Experiments are currently in progress to examine this possibility.

Although these experiments were performed with antibodies directed at multiple specificities within the entire *I* region, they clearly show that *I*-region products are expressed on T cells, and that a pure T-cell function can be eliminated by using anti-Ia sera and complement to lyse Ia-positive cells. Preliminary data using antisera of restricted specificity suggest that not all *I* subregions code for antigens expressed on Con-A-reactive T cells (Niederhuber, unpublished).

#### Summary

Genes mapping in the *I* region of the *H-2* complex control a system of lymphocyte alloantigens (Ia) which are expressed on a subpopulation of T cells and on most B cells. Specific anti-Ia serum in the presence of rabbit complement removed the splenic T-cell subpopulation responsive to Con-A, but did not affect the response to phytohemagglutinin (PHA) or Leucoagglutinin. Antibodies specific for Ia, H-2K, or H-2D membrane antigens were used without complement to pretreat spleen cells. These antibody pretreated cells responded normally to Con-A and PHA.

We thank Laura Mayo, Carolyn Rosio, and Deirdre Smith for their excellent technical assistance and Nancy Cuthbert for her secretarial assistance.

*Received for publication 16 October 1975.*



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