ANTIGEN PRESENTATION IN THE MURINE T-LYMPHOCYTE PROLIFERATIVE RESPONSE

I. Requirement for Genetic Identity at the Major Histocompatibility Complex

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The importance of the macrophage in the initiation of the immune response to soluble protein antigens has been demonstrated by several investigators (1–5). One of the clearest examples of this is in the guinea pig where the activation of proliferative responses by antigen-primed, thymus-dependent (T) lymphocytes has been shown to depend upon the presence of an auxilliary cell with the characteristics of a macrophage (1, 2). In this system, the critical function of the live macrophages appears to be the presentation of antigen. One of the striking features of this presentation is that it is only efficient when the antigen-presenting cell and the responding cell possess a common allelic form of the guinea pig equivalent of *I* subregions of the major histocompatibility complex (MHC)[1] (1, 2). A similar *I*-region histocompatibility restriction in the activation of T lymphocytes in the mouse has been suggested by studies of the transfer of delayed hypersensitivity (3) and of the priming of helper T lymphocytes (4, 5). In this communication, we directly demonstrate that the activation of proliferative responses of primed mouse peritoneal exudate T lymphocytes by antigen-pulsed spleen cells is histocompatibility restricted. Efficient activation of T lymphocytes from donors primed to 2,4-dinitrophenyl-ovalbumin (DNP-OVA) occurs only when the interacting cells possess a common allelic form of the MHC. In particular, identity at the *I-A* subregion of the MHC, in the face of differences in all other MHC regions, is sufficient for such activation.

Materials and Methods

Animals. Strains C57BL/10 Sn (B10), B10.A/SgSn (B10.A), B10.D2/nSn (B10.D2), B10.A(5R)/SgSn, B10.BR/SgSn, C57BL/6J (B6), A/HeJ (A), (B6 × A)F_1/J hybrids (B6A F_1), SJL/J, and DBA/2J were obtained from The Jackson Laboratory, Bar Harbor, Maine. A.TL/Sf mice were the progeny of breeding pairs kindly provided by Dr. Donald Shreffler and Dr. Chella David, Washington University, School of Medicine, St. Louis, Mo. Breeding pairs of the B10.A(4R)/Sg strain were kindly provided by Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Mont. BALB/c AnN mice were obtained from the Division of Research Services of the National Institutes

[1] *Abbreviations used in this paper:* DH, delayed-type hypersensitivity; DNP-OVA; 2,4-dinitrophenyl-ovalbumin; FCS, fetal calf serum; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PETLES, peritoneal exudate T-lymphocyte-enriched cells; PFC, plaque-forming cell.

of Health, Bethesda, Md. The (B10 × B10.D2)F_1 hybrids were bred in the Division of Research Services from the parental strains, C57BL/10SnN and B10.D2/nSnN. The (B10 × B10.A)F_1 hybrids were bred in our laboratory from the Jackson parental strains B10 and B10.A. Mice of both sexes were used between 6 and 18 wk of age.

Antigens. DNP derivatives of OVA were prepared by reacting 200 mg of twice recrystallized OVA (Mann Research Laboratories, New York) with 10 μl of 2,4-dinitrofluorobenzene (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in 5 ml of borate buffer, pH 8.0. The reaction was carried out in the dark for 24 h with constant stirring at room temperature. The supernate was dialyzed against phosphate-buffered saline, pH 7.2 and any precipitated protein removed by centrifugation (2,000 rpm × 10 min). These conjugates contained ~6 mol DNP/mol of protein and are designated DNP_6OVA.

Immunization. DNP_6OVA was emulsified in complete Freund's adjuvant containing 1 mg/ml of killed *Mycobacterium tuberculosis* (H37Ra) organisms (Difco Laboratories, Detroit, Mich.). Animals were immunized in the hind foot pads with 10 μg of antigen in a total of 0.1 ml of emulsion.

Preparation of Peritoneal Exudate T-Lymphocyte-Enriched Cells (PETLES). The original and slightly modified procedures for preparing PETLES are described in detail elsewhere (6–8). Briefly, 2–3 wk after immunization, thioglycollate-induced peritoneal exudate cells were harvested and passed over nylon wool columns. The PETLES population eluted from nylon columns contained an average of 5% macrophages, 40% lymphocytes, 53% eosinophils, and <2% B lymphocytes.

Preparation of Spleen Cells. Animals were sacrificed by cervical dislocation and the spleens removed aseptically. Spleen cells were flushed out of the capsule using a 21 gauge needle and a 12 ml syringe filled with $KHCO_3$-buffered NH_4Cl (9). The spleen was squeezed gently with a pair of forceps during the flushing to insure maximum release of cells; the erythrocytes were lysed during this procedure. The cell suspension was passed through a wire screen (no. 180 mesh) and washed once with Spinner's modified Eagle's minimal essential medium. The remaining nucleated cells were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N. Y.), 50 U/ml of penicillin G, 250 μg/ml of gentamicin (Schering Corp., Kenilworth, N. J.), and 2×10^{-5} M 2-mercaptoethanol. The cells were counted and kept on ice until ready for use. Although other tissues such as nonimmune PETLES and peritoneal exudate cells were used as sources of antigen-presenting cells, spleen cells were utilized in most of the experiments described in this paper because they presented DNP_6OVA well and were the easiest to prepare.

Procedure of Brief Antigen Pulsing. The spleen cells (10×10^6/ml) were incubated for 60 min at 37°C in RPMI-1640 with 10% FCS, containing appropriate concentrations of DNP_6OVA and 50 μg/ml of mitomycin-C (Sigma Chemical Co., St. Louis, Mo.). The cells were washed five times at 4°C with 1 ml of RPMI-1640 to remove unbound antigen and mitomycin-C, and resuspended in culture medium. These conditions for antigen pulsing were modeled on those previously described for guinea pig peritoneal exudate cells (10, 11). The cells were recounted and adjusted to appropriate concentrations for addition to the cell cultures. In some experiments, DNP_6OVA-pulsed or nonpulsed spleen cells were killed by incubating at 56°C for 60 min and then cooled to 37°C before mixing with PETLES.

Cell Cultures. The procedure for culturing PETLES has been described elsewhere (6, 7). Briefly, 100 μl of a 10% FCS-supplemented modified Eagle's-Hanks' medium (modified EHAA) containing $1–2 \times 10^5$ PETLES were placed in each well of a sterile, U bottom polystyrene, microculture plate (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). DNP_6OVA-pulsed or nonpulsed spleen cell suspensions were added in another 100 μl to give a total vol of 200 μl. As a control, soluble DNP_6OVA was added to some wells to give a total vol of 200 μl and a final free antigen concentration of 100 μg/ml. The cultures were incubated for 3–6 days at 37°C in a humidified atmosphere of 3% Co_2 and 97% air. Approximately 16–18 h before harvesting, the cultures were pulsed with 1 μCi of tritiated-methyl-thymidine (sp act 5 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). The cells were collected onto glass fiber filter paper strips (no. 934 AH, Whatman Inc., Clifton, N. J.) with a Mash II automated harvester (Microbiological Associates, Rockville, Md.), and washed with distilled water and 95% ethanol. The filter disk containing each sample was then placed in 2 ml of Hydromix scintillation fluid (Yorktown Research Inc., Hackensack, N. J.) and the radioactivity measured in a Beckman liquid scintillation

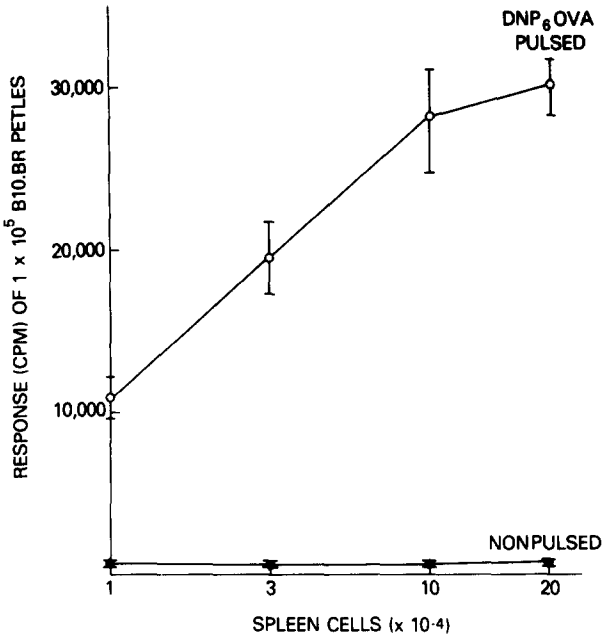

FIG. 1. Proliferative response (cpm ± SEM) of 1 × 10^5 B10.BR PETLES to varying numbers of syngeneic, mitomycin-C-treated spleen cells, pulsed (○—○), or not pulsed (●—●) with 30 μg/ml DNP_6OVA.

counter (Beckman Instruments Inc., Fullerton, Calif.). Determinations were done in triplicate or duplicate and the data are expressed either as mean counts per minute ± the standard error of the mean or as the difference between antigen-pulsed and antigen-nonpulsed responses.

Results

The Response of PETLES to Antigen Bound to Spleen Cells. PETLES represent a unique population of primed T lymphocytes in the mouse which is highly enriched for cells that can proliferate in culture in response to the continuous presence of the priming antigen (6–8). In the experiments to be described here, we investigated the parameters of PETLES stimulation when antigen was presented to the T lymphocytes in a cell-bound rather than a soluble form. This was achieved by exposing spleen cells from nonimmunized donors to antigen for 1 h at 37°C and then washing away unbound antigen. At the same time, the spleen cells were inactivated with mitomycin-C so that only the PETLES could incorporate ^{3}H-thymidine. The response of 1 × 10^5 B10.BR PETLES to DNP_6OVA bound to varying numbers of B10.BR spleen cells is shown in Fig. 1. The data indicate that a log linear relationship exists between the number of DNP_6OVA-pulsed spleen cells added and resultant DNA synthesis in the PETLES at a ratio of spleen cells to PETLES of 1:1 or less. The maximum proliferative response was achieved with 1–2 × 10^5 spleen cells. Larger numbers of spleen cells gave no greater response and in fact were often suppressive (data not shown).

The amount of antigen introduced into the system could also be varied by altering the concentration of antigen used to pulse the spleen cells. Fig. 2 shows

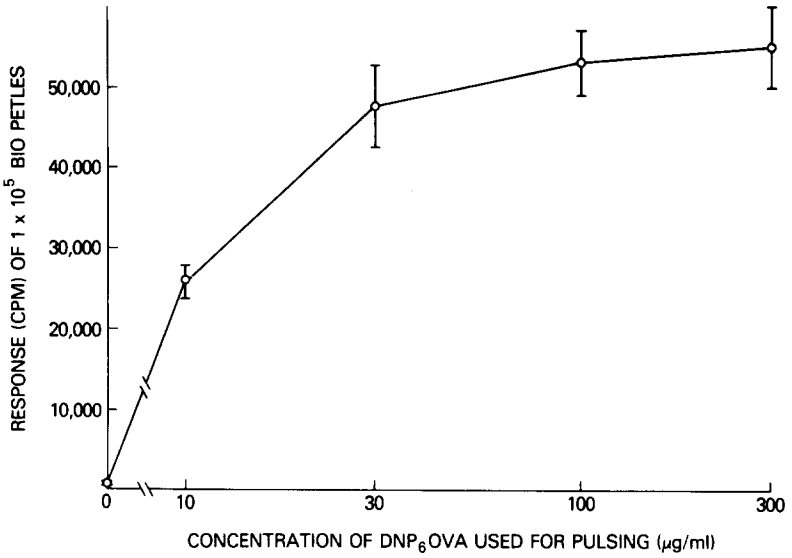

FIG. 2. Responses of 1×10^5 B10 PETLES to 1×10^5 syngeneic spleen cells that were pulsed with varying concentrations of DNP_6OVA. Results are expressed as cpm ± SEM. Each vertical bar represents the upper and lower limits of one SEM.

the response of PETLES to 1×10^5 syngeneic B10 spleen cells that had been exposed to various concentrations of DNP_6OVA. Thymidine incorporation into new DNA increased as the concentration of antigen increased, up to a dose of 100 μg/ml. Based on these results, all subsequent experiments employed 1×10^5 PETLES mixed with 1×10^5 spleen cells pulsed with 30–100 μg/ml of DNP_6OVA.

The activation of primed PETLES by antigen-pulsed spleen cells, under these conditions, appears to reflect an antigen-presentation function of the spleen cells and is not due to simple release of soluble antigen by these cells. This is illustrated both by the data presented below that a histocompatibility restriction exists in the capacity of spleen cells to activate primed PETLES and by the finding that antigen-pulsed spleen cells which have been washed and heat-treated before mixing with PETLES cause little or no stimulation in most experiments (Fig. 3). In 28 of the 38 experiments shown, stimulation by heat-killed spleen cells was 15% or less of the stimulation achieved with antigen-pulsed live cells. There were some experiments, however, which did show significant stimulation by heat-killed cells. In general, the data was disregarded from any experiment in which antigen-pulsed, heat-killed cells stimulated 15% or more of the response stimulated by live antigen-pulsed cells, because of the likelihood that soluble antigen might be carried over into such cultures.

Antigen Presentation by Syngeneic, Semisyngeneic, or Allogeneic Spleen Cells. Previous studies in the guinea pig (1, 2) have demonstrated the requirement for genetic identity at the MHC in order for peritoneal exudate cells to present antigen effectively to primed T lymphocytes. Experiments designed to test whether similar genetic restrictions applied to antigen presentation by murine spleen cells are shown in Table I. The experiments were set up in a crisscross fashion so that each presenting cell population was tested against both

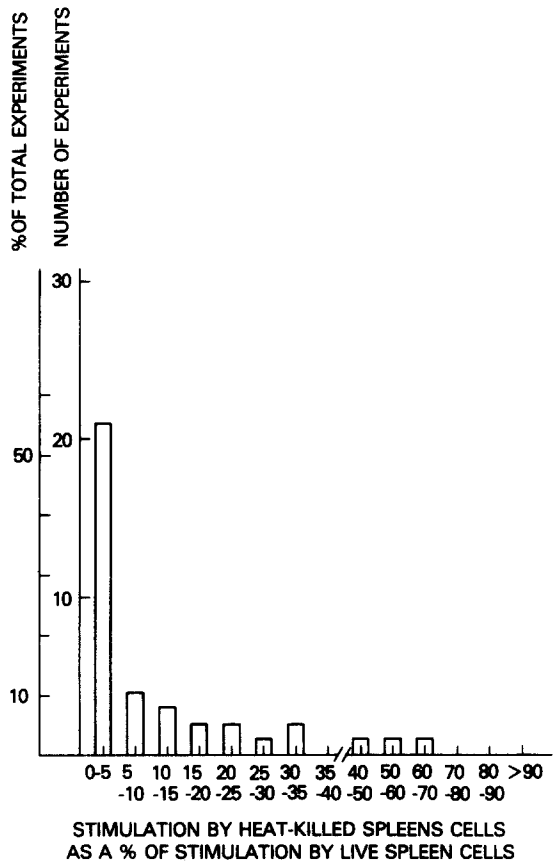

FIG. 3. A frequency histogram of 38 experiments in which spleen cells were pulsed with 10–100 μg/ml of DNP_6OVA and either added to syngeneic PETLES directly or heated to 56°C for 60 min, cooled to 37°C, and then added to the PETLES. The resulting proliferative responses are compared here by expressing the response to the heat-killed cells as a percentage of the response to the live cells.

syngeneic and allogeneic T lymphocytes. As shown in Table I, when PETLES from either B10 or B10.D2 mice primed to DNP_6OVA were stimulated in vitro with DNP_6OVA bound to spleen cells, use of syngeneic cells always resulted in greater antigen-specific proliferation (Δ cpm antigen), than use of allogeneic cells. Thus, antigen-pulsed B10 spleen cells presented well to syngeneic B10 PETLES (Δ cpm 30,000) but poorly to allogeneic B10.D2 PETLES (Δ cpm 1,400) while antigen-pulsed B10.D2 spleen cells presented well to syngeneic B10.D2 PETLES (Δ cpm 19,600) but poorly to allogeneic B10 PETLES (Δ cpm 2,200). Semisyngeneic antigen-pulsed (B10 × B10.D2)F_1 spleen cells gave intermediate levels of stimulation for both types of responding T cells (Δ cpm of 14,700 for B10 and 9,000 for B10.D2).

Stimulation of T-lymphocyte proliferation by antigen-pulsed allogeneic spleen cells was a strong function of the concentration of antigen used to pulse such cells. As shown in Fig. 4, stimulation of BALB/c PETLES by antigen-pulsed syngeneic spleen cells peaked when the spleen cells had been pulsed with 100

TABLE I
Antigen Presentation by Syngeneic, Semisyngeneic, or Allogeneic Spleen Cells

PETLES	Spleen cells	DNP_6OVA	Proliferative response on day 4		
			cpm ± SEM	Δ cpm (DNP_6-OVA)*	Δ cpm (MLR)‡
B10§	B10	Nonpulsed	1,600 ± 300		–
		Pulsed	31,600 ± 100	30,000	
	(B10 × B10.D2)F_1	Nonpulsed	13,600 ± 1,200		12,000
		Pulsed	28,300 ± 1,400	14,700	
	B10.D2	Nonpulsed	21,500 ± 100		19,900
		Pulsed	23,700 ± 4,400	2,200	
B10.D2§	B10.D2	Nonpulsed	2,600 ± 800		–
		Pulsed	22,100 ± 2,300	19,600	
	(B10 × B10.D2)F_1	Nonpulsed	29,900 ± 1,000		27,300
		Pulsed	38,900 ± 100	9,000	
	B10	Nonpulsed	26,400 ± 2,300		23,800
		Pulsed	27,800 ± 2,100	1,400	

* Δ cpm (DNP_6OVA); cpm obtained with antigen-pulsed presenting cells minus cpm obtained with nonpulsed presenting cells.

‡ Δ cpm (MLR); cpm obtained with nonpulsed allogeneic presenting cells minus cpm obtained with nonpulsed syngeneic presenting cells.

§ 1.5×10^5 DNP_6OVA-primed B10 and B10.D2 PETLES were cultured with 1×10^5 DNP_6OVA-pulsed or nonpulsed syngeneic, semisyngeneic, or allogeneic spleen cells for 4 days in microtiter plates.

μg/ml, whereas stimulation by antigen-pulsed allogeneic B10 spleen cells was still increasing at a pulsing concentration of 300 μg/ml. We suspect that the stimulation caused by allogeneic spleen cells pulsed with high concentrations of DNP_6OVA reflects "carryover" of antigen, a phenomenon which would tend to obscure the genetic restrictions present in the system. In order to minimize this putative antigen carryover and to maximize the genetic differences, most experiments were carried out using 30-50 μg/ml of antigen for pulsing.

The differences between syngeneic, allogeneic, and semisyngeneic presenting cells appeared not to be caused by differences in the time-course of the proliferative responses they stimulated. As shown in Fig. 5, the responses for all three types of cells peaked after 4–5 days of in vitro incubation, similar to what has been reported for cultures containing free antigen present continuously (6). In addition, the hierarchy of presenting activity (syngeneic better than semisyngeneic better than allogeneic) was observed at all time points from day 3 to day 6.

One might explain the genetic restrictions seen in this system by postulating a suppressive effect in cultures with an ongoing mixed lymphocyte reaction (MLR). Because only the allogeneic and semiallogeneic cells generate a MLR (Table I), cultures containing them would be the ones that would be suppressed. The finding that antigen-presenting F_1 cells were superior to allogeneic cells

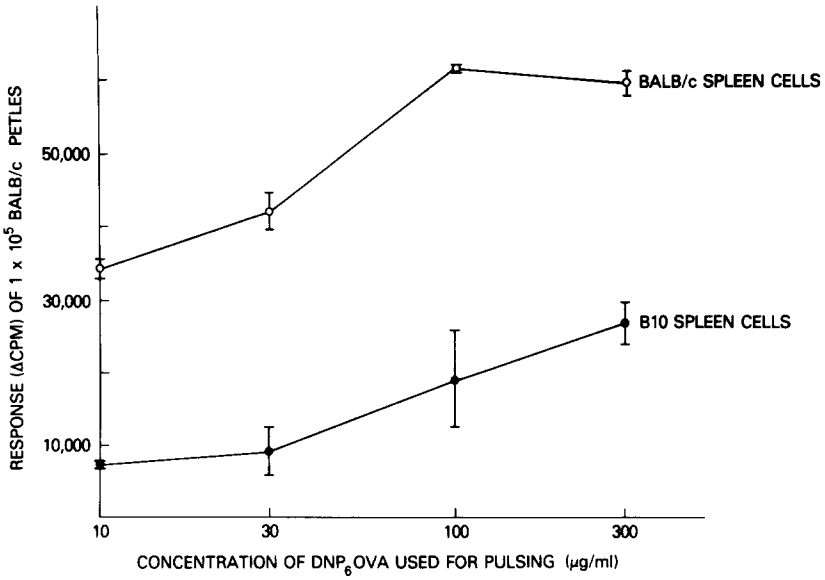

FIG. 4. Response of 1×10^5 BALB/c PETLES to varying concentrations of DNP_6OVA-pulsed BALB/c spleen cells (○—○) or DNP_6OVA-pulsed B10 spleen cells (●—●). The results are expressed as Δ cpm ± SEM.

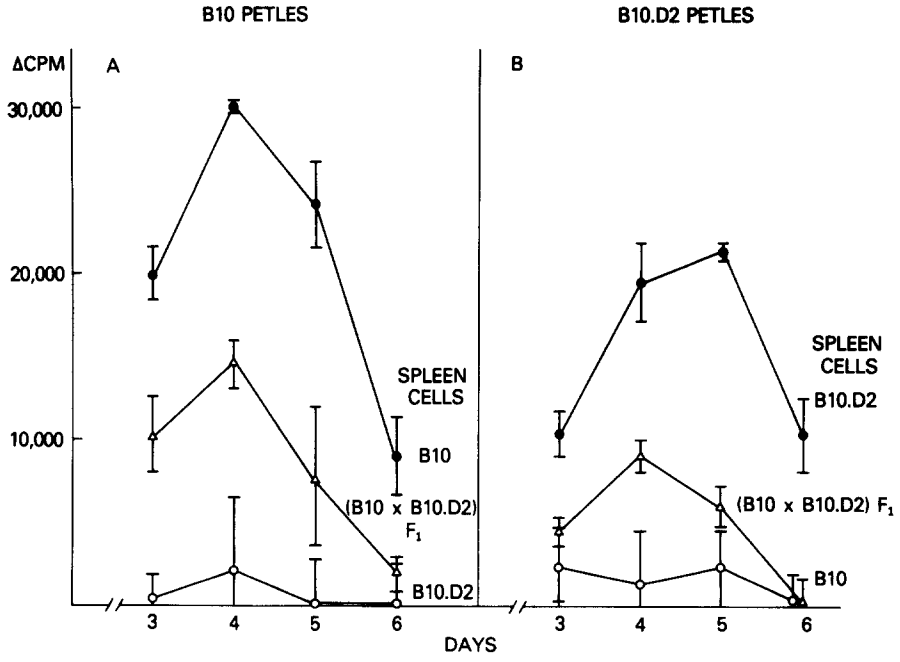

FIG. 5. Kinetics of antigen presentation by syngeneic (●—●), semisyngeneic (△—△), or allogeneic (○—○) spleen cells to 1×10^5 B10 PETLES (A) or 1×10^5 B10.D2 PETLES (B). The results are expressed as Δ cpm ± SEM.

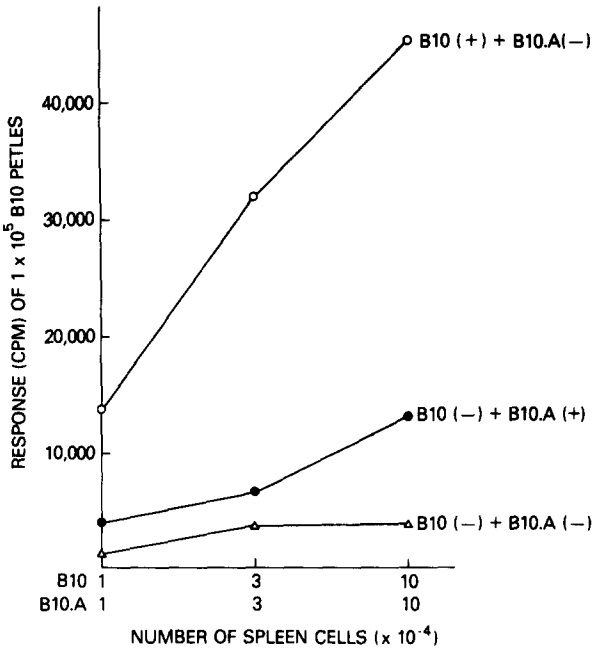

FIG. 6. Syngeneic cells present antigen better than allogeneic cells in the presence of the same MLR. 1×10^5 DNP_6OVA-primed B10 PETLES were cultured with mixtures of equal numbers of DNP_6OVA-pulsed B10 spleen cells [B10(+)] and nonpulsed B10.A spleen cells [B10.A(−)] (○—○), nonpulsed B10 spleen cells [B10(−)] and DNP_6OVA-pulsed B10.A spleen cells [B10.A(+)] (●—●), or nonpulsed B10 spleen cells and nonpulsed B10.A spleen cells (△—△). Stimulation was assessed 5 days later by measuring the incorporation of a 16 h pulse of tritiated methyl-thymidine. The results are expressed as cpm.

could be accounted for by a less vigorous suppressive effect because they possess only 1/2 the number of MLR-stimulating determinants. Although this last point was not always supported by the data, experiments were set up to test directly the possibility that a suppressive effect of an ongoing MLR accounted for the genetic restrictions which we observed. These experiments involved mixing equal numbers of syngeneic and allogeneic spleen cells or equal numbers of syngeneic and semisyngeneic spleen cells and comparing the ability of these mixtures to stimulate PETLES when antigen was bound to one or the other type of spleen cell. Thus, in this form of experiment, an identical MLR existed for both types of antigen presentation. As shown in Figs. 6 and 7, the same patterns of stimulation were observed, namely syngeneic cells presented better than allogeneic cells (Fig. 6) or semisyngeneic cells (Fig. 7). Therefore, we conclude that MLR suppression cannot account for the genetic differences observed in the ability of antigen-pulsed spleen cells to activate PETLES from primed donors.

An Mls Disparity between MHC Compatible Strains does not Inhibit Antigen Presentation. The results presented in Figs. 6 and 7 demonstrate that a MLR induced by *H-2*-incompatible spleen cells does not suppress antigen presentation by spleen cells syngeneic to the responding PETLES population. The conclusion suggested by these results, that MLRs per se are not suppressive for antigen presentation, was supported by further experiments in which Mls-incompatible,

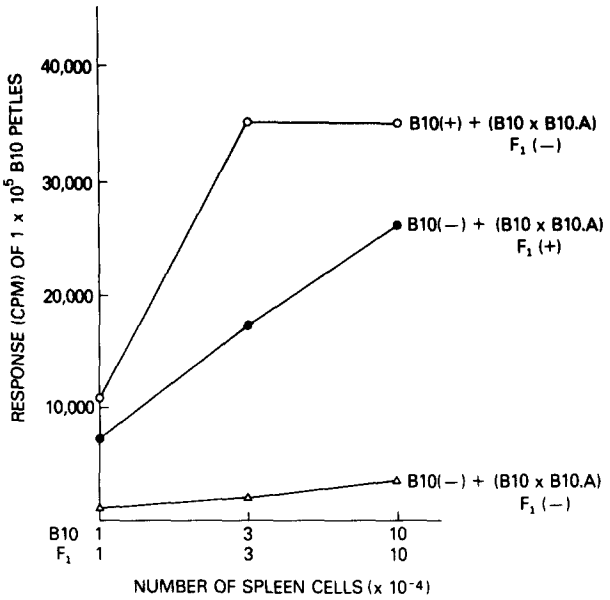

FIG. 7. Syngeneic spleen cells present antigen better than semisyngeneic spleen cells in the presence of the same MLR. 1×10^5 DNP_6OVA-primed B10 PETLES were cultured with mixtures of equal numbers of DNP_6OVA-pulsed B10 spleen cells [B10(+)] and nonpulsed (B10 × B10.A)F_1 spleen cells [F_1(−)] (○—○), nonpulsed B10 spleen cells [B10(−)] and DNP_6OVA-pulsed F_1 spleen cells [F_1(+)] (●—●), or nonpulsed B10 spleen cells and nonpulsed F_1 spleen cells (△—△). Stimulation was assessed 5 days later by measuring the incorporation of a 16 h pulse of tritiated methyl-thymidine. The results are expressed as cpm.

MHC-identical spleen cells were used to present antigen (Table II). When BALB/c PETLES (*H-2*^{d}, *Mls*^{b}) primed to DNP_6OVA were stimulated with DNP_6OVA-pulsed DBA/2 spleen cells (*H-2*^{d}, *Mls*^{a}), the antigen-specific proliferative response achieved was almost as great as that obtained when syngeneic BALB/c spleen cells were used for antigen presentation, despite the large MLR. B10.D2 spleen cells (*H-2*^{d}, *Mls*^{b}), which are identical to BALB/c PETLES at both the *H-2* and *Mls* loci but different at many other loci, also presented DNP_6OVA. In contrast, B10 spleen cells (*H-2*^{b}, *Mls*^{b}), which are compatible at the *Mls* locus but incompatible at the MHC, did not present DNP_6OVA effectively. Note that the magnitudes of the two types of MLR were similar (Δ cpm 19,900 for the MHC incompatibility and Δ cpm 27,300 for the Mls incompatibility), yet antigen presentation occurred only in the case of MHC compatibility. These results and those of the previous section are interpreted as indicating that a MLR, in and of itself, does not suppress nor enhance the ability of spleen cells to present antigen to T lymphocytes. The failure of MHC allogeneic spleen cells to present antigen well is, therefore, interpreted as a requirement for genetic identity at a MHC locus in order for effective interaction between antigen-pulsed spleen cells and primed PETLES to occur.

Genetic Mapping of the MHC Genes Required for Presentation of DNP_6OVA. In order to determine at which MHC loci common alleles were necessary for effective interactions between primed T lymphocytes and antigen-

TABLE II
Mls Incompatibility does not Affect Antigen Presentation

Spleen Cells*	*Mls*	*H-2*	DNP_6OVA	cpm ± SEM	Δ cpm (DNP_6-OVA)‡	Δ cpm (MLR)§
BALB/c	*b*	*d*	Nonpulsed	1,300 ± 200		–
			Pulsed	47,000 ± 1,900	45,700	
DBA/2	*a*	*d*	Nonpulsed	28,600 ± 400		27,300
			Pulsed	63,900 ± 4,400	35,300	
B10.D2	*b*	*d*	Nonpulsed	2,700 ± 700		1,400
			Pulsed	59,500 ± 500	56,800	
B10	*b*	*b*	Nonpulsed	21,200 ± 1,800		19,900
			Pulsed	25,100 ± 1,200	3,900	

* 1×10^5 DNP_6OVA-primed BALB/c PETLES were cultured with 1×10^5 DNP_6OVA-pulsed or nonpulsed BALB/c, DBA/2, B10.D2, or B10 spleen cells for 5 days in microtiter plates.
‡ See footnote (*) to Table I.
§ See footnote (‡) to Table I.

pulsed spleen cells to occur, the congenic resistant lines B10 and B10.A and their associated recombinant strains, B10.A(4R) (*kkbbbbbbb*)[2] and B10.A(5R) (*bbbkkdddd*) were utilized as well as the recombinant strain A.TL (*skkkkkkkd*). B10 and B10.A PETLES obtained from mice primed to DNP_6OVA, were cultured with antigen-pulsed or nonpulsed spleen cells from B10, B10.A, B10.A(4R), B10.A(5R), or A.TL mice. As shown in Table III, DNP_6OVA-pulsed B10 spleen cells stimulated the proliferation of B10 PETLES (Δ cpm 20,100), while these same spleen cells were ineffective in stimulating B10.A PETLES (Δ cpm 1,000). DNP_6OVA-pulsed B10.A spleen cells gave opposite results; they stimulated B10.A PETLES (Δ cpm 11,100) more effectively than B10 PETLES (Δ cpm 3,100). The B10.A(5R) recombinant spleen cells behaved like B10 spleen cells in that they stimulated B10 PETLES (Δ cpm 12,400) more effectively than they stimulated B10.A PETLES (Δ cpm 1,600). As summarized in Table V, this result suggests that genetic identity at the *K* region or the *I-A* or *I-B* subregion is necessary to achieve effective interaction, but that identity at the subregions of *I* to the right of *I-B*, as well as at the *G*, *S*, and *D* regions, is not sufficient. B10.A(4R) spleen cells (Table III) behaved like B10.A spleen cells in that they stimulated B10.A PETLES (Δ cpm 14,000) more effectively than they stimulated B10 PETLES (Δ cpm 3,300). These results map the gene(s) controlling the interaction to the *K* region or *I-A* subregion (Table V). In order to test whether identity at the *K* region was necessary, A.TL spleen cells were used (Table III). These cells, when pulsed with DNP_6OVA, stimulated B10.A PETLES well (Δ cpm 10,400) but B10 PETLES poorly (Δ cpm 3,000). Thus, compatibility at the *K* region is not essential for presentation of DNP_6OVA.

The above results, taken collectively, suggest that effective presentation can be achieved when compatibility at the *I-A* subregion is present. However, the

[2] Letters in parentheses refer to the haplotype source of the *K*, *I-A*, *I-B*, *I-J*, *I-E*, *I-C*, *S*, *G*, and *D* alleles of the MHC.

TABLE III

Responses of B10 and B10.A PETLES to DNP_6OVA-Pulsed Spleen Cells from Congenic Resistant Lines and Recombinant Strains

Spleen cells	PETLES					
	B10			B10.A		
	Nonpulsed	Pulsed	Δ cpm*	Nonpulsed	Pulsed	Δ cpm*
B10	1,900 (500)‡	22,000 (200)	20,100	10,200 (700)	11,200 (700)	1,000
B10.A	10,500 (400)	13,600 (400)	3,100	1,200 (500)	12,300 (1,800)	11,100
B10.A(5R)	6,300 (2,800)	18,700 (1,300)	12,400	21,100 (1,400)	22,700 (4,000)	1,600
B10.A(4R)	13,400 (200)	16,700 (2,800)	3,300	1,900 (100)	15,900 (2,300)	14,000
A.TL	8,800 (600)	11,800 (500)	3,000	13,100 (600)	23,500 (5,400)	10,400

* Δ cpm, cpm obtained with DNP_6OVA-pulsed spleen cells minus the cpm obtained with nonpulsed spleen cells.

‡ cpm; (1 SEM in parentheses).

TABLE IV

Responses of A.TL and A.TH PETLES to DNP_6OVA-Pulsed Spleen Cells from Congenic Resistant Lines and Recombinant Strains

Spleen cells	PETLES					
	A.TL			A.TH		
	Nonpulsed	Pulsed	Δ cpm*	Nonpulsed	Pulsed	Δ cpm*
A.TL	1,600 (200)‡	12,500 (1,400)	10,900	16,700 (1,600)	19,300 (1,800)	2,600
A.TH	14,100 (700)	14,100 (1,500)	0	3,900 (1,000)	45,100 (600)	41,200
SJL	14,900 (900)	17,400 (400)	2,500	12,200 (2,200)	55,800 (1,600)	43,600
B10.A(4R)	10,900 (1,300)	25,100 (100)	14,200	16,200 (200)	20,800 (1,100)	4,600

* cpm obtained with DNP_6OVA-pulsed spleen cells minus the cpm obtained with nonpulsed spleen cells.

‡ cpm; (1 SEM in parentheses).

definitive proof of this hypothesis is presented in Table IV. A.TH (*sssssssd*) and A.TL (*skkkkkkd*) PETLES were obtained from mice primed to DNP_6OVA. B10.A(4R) (*kkbbbbbbb*) spleen cells pulsed with DNP_6OVA presented as well to A.TL PETLES (Δ cpm 14,200) as A.TL spleen cells (Δ cpm 11,000), whereas SJL (*sssssssss*) spleen cells (Δ cpm 2,500) and A.TH spleen cells (Δ cpm 0) were less effective. In contrast, the same SJL and A.TH spleen cells presented well to A.TH PETLES (Δ cpm 43,600 for SJL and 41,200 for A.TH), demonstrating that they could function in antigen presentation. In this case it was the B10.A(4R) and A.TL spleen cells which were ineffective in presenting DNP_6OVA to A.TH PETLES. These results demonstrate that compatibility at only *I-A* [B10.A(4R) and A.TL] is sufficient for effective presentation of DNP_6OVA, whereas compatibility at only *K* (SJL and A.TL) is not (Table V).

Discussion

The present studies have demonstrated the capacity of spleen cells from nonimmunized donors to bind and retain antigen in an immunogenic form and to effectively use this antigen to stimulate a proliferative response by primed T

TABLE V

The I-A Subregion Controls the Interaction between DNP_6OVA-Pulsed Spleen Cells and Primed T Lymphocytes

Spleen Cells	B10 PETLES		B10.A PETLES	
	H-2 region identity*	% Syngeneic Response‡	*H-2* region identity*	% of Syngeneic Response‡
B10	*K,A,B,J,E,C,S,G,D*	100	None	9
B10.A	None	16	*K,A,B,J,E,C,S,G,D*	100
B10.A(5R)	*K,A,B*	62	*J,E,C,S,G,D*	14
B10.A(4R)	None	16	*K,A*	127
A.TL	None	15	*A,B,J,E,D*	94
	A.TL PETLES		A.TH PETLES	
A.TL	*K,A,B,J,E,C,S,G,D*	100	*K,D*	6
A.TH	*K,D*	0	*K,A,B,J,E,C,S,G,D*	100
SJL	*K*	23	*K,A,B,J,E,C,S,G*	106
B10.A(4R)	*A*	130	None	11

* Letters refer to regions or subregions of the MHC which are shared by the stimulating spleen cells and the responding PETLES. *A,B,J,E,* and *C* represent the subregions of *I*.

‡ (Δ cpm of PETLES to DNP_6OVA-pulsed allogeneic spleen cells/Δ cpm of the same PETLES to DNP_6OVA-pulsed syngeneic spleen cells) × 100. Underlined values indicate substantial responses.

lymphocytes in vitro. The nature of the cell type within the spleen cell population which is responsible for antigen presentation is now under active study. Based on results from guinea pig systems, we anticipate that this cell will prove to be an adherent, Ia-bearing macrophage.

In this paper we have demonstrated that there are certain genetic requirements for effective antigen presentation. Spleen cells histoincompatible at the MHC did not present antigen as well as syngeneic spleen cells. F_1 spleen cells were intermediate in presenting ability. Fine structure mapping indicated that identity of genes in the *I-A* subregion of the MHC was sufficient for effective presentation of the antigen, DNP_6OVA. Identity at the *K* region only or at the *I-B* through *D* regions was not sufficient. However, we were unable to rule out the formal possibility that some combination of *K* and *I-B* through *G* genes might allow effective antigen presentation since the appropriate recombinants for testing this possibility do not exist. Thus, we can not conclude that identity of the *I-A* subregion is necessary, only that it is sufficient.

The histocompatibility requirements did not appear to be the result of suppressive or enhancing effects generated by MLRs for the following reasons. Mixtures of syngeneic and allogeneic or semisyngeneic spleen cells, in which all cultures developed the same MLR, still showed that syngeneic cells were superior in antigen-presenting ability. In addition, the presence of a MLR generated by a difference at the *Mls* locus did not affect antigen presentation if the cells were histocompatible at the MHC. Thus, some other mechanism must account for the observations.

At the present time, we favor the interpretation that primed T lymphocytes recognize antigen in association with gene products of the MHC expressed on

living cells. For cytotoxic cells nominally specific for viral (12), simple chemical (13), minor histocompatibility (14), or tumor-associated (15, 16) antigens, the association appears to be with *K* and *D* region gene products. For proliferating T cells in the guinea pig (1, 2) and delayed-typed hypersensitivity (DH) transferring cells in the mouse (3), the association would appear to be principally with *I* region products. In our system, we postulate that the PETLES population from immunized donors contain T cells which have been primed to DNP_6OVA in association with syngeneic *I-A* region gene products. Stimulation with DNP_6OVA on allogeneic spleen cells does not present the same combination of antigen and *I-A* subregion product and, therefore, would not be stimulatory. F_1 spleen cells do contain the correct allelic product and should be able to present DNP_6OVA. The fact that they did not present antigen as well as syngeneic spleen cells we attribute to a gene dose effect (they possess only one copy of the syngeneic allele) although this remains to be proven.

This interpretation of the results leaves several unresolved questions. One is why the allogeneic cells should present at all since they do not possess the correct MHC allelic products. We believe this is the result of a phenomenon we call "antigen carryover." By this we mean that antigen-pulsed allogeneic spleen cells transfer or release antigen after being placed in culture. This antigen is accepted or bound by residual syngeneic antigen-presenting cells which in turn stimulate primed T lymphocytes. Our reasons for thinking this phenomenon is occurring are threefold. One is that heat-killed cells do release stimulatory antigen albeit only to a small degree (Fig. 3). Second, allogeneic stimulation occurs most often when the spleen cells are pulsed with high concentrations of antigen (Fig. 4) or when cell interactions are maximized by using larger numbers of PETLES in round bottom plates (R. H. Schwartz, unpublished observations). Third, recent observations on antigen-specific T-cell populations which have been selected in vitro and are devoid of antigen-presenting cells, indicate that, under such conditions, allogeneic spleen cells do not present at all (A. Yano, unpublished observations). On the other hand, it is possible that subpopulations of T lymphocytes exist which principally recognize free antigen; such cells might be triggered by antigen presented on allogeneic spleen cells. Indeed, Rosenwasser and Rosenthal (17) have recently studied the proliferative responses of lymph node T lymphocytes, depleted of syngeneic macrophages and reconstituted with syngeneic or allogeneic peritoneal exudate cells. In this system, no MHC restriction was observed for stimulation by purified protein derivative of tuberculin or DNP-OVA. It is also possible, based on the recent report of Gorczynski (18) that different subpopulations of antigen-presenting cells (macrophages) exist, some of which do and some of which do not show histocompatibility restriction in antigen presentation. Finally, it is possible that *I-A* gene products which are serologically distinct may "cross-react" to some extent in their antigen-presentation function.

The other unresolved problem is the failure of some workers to demonstrate histocompatibility requirements for helper T cell-macrophage interactions. Although the very early studies of Mitchison (19) using an adoptive transfer system, suggested that bovine serum albumin bound to allogeneic macrophages was ineffective in eliciting antibody formation, the subsequent detailed studies of Katz and Unanue (20) in an in vitro secondary anti-keyhole limpet hemocya-

nin antibody-forming system indicated that allogeneic macrophages and even fibroblasts, could present antigen effectively. This was despite the fact that these same workers could demonstrate allogeneic restrictions in T-B cell interactions (21, 22). More recently, however, Pierce et al. (5), Erb and Feldmann (4), and Kappler and Marrack (23) have been successful in demonstrating histocompatibility requirements for helper cell induction and antibody formation in vitro and in vivo. Although this recent work suggests that helper T cells show the same histocompatibility restrictions in their interactions with macrophages as do the proliferating T cells (1, 2) and the DH T cells (3), no adequate explanation for the lack of agreement has yet been offered. Even amongst those who agree that a histocompatibility restriction exists in the interaction of macrophages and helper T lymphocytes a consensus on the mechanism of the restrictions does not exist. Pierce et al. (5) have argued that the restriction occurs only in secondary antibody responses since allogeneic and syngeneic adherent cells work equally well in generating a primary plaque-forming cell (PFC) response to L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}, whereas syngeneic cells are markedly better than allogeneic cells in a secondary PFC response. The data of Cosenza and Leserman (24) on sheep erythrocyte primary PFC responses as well as the primary proliferation data of Thomas and Shevach (25, 26) support this concept. On the other hand, Erb and Feldmann (4) have isolated a macrophage factor which is necessary for primary helper cell induction in their system. This factor displays genetic restrictions despite the fact that it is generated by macrophages from unprimed donors. At the present time there is no obvious resolution of these conflicting results.

In conclusion, the results presented in this paper add to the growing body of evidence that suggests that most T cells recognize antigen in association with gene products of the MHC. The mechanisms by which this might occur have been discussed adequately in other publications from this laboratory (27, 28) as well as from other laboratories (3, 29, 30). Suffice it to say that the uniqueness of T-cell recognition and responsiveness is felt to be epitomized by the requirement for histocompatible cell interactions. That this might be the basis for *Ir* gene control of the immune response is currently being investigated.

Summary

A method is described for stimulating proliferation in primed populations of murine T lymphocytes using antigen bound to mitomycin-C-treated spleen cells. This form of antigen presentation appears to be an active process because heat-killed spleen cells are ineffective, and because genetic similarity at the major histocompatibility complex (MHC) between the responder T cells and the presenting spleen cells is required for effective interactions. At all times examined, from day 3 to day 6 of the proliferative response, syngeneic spleen cells presented antigen better to peritoneal exudate T-lymphocyte-enriched cells (PETLES) than semisyngeneic F_1 spleen cells, which in turn could present antigen better than totally allogeneic spleen cells. Spleen cell mixing experiments demonstrated that these genetic restrictions were not the result of suppression by the ongoing mixed lymphocyte reactions (MLR) in the allogeneic and F_1 cases. Furthermore, incompatibility at the *Mls* locus generated a strong MLR but failed to prevent antigen presentation if the spleen cells and PETLES were *H-2*

compatible. Genetic mapping studies demonstrated that compatibility at only the *I-A* subregion of the MHC was sufficient for effective presentation of the antigen, dinitrophenylated ovalbumin. Compatibility at only the *K* region, or the *K* and *D* regions was not sufficient. These results support the concept that functional activation of primed, proliferating T lymphocytes requires the participation of gene products coded for by the *I* region of the MHC. This conclusion is consistent with a growing body of evidence which suggests that most T cells recognize antigen in association with MHC gene products.

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