

T-LYMPHOCYTE-ENRICHED MURINE PERITONEAL EXUDATE CELLS

II. Genetic Control of Antigen-Induced T-Lymphocyte Proliferation

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Immune responses to a large number of antigens have been shown to be controlled by specific immune response (*Ir*) genes encoded within the major histocompatibility complex (MHC)¹ in several species (1). In the mouse, *Ir* genes have been mapped to a particular part of the MHC, termed the *I* region (2). Although it has been proposed that specific *Ir* genes exert some or all of their effects at the level of activation of thymus-dependent (T) lymphocytes (3), this issue has been difficult to study directly in the mouse because of the lack of a reproducible assay system which exclusively measures T-lymphocyte function. Recently, however, we have introduced a reliable antigen-specific murine T-lymphocyte proliferation assay which allows for direct in vitro assessment of this aspect of T-lymphocyte function (4). In this procedure, an enriched population of T lymphocytes is purified from thioglycollate-induced peritoneal exudates of immunized mice by passage over nylon wool columns. This population, termed PETLES, is exquisitely sensitive to antigen and yields large degrees of proliferation when challenged in vitro with the immunizing antigen. The response is sensitive to pretreatment with anti-Thy 1 and complement(C), and exhibits carrier specificity. Moreover, as we show in this paper, greater than 85% of the transformed cells bear the Thy 1 antigen while less than 5% bear surface immunoglobulin. Thus, the assay can be regarded as almost entirely a measure of T-lymphocyte function.

In the present communication, we utilize the assay to examine the T-lymphocyte proliferative responses to seven different antigens; the antibody responses to these antigens have previously been shown to be under the control of *Ir* genes. We demonstrate here that a close parallel exists between the genetic control of T-lymphocyte proliferation and that of antibody formation, suggesting that the same MHC gene controls both types of immune responsiveness. These observations make it appear highly unlikely that *Ir* genes are expressed solely in B lymphocytes, because one would expect that mice with a defect limited to B lymphocytes would show a normal T-lymphocyte proliferative response.

¹ *Abbreviations used in this paper:* BSA-azide buffer, 2% bovine serum albumin and 0.2% NaN₃; FCS, fetal calf serum; Fl-RαMIG, fluorescein-conjugated rabbit antimouse IgG; GAT, poly(Glu⁶⁰-Ala³⁰Tyr¹⁰); GLT, poly(Glu¹⁸Lys³⁸Tyr⁴); LDH_B, porcine lactate dehydrogenase H₄; LPS, lipopolysaccharide; MHC, major histocompatibility complex; Nase, staphylococcal nuclease; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PETLES, peritoneal exudate T-lymphocyte-enriched cells; PPD, purified protein derivative of tuberculin; (ϕ,G)-A--L, poly-(Phe,Glu)-poly-D,L-Ala--poly-Lys; (T,G)-A--L, poly-(Tyr,Glu)-poly-D,L-Ala--poly-Lys.

Materials and Methods

Animals. BALB/c AnN and C3H/HeN mice were obtained from the Division of Research Services of the National Institutes of Health, Bethesda, Md. Strains B10.A(18R) B10.HTT, B10.S(7R), and B10.S(9R) were kindly provided by Dr. Martin Dorf, Department of Pathology, Harvard Medical School, Boston, Mass. All other inbred and congenic resistant lines were obtained from The Jackson Laboratory, Bar Harbor, Maine. Reciprocal F₁ hybrid mice were bred in our own laboratory from the Jackson parental strains, C57BL/10Sn(B10) and B10.A/SgSn. No maternal influence was noted in the ability of these F₁ hybrids to respond to antigenic challenge. Mice were used between 6 and 24 wk of age, and in any given experiment, mice of only one sex were used.

Antigens. The linear random terpolymers poly(Glu⁴⁰Ala³⁰Tyr¹⁰) (GAT) and poly(Glu³⁸Lys³⁸Tyr⁴) (GLT) were synthesized from the *N*-carboxyanhydrides of the amino acids (5) by Pilot Chemical Co., Inc., Boston, Mass.; and Miles-Yeda, Rehovot, Israel, respectively. They were dissolved in 1 N NaOH, neutralized to pH 7.2 and stored at concentrations of 2-10 mg/ml at -20°C. The branched chain synthetic copolymers poly-(Tyr,Glu)-poly-D,L-Ala--poly-Lys [(T,G)-A--L] and poly-(Phe,Glu)-poly-D,L-Ala--poly-Lys [(ϕ,G)-A--L] were the kind gifts of Doctors Michael Sela and Edna Mozes, The Weizmann Institute of Science, Rehovot, Israel (6). These two antigens were dissolved directly in phosphate-buffered saline (PBS), pH 7.2, and stored at -20°C. Staphylococcal nuclease (Nase), was purified from the extracellular broth of cultures of *Staphylococcus aureus*, Foggi strain, as previously described (7) and a 3 mg/ml stock solution in distilled water was the kind gift of Dr. C. Garrison Fathman, NCI, NIH, Bethesda, Md. The IgA myeloma protein, TEPC 15, was obtained from an ascites tumor (Litton Bionetics, Rockville, Md.) and purified by affinity chromatography on a phosphorylcholine column according to the procedure of Chesebro and Metzger (8). The *p*-nitrophenyl phosphorylcholine reagent was kindly provided by Dr. H. Metzger, NIAMDD, NIH, Bethesda, Md., and the organic coupling reactions were performed with the help of Dr. J. K. Inman, NIAID, NIH, Bethesda, Md. Porcine lactate dehydrogenase H₄ (LDH₄) was purchased from Boehringer Mannheim Corp., New York, dialyzed against PBS, centrifuged to remove aggregated material, sterilized by filtration through a 0.45 μm Millipore filter, and stored at 4°C (9). Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratory, Willowdale, Ontario, as a 2 mg/ml solution and stored at -20°C. All antigens were diluted with culture medium to appropriate concentrations just before use. The mitogen, lipopolysaccharide (LPS), isolated from *Salmonella typhosa*, was purchased from Difco Laboratories, Detroit, Mich. It was dissolved in PBS, sterilized by filtration, diluted with culture medium, and used at a final concentration of 10 μg/ml.

Immunizations. Mice were immunized with 1-20 μg of antigen emulsified in complete Freund's adjuvant containing 1 mg/ml *Mycobacterium tuberculosis*, strain H37Ra (Difco Laboratories). Each mouse received 0.1 ml of emulsion distributed equally between the two hind foot pads.

Preparation of PETLES. 2.5 wk after immunization, mice were injected intraperitoneally with 1 ml of 10% Brewer's thioglycollate (Difco Laboratories). 5 days later the peritoneal exudate cells (PEC) were harvested as described in detail elsewhere (4). Purification of the lymphoid population previously involved adherence to glass followed by passage of the nonadherent cells over nylon wool columns. In the present studies this procedure was greatly simplified by eliminating the glass adherence step and increasing the size of the nylon wool columns. 3.5 g of washed nylon wool were packed into a 35 ml syringe and used to fractionate 4-6 × 10⁸ PEC; 2.5-g columns in a 20 ml syringe were used for 2-3 × 10⁸ PEC. The cells were incubated on the column for 1 h, with two 3- to 4-ml washes into the column at 15- and 30-min intervals. The nonadherent cells were eluted with 100 ml of EHAA culture medium (10) containing 10% heat-inactivated (56°C, 45 min) fetal calf serum (FCS). This population contained approximately twice as many macrophages as the peritoneal exudate T-lymphocyte-enriched cells (PETLES) described previously (4), but otherwise was very similar in composition. The mean composition using the new procedure was 13% macrophages, 55% lymphocytes, 2% B lymphocytes (identified by staining with fluorescein-conjugated rabbit antimouse immunoglobulin), and 32% eosinophils for 107 experiments. The mean composition, using the old procedure was 7% macrophages, 54% lymphocytes, 2% B lymphocytes, and 39% eosinophils. The slight enrichment of macrophages in the new PETLES did not affect their responsiveness to antigen. Proliferation ensued over as wide a dose range of antigen as before and the response was almost completely eliminated by pretreatment of the population with anti-Thy 1 antiserum plus C.

Cell Cultures. 1×10^5 PETLES were cultured in sterile, U bottom, polystyrene, microculture plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) containing 0.2 ml of EHAA medium supplemented with 10% FCS and 10–50 $\mu\text{g/ml}$ of gentamycin instead of streptomycin. Antigens were diluted to twice optimal concentrations (100 $\mu\text{g/ml}$ for all antigens except PPD, which was 20 $\mu\text{g/ml}$, and Nase which was 1 $\mu\text{g/ml}$) with supplemented EHAA medium; 0.1 ml of diluted antigen was then mixed with 0.1 ml of cells.

Cells were cultured for 5 days at 37°C in a humidified atmosphere of 2% CO₂ and 98% air. Approximately 16–18 h before harvesting the cultures were pulsed with 1 μCi of tritiated-methylthymidine (sp act 5 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) in 10 μl of PBS. The cultures were harvested onto glass fiber filter paper strips with a MASH II automated harvester (Microbiological Associates, Rockville, Md.) and washed with distilled water and 95% ethanol. The filter discs for each sample were placed in 2 ml of Hydromix scintillation fluid (Yorktown Research Inc., Hackensack, N. J.) and the radioactivity monitored in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). All determinations were done in triplicate and the data are expressed as counts per minute \pm standard error of the mean (SEM).

Identification of Dividing Cells in Antigen-Stimulated PETLES. 2×10^5 PETLES per well were cultured in EHAA medium plus 10% FCS for 4 or 5 days in the presence of PPD or LPS. The cells were harvested by suspending with a Pasteur pipette. The pool of cells from 10 to 20 wells was separated on a Ficoll-Hypaque density gradient ($\rho = 1.077$) for 35 min at room temperature (11) to remove dead cells and debris. The interface population was recovered, washed twice with Eagle's minimal essential medium, and the cells counted. Recovery of live cells ranged from 10 to 33% of the initial number of PETLES plated. The cells were then washed twice at 4°C with a 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.2, containing 2% bovine serum albumin and 0.2% NaN₃ (BSA-azide buffer) (12) and divided into two groups. One was treated with anti-Thy 1.2 serum [raised in AKR mice by injection of C3H thymocytes as described by Reif and Allen (13)], the other with normal mouse serum. In each case 50 μl of neat serum was used/ 10^6 cells. The cells were incubated on ice for 30 min. They were then washed three times with the BSA-azide buffer and reacted for 30 min at 4°C with fluorescein-conjugated rabbit antimouse IgG (Fl-R α MIG) (both antiheavy and light chain specificities) (lot no. 6117; Cappell Laboratories, Inc., Dowingtown, Pa.). After another three washes wet mounts were prepared and read. Blast cells were identified mainly on the basis of their large size and the absence of numerous phagocytic vesicles (to distinguish them from macrophages). These cells were then scored as positive or negative for fluorescence using a Leitz orthoplan incident-illumination microscope (E. Leitz, Inc., Rockleigh, N. J.), the details of which have been described previously (12). In the population stained with normal mouse serum and then Fl-R α MIG, positive cells were classified as B lymphocytes. In the population stained with anti-Thy 1.2 followed by Fl-R α MIG, positive cells constituted both T and B lymphocytes. The percentage of T-cell blasts was obtained by subtraction of the first from the second determinations.

Results

Demonstration that Proliferating Cells are Principally T Lymphocytes. We have previously demonstrated that antigen-induced proliferation of PETLES is sensitive to pretreatment with anti-Thy 1 antiserum and C, and that the response exhibits carrier specificity (4). In addition, since B lymphocytes, as defined by staining with Fl-R α MIG, are relatively rare in PETLES ($2.1 \pm 0.3\%$, $n = 32$), it seemed reasonable to conclude that the proliferative response measured principally the participation of T lymphocytes. However, because this assertion is particularly important for the use of the assay in the study of *Ir*-gene-controlled systems, it seemed necessary to directly rule out any significant contribution of T-cell-dependent, B-lymphocyte proliferation to the total magnitude of the proliferative response. The question was addressed by examining the surface markers of the cells dividing in response to antigen. Blast cells obtained 4 or 5 days after the stimulation of PETLES with PPD were examined for either immunoglobulin or Thy 1 on their surfaces (see Materials and Methods). In four

experiments using immunofluorescence to identify surface markers and morphological criteria to identify blasts, a mean of 5% of the blast cells was found to bear immunoglobulin, a mean of 85% bore Thy 1.2, and 10% bore neither marker. In contrast, when PETLES were stimulated with the B-cell mitogen, LPS, 80% of the small number of recovered blasts bore immunoglobulin, demonstrating that if immunoglobulin-bearing cells made a measurable contribution to the antigen-induced proliferative response, they should have been detected. We conclude from this study that 85% or more of the PETLES which proliferate in response to antigen are members of the T-lymphocyte line.

Genetic Control of Antigen-Induced T-Lymphocyte Proliferation. Murine antibody responses to a variety of antigens have been shown to be under the control of specific *Ir* genes located in the *I* region of the MHC (1, 2). We evaluated the T-lymphocyte proliferative responses to seven of these antigens in a series of inbred mouse strains in order to study the genetic expression of *Ir* genes in a system devoid of B lymphocytes. Three classes of antigens were examined: the linear synthetic random terpolymers, GAT and GLT; the branched chain synthetic copolymers, (T,G)-A--L and (ϕ ,G)-A--L; and the protein antigens, LDH_B, Nase, and TEPC-15. Mice were immunized with 1-20 μ g of antigen, emulsified in complete Freund's adjuvant. PETLES were prepared 3 wk later and 5×10^4 , 1×10^5 , and 2×10^5 cells were challenged in vitro with 1, 10, or 100 μ g/ml of the immunizing antigen or with a maximal stimulating concentration of PPD (20 μ g/ml). The PPD stimulation served as a positive control in each experiment to demonstrate that the failure of the cells to respond to an antigen in a genetically controlled system could not be ascribed to a nonspecific failure of the assay. Representative results for each of the seven antigens, using 1×10^5 PETLES and the maximum stimulating antigen concentration (usually 100 μ g/ml), are shown in Tables I-III.

Antigens in all three classes proved satisfactory for use in the proliferation assay, evoking significant tritiated thymidine incorporation into PETLES from responder strains, although generally less than the incorporation seen by the same cells in response to PPD. Strains designated as nonresponders to these antigens showed no statistically significant difference (Student's *t* test) in the counts per minute incorporated in the presence of antigen, compared to the counts per minute incorporated in the presence of medium alone. In all cases, nonresponder strains showed excellent responses to PPD. Furthermore, strains which were nonresponders to any one of the seven antigens were always responders to several of the other antigens.

It was usually possible to unambiguously designate strains tested as responders or nonresponders; however, in the case of the protein antigens LDH_B and Nase, some strains mounted a weak but significant response at higher immunizing doses. LDH_B elicited no response from B10.A/SgSn mice when these animals were immunized with a low dose of the antigen (1 μ g) but it did elicit a weak response when the mice were immunized with a higher dose of antigen (10 μ g). This response was always less than that given by a responder strain at either immunizing dose [compare B10.A(10): Δ cpm of 2,800; E/C-1 of 1.5 with B10(1): Δ cpm of 41,100, E/C-1 of 4.4 or with B10(10): Δ cpm of 17,400; E/C-1 of 5.9]. Similar observations were made with Nase in the C57BL/10Sn strain. In such

TABLE I
Genetic Control of the T-Lymphocyte Proliferative Response to GAT, GLT, Nase, LDH_B, and (Φ,G)-A--L*

Antigens	Strains†	Medium	Antigen	PPD	Responders‡
GAT	C57BL/10Sn (10)	2,800 ± 800	22,800 ± 2,400	71,900 ± 1,500	++
	C3H/HeN (10)	580 ± 130	18,000 ± 1,200	42,400 ± 900	++
	SJL/J (10)	4,100 ± 1,700	3,200 ± 500	69,200 ± 9,000	-
	DBA/1J (10)	3,600 ± 500	3,800 ± 300	44,300 ± 2,300	-
GLT	BALB/cAnN (10)	2,400 ± 100	18,900 ± 2,300	124,700 ± 11,100	++
	B10.D2/nSn (10)	450 ± 60	32,500 ± 4,500	59,600 ± 4,600	++
	SJL/J (10)	5,200 ± 1,300	3,600 ± 700	48,100 ± 2,400	-
	C57BL/10Sn (10)	1,600 ± 500	2,200 ± 600	23,300 ± 2,600	-
Nase	SJL/J (10)	2,200 ± 300	134,900 ± 8,300	179,000 ± 5,000	++
	SJL/J (1)	3,900 ± 1,300	90,900 ± 11,600	170,000 ± 4,100	++
	BALB/c AnN (10)	2,200 ± 400	61,000 ± 5,200	112,000 ± 1,600	++
	DBA/1J (10)	2,300 ± 1,100	2,200 ± 500	29,100 ± 50	-
	C57BL/10Sn (10)	1,100 ± 400	6,000 ± 500	46,900 ± 2,500	+
	C57BL/10Sn (1)	1,400 ± 400	1,300 ± 200	40,500 ± 800	-
LDH _B	C57BL/10Sn (10)	2,600 ± 800	18,000 ± 2,000	43,500 ± 3,500	++
	C57BL/10Sn (1)	9,400 ± 800	50,500 ± 2,600	79,500 ± 4,900	++
	SJL/J (10)	3,700 ± 1,300	28,500 ± 3,900	67,800 ± 7,000	++
	AKR/J (10)	1,000 ± 200	3,800 ± 1,100	36,400 ± 900	+
	B10.A/SgSn (10)	1,900 ± 800	4,700 ± 1,300	92,000 ± 4,700	+
	B10.A/SgSn (1)	2,300 ± 700	2,900 ± 900	163,500 ± 6,500	-
(Φ,G)-A--L	C57BL/10Sn (20)	1,800 ± 200	28,500 ± 1,700	53,000 ± 9,300	++
	B10.BR/SgSn (20)	1,300 ± 300	51,900 ± 400	78,800 ± 4,400	++
	B10.A/SgSn (10)	370 ± 10	16,800 ± 1,000	45,700 ± 4,600	++
	B10.M/Sn (20)	1,000 ± 100	1,100 ± 200	18,000 ± 1,200	-
	SJL/J (10)	3,200 ± 250	4,900 ± 1,900	49,300 ± 9,600	-

* Mice were immunized with 1–20 μg of antigen in CFA 3–5 wk before collection and preparation of PETLES. 1×10^5 cells were cultured with 1, 10, or 100 μg/ml of the immunizing antigen, with 20–40 μg/ml of PPD, or with medium alone. Stimulation was assessed 5 days later by measuring the incorporation of an 18 h pulse of tritiated thymidine. The data are expressed as the mean counts per minute ± the standard error of the mean for triplicate determinations. For the antigen-stimulated cultures only the data from the concentration giving the largest stimulation are shown. This was generally 100 μg/ml for GAT, GLT, LDH_B, and (Φ,G)-A--L, and 1 μg/ml for Nase.

† The numbers in parentheses after each strain indicate the immunizing dose of the antigen in micrograms.

‡ Strains were assigned a nonresponder status (-) if the incorporation of thymidine in the presence of the immunizing antigen was not significantly different by a Student's *t* test from the incorporation of thymidine in the medium controls. Responder strains were divided into weak (+) and strong (++) categories depending on the magnitude of the statistically significant response. Strong responders gave E/C-1 of 4 or more while weak responders gave E/C-1 of <4.

cases it seems more appropriate to refer to the strains as "low responders."

In each instance, with one exception to be discussed subsequently, the designation of responder or nonresponder assigned to a strain based on the capacity of its T lymphocytes to proliferate in vitro was the same as the designation previously given the strain on the basis of its in vivo antibody response (1, 2). This is shown most clearly in Tables II and III, which present studies of responsiveness to (T,G)-A--L and TEPC-15 by an extensive series of mouse strains, including congenic mice bearing *H-2* recombinant chromosomes. For both antigens it is possible to map the locus of the *Ir* gene controlling the proliferative response in the same manner as was done for the antibody response (14, 15). The strain distribution patterns of responsiveness demonstrate an association between *Ir* genes and the MHC. For example, strains having the *b* and *d H-2* haplotypes were responders to (T,G)-A--L, whereas strains with the *a*, *k*, *r*, and *s* haplotypes were nonresponders. The fact that responsiveness is

TABLE II
Genetic Control of the T-Lymphocyte Proliferative Response to (T,G)-A--L*

Strain	MHC alleles† K, A, B, C, S, D	Proliferative response to:			Responders‡
		Medium	(T,G)-A--L	PPD	
		<i>cpm ± SEM</i>			
A/WySn	k k k d d d	3,400 ± 2,200	3,800 ± 2,400	57,600 ± 3,400	-
C57BL/10Sn	b b b b b b	800 ± 200	65,500 ± 6,700	71,200 ± 4,000	++
BALB/cAnN	d d d d d d	2,400 ± 200	22,200 ± 4,800	126,900 ± 17,000	++
DBA/2J	d d d d d d	5,500 ± 2,800	38,100 ± 6,600	83,300 ± 10,200	++
C3H/HeN	k k k k k k	3,000 ± 700	3,300 ± 200	36,600 ± 700	-
AKR/J	k k k k k k	2,800 ± 200	4,300 ± 600	21,700 ± 4,100	-
RIII	r r r r r r	10,500 ± 2,300	12,200 ± 500	50,900 ± 4,200	-
SJL/J	s s s s s s	3,400 ± 1,900	4,200 ± 400	188,800 ± 11,000	-
A.By/Sn	b b b b b b	1,100 ± 300	10,900 ± 1,500	7,600 ± 2,000	++
B10.A/SgSn	k k k d d d	3,500 ± 400	4,000 ± 800	81,800 ± 3,900	-
B10.D2/nSn	d d d d d d	3,200 ± 500	46,600 ± 2,800	64,600 ± 5,000	++
B10.BR/SgSn	k k k k k k	2,300 ± 50	2,100 ± 600	69,900 ± 2,500	-
(B10 × B10.A)F ₁	k k k d d d/ b b b b b b	5,700 ± 1,300	76,000 ± 8,700	163,500 ± 22,000	++
B10.A(18R)	b b b b b d	6,200 ± 400	47,600 ± 2,000	121,200 ± 5,600	++
B10.A(5R)	b b b d d d	1,200 ± 300	33,300 ± 1,800	50,600 ± 4,200	++
B10.A(4R)	k k b b b b	4,400 ± 1,500	5,300 ± 1,600	239,700 ± 10,500	-
B10.A(2R)	k k k d d b	1,900 ± 100	3,000 ± 800	38,200 ± 1,500	-

* Mice were immunized with 20 µg of (T,G)-A--L in CFA 3 wk before collection and preparation of PETLES. 1×10^6 cells were cultured for 5 days with 100 µg/ml of (T,G)-A--L, 20 µg/ml of PPD, or medium alone. Stimulation was assessed by measuring the incorporation of an 18 h pulse of tritiated thymidine. The data are expressed as the mean counts per minute ± the standard error of the mean for triplicate determinations.

† Letters indicate the haplotype source of the genetic information for each region or subregion of the major histocompatibility complex (MHC). A, B, and C designate the I-A, I-B, and I-C subregions, respectively.

‡ See last legend of Table I.

TABLE III
Genetic Control of the T-Lymphocyte Proliferative Response to TEPC 15*

Strain	MHC alleles† K, A, B, C, S, D	IgA _{C_H} ‡ allotype	Proliferative response (cpm ± SEM) to:			Responders
			Medium	TEPC 15	PPD	
C57BL/10Sn	b b b b b b	A ¹⁵	2,000 ± 200	1,900 ± 200	31,100 ± 1,600	-
BALB/cAnN	d d d d d d	A ^{12, 13, 14}	1,300 ± 400	2,400 ± 500	67,200 ± 2,800	-
AL/AnN	k k k k k d	A ^{13, 17}	4,100 ± 1,100	20,700 ± 3,000	32,600 ± 2,400	++
AKR/J	k k k k k k	A ^{13, 17}	1,900 ± 800	29,300 ± 1,100	96,600 ± 4,200	++
C3H/HeN	k k k k k k	A ^{12, 13, 14}	2,200 ± 200	3,000 ± 500	94,400 ± 10,400	-
RIII	r r r r r r	A ⁻	14,100 ± 800	36,900 ± 400	113,600 ± 4,600	+
SJL/J	s s s s s s	A ¹⁵	300 ± 40	400 ± 50	81,300 ± 12,500	-
A.By/Sn	b b b b b b	A ^{13, 17}	4,100 ± 700	5,200 ± 1,400	42,900 ± 1,000	-
B10.A/SgSn	k k k d d d	A ¹⁵	1,800 ± 800	49,800 ± 3,000	129,700 ± 6,700	++
B10.D2/nSn	d d d d d d	A ¹⁵	580 ± 110	670 ± 60	12,300 ± 1,400	-
B10.BR/SgSn	k k k k k k	A ¹⁵	3,900 ± 1,400	36,700 ± 4,400	148,100 ± 3,300	++
B10.HTT	s s s k k d	A ¹⁵	3,300 ± 1,300	17,100 ± 800	35,300 ± 6,500	++
B10.S (9R)	s s s d d d	A ¹⁵	5,700 ± 1,100	14,500 ± 1,300	41,100 ± 2,100	+
B10.S (7R)	s s s s s d	A ¹⁵	9,800 ± 600	19,100 ± 1,800	56,700 ± 1,700	+
(B10 × B10.A)F ₁	k k k d d d/ b b b b b b	A ¹⁵	1,600 ± 400	19,400 ± 2,000	63,700 ± 3,700	++
B10.A (5R)	b b b d d d	A ¹⁵	2,600 ± 400	3,300 ± 800	72,200 ± 11,800	-
B10.A (4R)	k k b b b b	A ¹⁵	2,200 ± 300	23,100 ± 700	85,400 ± 10,000	++
B10.A (2R)	k k k d d b	A ¹⁵	1,700 ± 700	44,500 ± 1,200	75,000 ± 3,800	++

* Mice were immunized with 20 µg of the BALB/c IgA myeloma protein, TEPC-15, in CFA 3 wk before collection and preparation of PETLES. 1×10^6 cells were cultured for 5 days with 200 µg/ml of TEPC-15, 20 µg/ml of PPD, or medium alone. Stimulation was assessed by measuring the incorporation of an 18 h pulse of tritiated thymidine. The data are expressed as the mean counts per minute ± the standard error of the mean for triplicate determinations.

† See same legend of Table II.

‡ IgA_{C_H}-allotypic determinants are designated according to the Potter-Lieberman nomenclature (16). The BALB/c IgA myeloma protein, TEPC-15 expresses A^{12, 13, 14} allotypic determinants.

|| See last legend of Table I.

indeed coded for by genes in the MHC was shown in the experiments with congenic resistant lines. For (T,G)-A--L, the B10 strain was a responder, whereas the congenic lines B10.A and B10.BR, which possess *a* and *k* MHC haplotypes, respectively, on a B10, non-MHC background, were nonresponders. Thus, the B10 MHC genes are essential for responsiveness to (T,G)-A--L. Conversely, when the MHC genes of B10 were associated with the non-MHC genes of nonresponder A mice, as is found in the congenic strain, A.BY, the mice were responders. Thus, MHC genes are sufficient in themselves to confer responsiveness to the animal. A similar analysis can be made for the response to TEPC 15. Responsiveness appeared to be a dominant genetic trait as shown by the ability of (B10 × B10.A)_{F₁} mice to respond to both (T,G)-A--L and TEPC 15.

Mapping of the Ir Genes Controlling the T-Lymphocyte Proliferative Responses to (T,G)-A--L and TEPC-15 to the K or I-A Region of the MHC. A more precise localization of the genes controlling immune responsiveness was made by examining the responses of *a/b* MHC haplotype recombinant strains (Tables II and III). B10.A (5R) mice derive the *K*, *I-A*, and *I-B* regions of their MHC from the *b* haplotype and the *I-C*, *S*, and *D* regions from the *a* haplotype. This strain was a responder to (T,G)-A--L, a trait associated with the *b* haplotype. This indicates that one *Ir* gene, which allows the *b* haplotype to respond, lies to the left of *I-C*. B10.A(2R) and B10.A(4R) mice, on the other hand, failed to respond to (T,G)-A--L. The latter recombinant was the most important since it possesses *K* and *I-A* regions derived from the *a* haplotype and *I-B*, *I-C*, *S*, and *D* regions derived from the *b* haplotype. The failure of the B10.A(4R) to respond indicates that one gene allowing the *b* haplotype to respond must lie to the left of *I-B*, presumably in the *K* or *I-A* regions, but possibly to the left of *K*, outside of MHC. By applying similar reasoning to the responses of these mice to TEPC-15, one can locate one locus controlling responsiveness to this antigen as also being to the left of *I-B*. In this case, however, responsiveness is determined by the *a* haplotype and nonresponsiveness by the *b* haplotype. These results are precisely the same as those which were obtained by examining the antibody responses of these strains to both (T,G)-A--L and TEPC-15 (14, 15).

Antibody responses to TEPC-15, which is a BALB/c IgA myeloma protein, are actually determined by two genetic loci, the MHC-linked *Ir* genes, in which possession of a responder allele allows a response to occur, and the IgC_H region, in which sharing of the BALB/c IgA allotype prevents a response (16). Table III shows that the same is true for T-lymphocyte proliferation. AKR and B10.BR mice possess the *H-2^k* haplotype as well as an allotypic form of IgA different from that of the BALB/c. These strains both show substantial proliferative responses to TEPC-15. On the other hand, C3H mice, although bearing the same responder allele in the MHC as AKR and B10.BR, nevertheless fail to respond to TEPC-15 because they possess IgA molecules of the same allotype as BALB/c.

One other point should be noted about the data in Table III. The SJL/J strain failed to respond to TEPC-15 (despite multiple attempts, including boosting), yet recombinant strains carrying the *H-2^s* genes in the *K* and *I-A* regions, such as B10.HTT, B10.S(7R), and B10.S(9R), were responders, although rather weak ones. The results obtained with recombinant strains parallel the findings for the antibody responsiveness of *H-2^s* haplotype strains to TEPC-15, whereas the

result with the SJL strain does not. The reason for this single exception is unknown.

The major conclusion to be drawn from these experiments is that the genetic control of the T-lymphocyte proliferative response in mice closely parallels the genetic capacity of these animals to generate an antibody response. This correlation suggests that the same immune response gene controls both types of responses. Because the proliferative assay is almost completely devoid of any contribution from B cells, it seems quite unlikely that any of the *Ir* genes controlling responses to the seven antigens studied can be expressed solely in B lymphocytes.

Discussion

In this paper we have used the antigen-induced proliferative response of mouse PETLES to assay T-lymphocyte function in *Ir*-gene-controlled systems. Although studies of this type have been reported previously, notably for DNA synthetic responses of guinea pig T lymphocytes (17), the superior understanding of MHC genetics in the mouse makes an assessment of T-cell function in this species particularly important. Furthermore, recent studies of collaboration between B cells and helper T cells (or T-cell factors), in immune responses controlled by specific *Ir* genes, have suggested that the locus of expression of at least some *Ir*-gene products is in the B lymphocyte rather than in the T lymphocyte (18, 19). The proliferative response of PETLES allows a direct examination of T-lymphocyte function in mice with such proposed B-lymphocyte defects.

As a prelude to the analysis of the proliferative response of PETLES in *Ir*-gene-controlled systems, it was mandatory to establish that this assay, indeed, measured only T-lymphocyte function. We have previously shown that very few B cells are present in PETLES (2%), that the response of PETLES to antigen can be essentially eliminated by pretreatment with anti-Thy 1.2 and C, and that the responding cells exhibit carrier specificity. In this paper we further show that at least 85% of the blast cells which appear after antigen stimulation bear the Thy 1.2 marker and that only 5% bear detectable surface immunoglobulin. Thus, from this extensive series of criteria it seems quite clear that the proliferative response of PETLES to antigen can be regarded as predominantly reflecting T-lymphocyte function.

We then studied the responsiveness of PETLES from mice immunized to seven individual antigens, the antibody responses to which are known to be under the control of specific *Ir* genes. In each case, PETLES from animals previously designated as responders by serum antibody responses (1, 2) displayed substantial incorporation of tritiated thymidine when stimulated in vitro. Similarly, PETLES from animals previously classified as low or nonresponders on the basis of serum antibody titers showed either no or only modest net incorporation of tritiated thymidine when confronted with antigen in vitro. This concordance in assignment of responder status by examining antibody production and T-cell proliferative responses suggests that the same *Ir* genes (or very closely linked genes) control both types of immune responses. Furthermore, if an *Ir*-gene product were expressed exclusively in B lymphocytes,

PETLES from such "nonresponders" should mount a normal T-lymphocyte proliferative response. Since this was not the case, we conclude that none of the *Ir* genes studied in these experiments are expressed exclusively in B lymphocytes.

This point seems especially germane for the immune response to (T,G)-A--L. In our hands, all mice classified as nonresponders by antibody assay proved to also be nonresponders in the proliferation assay. In particular, mice of the *H-2^k* haplotype, including B10.BR/SgSn, AKR/J, and C3H/HeN were all nonresponders. Similarly, Lonai and McDevitt (20) have previously reported that lymph node cells from (T,G)-A--L immunized C3H/DiSn mice, which are also *H-2^k*, fail to proliferate when cultured in vitro with (T,G)-A--L. On the other hand, Lichtenberg et al. (18) have demonstrated by limiting dilution adoptive transfers that bone marrow of *H-2^k* mice contains reduced numbers of B-cell precursors with specificity for (T,G)-A--L, whereas precursors of specific T cells derived from thymuses of strains of this haplotype appear to be present in normal amounts. In addition, Taussig et al. (19) have reported that spleen cells from irradiated *H-2^k* mice, reconstituted with syngeneic thymocytes and immunized with (T,G)-A--L, produce a specific factor which aids bone marrow cells from other strains to respond. In the latter experiments, *H-2^k* mice appear to be nonresponders because their B cells are unable to bind the cooperating factor (21). Thus, both Lichtenberg et al. and Taussig et al. classify *H-2^k* mice as nonresponders because they appear to have a defect in their B-lymphocyte function, while we classify *H-2^k* mice as nonresponders because they fail to develop a T-lymphocyte proliferative response.

Although these results are in apparent contrast to each other, several ways to make them consistent can be proposed. It is possible that the nonresponder allele for the (T,G)-A--L *Ir* gene, which mice of the *H-2^k* haplotype have, is expressed in both B lymphocytes and T lymphocytes, but that some T-lymphocyte functions, such as the production of collaborative factors, are not controlled by this *Ir*-gene product, while others, such as T-lymphocyte proliferation, are critically dependent on the expression of a responder allele. As a variant on this approach, it might be postulated that the (T,G)-A--L nonresponder allele in the *H-2^k* haplotype is expressed in B lymphocytes and in certain subclasses of T lymphocytes (22). The cells important in factor production and perhaps in helper function might be a set of T lymphocytes whose activation does not require the expression of the responder allele. Proliferation, on the other hand, might be the initial response of a set of T lymphocytes which required the function of this *Ir*-gene product. Alternatively, the proliferative response might require the action of a T cell analogous to the factor producing cell, in order to activate the cell capable of proliferation. In this case, an analogy between the proliferating T cell and the B cell could be drawn in that both would be "helped" by the factor-producing cell.

Another possibility is that the (T,G)-A--L nonresponder allele in *H-2^k* mice is expressed in macrophages, as well as B lymphocytes, but not at all in T lymphocytes. The antigen-induced proliferation of guinea pig T lymphocytes has been shown to be dependent upon the presence of macrophages (23). Furthermore, in the activation of T lymphocytes from (responder \times nonresponder) F_1

guinea pigs, macrophages from the responder parent are much more effective at presenting antigen than macrophages from the nonresponder parent (24). If similar macrophage dependence can be shown for the proliferation of mouse PETLES, then having macrophages express *Ir*-gene functions would seem to be a most attractive possibility. However, in order to explain the differing results of the factor production and the proliferation assays, it would also be necessary to postulate that both priming and in vitro activation of factor-producing T lymphocytes are not macrophage dependent.

In this discussion of the (T,G)-A--L system, we have not considered mice which possess a nonresponder allele at the *Ir* locus that functions in factor producing T lymphocytes, as defined by Taussig et al. (21). Mice, such as B10.M (*H-2^f*), fail to produce-T-cell factor but display normal B-cell acceptor function (21). Studies on the proliferative responses of *H-2^f* mice and of F₁ hybrids between the two types of nonresponders, such as (*H-2^k* × *H-2^f*), are now in progress.

Summary

The recent introduction of a reliable, T-lymphocyte proliferation assay, which utilizes thioglycollate-induced, nylon wool column-passed, peritoneal exudate lymphocytes from immune mice (PETLES), allowed us to investigate the genetic control of murine immune responses at the T-lymphocyte level. Examination of the blast cells generated in this population 5 days after stimulation with antigen, revealed that 85% of the cells bore the Thy 1 antigen on their surface, whereas only 5% bore immunoglobulin. Thus, the assay can be considered to measure almost exclusively T-lymphocyte function. This assay was used to examine the T-lymphocyte proliferative responses to seven different antigens: poly(Glu⁶⁰Ala³⁰Tyr¹⁰), poly(Glu⁵⁸Lys³⁸Tyr⁴), poly-(Tyr,Glu)-poly-D,L-Ala--poly-Lys, poly-(Phe,Glu)-poly-D,L-Ala--poly-Lys, staphylococcal nuclease, lactate dehydrogenase H₄, and the BALB/c IgA myeloma protein, TEPC-15. PETLES from a large number of different inbred mouse strains, including *H-2* congenic resistant lines and *H-2* recombinants, were studied. The strains could be classified as high responders, low responders, or nonresponders to a particular antigen as judged by the magnitude of the T-lymphocyte proliferative response. In every case but one this classification corresponded to the responder status given the strain based on its ability to mount an in vivo antibody response to the same antigen. For two of the antigens, poly-(Tyr,Glu)-poly-D,L-Ala--poly-Lys and TEPC-15, the immune response genes controlling the T-lymphocyte proliferative response were mapped to the *K* region or *I-A* subregion of the major histocompatibility complex, as had previously been shown for the control of the antibody responses to these antigens. This tight linkage of the two phenotypic responses very strongly suggests that the same immune response gene controls the expression of both the proliferative and antibody responses. Since there is essentially no contribution from B lymphocytes in the T-lymphocyte proliferation assay, it seems reasonable to conclude that none of the seven immune response genes studied are expressed solely in B lymphocytes.

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