T-LYMPHOCYTE-ENRICHED MURINE PERITONEAL EXUDATE CELLS

IV. Genetic Control of Cross-Stimulation at the T-Cell Level

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Antibodies to many structurally related antigens have been shown to cross-react extensively. In the case of the linear synthetic polypeptides, antibodies to the terpolymer poly $(Glu^{53}Lys^{36}Phe^{11})_n$ [GLΦ][1] combine with the terpolymer poly $(Glu^{57}Lys^{38}Tyr^{5})$ [GLT] and the copolymer poly $(Glu^{60}Lys^{40})_n$ [GL] (1) while antibodies to the terpolymer poly $(Glu^{60}Ala^{30}Tyr^{10})_n$ [GAT] combine with the copolymers, poly $(Glu^{60}Ala^{40})_n$ [GA] and poly $(Glu^{90}Tyr^{10})_n$ [GT] (2). In the case of the branched-chain copolymers, antibodies to poly (Tyr, Glu)-poly D, L-Ala--poly Lys [(T,G)-A--L] combine with poly (Phe, Glu)-poly D, L-Ala--poly Lys [(Φ,G)-A--L], poly (His, Glu)-poly D, L-Ala--poly Lys [(H,G)-A--L] (3), and even poly (Tyr, Glu)-poly Pro--poly Lys [(T,G)-Pro--L] (4). In contrast, manifestations of T-cell immunity often appear to be more restricted in their cross-reactions. Experiments involving both skin testing for delayed hypersensitivity and lymphocyte proliferation in vitro have shown that guinea pig T cells can distinguish such small differences as those displayed by nona-L-lysines substituted in various positions with a single dinitrophenyl group (5) or the position of a nitro group on the dinitrophenyl moiety (6). Recently, this T-cell discrimination has been further explored in both the mouse (7, 8) and rat (9) using proliferation assays.

Although several laboratories have confirmed the restricted nature of T-cell responses (7, 9), conflicting reports using branched-chain copolymers to stimulate mouse cells have appeared (7, 8). It seemed to us that these apparent contradictions might have a genetic basis. Therefore, we undertook an extensive analysis of cross-reactions among the branched-chain copolymers in a variety of mouse strains using the highly sensitive peritoneal exudate T-lymphocyte-enriched cells (PETLES) proliferation assay (10, 11). The results revealed some surprising cross-reactions as well as demonstrating the importance of immune response genes in these phenomena. As a general rule, it was found that in order for immunization with one antigen to prime for cross-stimulation to a second structurally related antigen, the immunized strain had to possess responder alleles of immune response (*Ir*) genes for both antigens.

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[1] *Abbreviations used in this paper:* Δcpm, difference between antigen-stimulated cpm and control cpm; EHAA, Eagle's high amino acids medium; GA, poly $(Glu^{60}Ala^{40})_n$; G-A--L, poly Glu-poly D,L-Ala--poly. Lys; (Φ,G)-A--L, poly (Phe,Glu)-poly D,L-Ala--poly Lys; GAT, poly $(Glu^{60}Ala^{30}Tyr^{10})_n$; GL, poly $(Glu^{60}Lys^{40})_n$; GLΦ, poly $(Glu^{53}Lys^{36}Phe^{11})_n$; GLT, poly $(Glu^{57}Lys^{38}Tyr^{5})_n$; GT, poly $(Glu^{90}Tyr^{10})_n$; (H,G)-A--L, poly (His,Glu)-poly D,L-Ala--poly Lys; MHC, major histocompatibility complex; (T,G)-A--L, poly (Tyr,Glu)-poly D,L-Ala--poly Lys; (T,G)-Pro--L, poly (Tyr,Glu)-poly Pro--poly Lys; PETLES, peritoneal exudate T-lymphocyte-enriched cells; PPD, purified protein derivative of tuberculin.

Materials and Methods

Animals. BALB/cAnN mice were obtained from the Division of Research Services of the National Institutes of Health. 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. B10.A(4R)/Sg mice were the progeny of breeding pairs kindly provided by Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Mont. All other inbred and congenic resistant lines were obtained from The Jackson Laboratory, Bar Harbor, Maine. The (B10 × B10.A)F_1 hybrids were bred in our laboratory from the Jackson parental strains C57BL/10Sn (B10) and B10.A/SgSn (B10.A). Mice of both sexes were used between 6 and 30 wk of age.

Antigens. The linear random terpolymer poly $(Glu^{60}Ala^{30}Tyr^{10})_n$ [GAT] was synthesized from the *N*-carboxyanhydrides of the L-amino acids (12) by Pilot Chemical Co., Watertown, Mass. (lot no. M-18-H). It was dissolved in 1% (wt/vol) Na_2CO_3 in saline, neutralized to pH 7.2 with 1 N HCl, and stored at −20°C at a concentration of 2 mg/ml. The branched chain synthetic copolymers (13-15) poly (Tyr, Glu)-poly D, L-Ala--poly Lys [(T,G)-A--L] (lot no. 1383), poly (Phe,Glu)-poly D, L-Ala--Poly Lys [(Φ,G)-A--L] (lot no. 1501), poly (Glu)-poly D, L-Ala--poly Lys [G-A--L] (lot no. 940), and poly (Tyr, Glu)-poly Pro,--poly Lys [(T,G)-Pro--L] (lot no. 946) were the kind gifts of Dr. Michael Sela, Dr. Edna Mozes, and Dr. Anne-Marie Verhulst-Schmidt. Poly (His, Glu)-poly D, L-Ala,--poly Lys [(H,G)-A--L] (lot no. 14) was the generous gift of Dr. Erwin Rüde and Dr. Peter Krammer. All five copolymers were dissolved directly in phosphate-buffered saline, pH 7.2 (PBS), and stored at −20°C at a concentration of 2 mg/ml. Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Labs., Willowdale, Ontario, as a 2 mg/ml solution and stored at −20°C. All antigen solutions were sterilized by filtration through a 0.45 μm Millipore filter. They were diluted with culture medium to appropriate concentrations just before use.

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

Preparation of Cells. The preparation and culture of PETLES has been described in detail elsewhere (10, 11). Briefly, 3 wk after immunization, thioglycollate-induced peritoneal exudate cells were harvested and passed over nylon wool columns. Because of a shortage of nylon wool, the fiber was "recycled" after use. This entailed washing in distilled water to remove the cells and medium, and storing in 0.02% NaN_3 until a large batch of nylon had been accumulated. The nylon was then washed free of the NaN_3, boiled in 10 mM EDTA for 30 min, washed free of the EDTA, and soaked in double-distilled water for 5 days at either 37°C or 4°C, changing the water each day. The nylon was then dried and packaged as previously described (10, 11). PETLES were eluted from the columns with 40–50 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum, antibiotics, and 2-mercaptoethanol. The population eluted from standard nylon columns contained an average of 13% macrophages, 55% lymphocytes, 32% eosinophils, and only 2% B lymphocytes (identified by staining with fluorescein-conjugated rabbit anti-mouse immunoglobulin), although these percentages varied significantly depending on the mouse strain used (10, 11). The recycled nylon on the other hand was more effective in trapping macrophages and less effective in trapping eosinophils. For 25 experiments with B10 mice or H-2 congenic mice on a B10 background, the mean PETLES population was composed of 3.5 ± 0.5% macrophages, 29 ± 2% lymphocytes, and 67 ± 2% eosinophils, with <2% B cells. PETLES obtained from recycled nylon columns appeared to be slightly less responsive to antigen, particularly in those populations containing over 75% eosinophils. However, this problem could be compensated for by increasing the number of cells cultured per well. In all other respects, such as the T-cell dependence of the assay, the PETLES behaved as previously described (10,11).

Cell Cultures. 2×10^5 PETLES were cultured in each well of a sterile, U bottom, microculture plate (Cooke Engineering, Alexandria, Va.) containing 0.2 ml Eagle's high amino acids (EHAA) medium supplemented with 10% fetal calf serum (FCS). The EHAA was modified from the original description of the medium by Click et al. (16) to include 50 μg/ml of gentamicin instead of streptomycin, 10 mM HEPES plus 15 mM $NaHCO_3$ instead of 15 mM $NaHCO_3$, and 240 mg/liter of L-leucine instead of 130 mg/liter. The complete medium was made up and stored at 4°C except for the antibiotics, 5×10^{-5} M 2-mercaptoethanol, and 4 mM glutamine which were added just before use. The cells were added to the wells first in 0.1 ml of medium, and then the antigens were

added in 0.1 ml to give a final concentration of 0.01 to 500 μg/ml. The cultures were incubated for 5 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. 934AH, Whatman Inc., Clifton, N. J.) with a MASH II automated harvestor (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, Hackensack, N. J.) and the radioactivity measured in a Beckman liquid scintillation counter. Most determinations were done in triplicate except for dose-response curves in which case each point was done in duplicate. The data are expressed as cpm ± the standard error of the mean (SEM) and plotted for the dose-response curves as the difference between the antigen-stimulated cultures and control cultures without antigen (Δcpm) vs. the $\log_{10}$ of the antigen concentration. Statistical analysis was done with a two-tailed Student's *t* test.

Results

Initial studies by Lonai and McDevitt (7) of cross-reactions among the branched-chain copolymers at the T-cell level had indicated no detectable cross-stimulation between (Φ,G)-A--L and (H,G)-A--L in lymphocytes from primed C3H/DiSn or C3H.Q mice, suggesting that the pattern of cross-reactivity of T-lymphocyte receptors and that of antibodies were quite different. A subsequent study by Oppenheim et al. (8), however, found that (T,G)-A--L and (Φ,G)-A--L gave complete cross-stimulation in both directions using cells from C57BL/6 mice, suggesting that a similar pattern of cross-reactivity was expressed by T cells and antibody. These apparently discrepant conclusions prompted us to more fully explore the question of cross-reactions among the branched-chain copolymers in a variety of different mouse strains, principally of the B10 congenic series. Mice of this series were chosen so that any differences observed could be ascribed to the action of major histocompatibility complex (MHC) gene products. Table I shows the capacity of mice of different H-2 types to respond to each of the immunogens studied.

The results of our studies of immunogenicity and cross-stimulation at the T-cell level are presented in Tables II–V. Representative experiments for each strain and antigen are shown. In a few cases, where a large variation in the degree of cross-reaction for a particular antigen was observed, two experiments are presented.

PETLES from mice of *H-2*^{b} and *H-2*^{d} haplotypes, which had been immunized with (T,G)-A--L, could be stimulated in vitro with (T,G)-A--L, (Φ,G)-A--L, and GAT, but no significant stimulation was observed with (H,G)-A--L, (T,G)-Pro--L, or G-A--L (Table II). The (Φ,G)-A--L cross-reactions ranged from 50–80%, and the GAT cross-reactions ranged from 30–100%. PETLES from *H-2*^{a}, *H-2*^{k}, *H-2*^{q}, and *H-2*^{s} mice immunized with (T,G)-A--L did not respond to (T,G)-A--L and also showed no response to any of the potentially cross-reactive antigens. They did respond to PPD, however, indicating that the failure to respond to the branched-chain copolymers was a selective nonresponsiveness.

PETLES from *H-2*^{a}, *H-2*^{b}, *H-2*^{d}, *H-2*^{k}, and *H-2*^{q} mice immunized with (Φ,G)-A--L, showed a variety of different cross-reaction patterns (Table III). *H-2*^{b} and *H-2*^{d} mice, which had been immunized with (Φ,G)-A--L, responded to (T,G)-A--L and GAT, but not to (H,G)-A--L, (T,G)-Pro--L, and G-A--L. The (T,G)-A--L cross-stimulation in *H-2*^{b} mice was 80–100% of the (Φ,G)-A--L stimulation; in *H-2*^{d} mice the cross-reaction was less complete ranging from 10–60% with an

TABLE I

*Ir Gene Control of the T-Cell Proliferative Response to the Synthetic Polypeptides**

Polypeptide	H-2 haplotypes of	
	Responder strains	Nonresponder strains
(T,G)-A--L	b,d,i5	a,k,q,s,h4
(Φ,G)-A--L	a,b,d,k,q,h4,i5	s
(H,G)-A--L	a,k,h4,t1	b,d,s,i5
GAT	a,b,d,k	q,s

* Data summarized from the present paper and references 7 and 11.

TABLE II

Cross-Stimulation of PETLES from Mice Immunized with (T,G)-A--L

H-2 type	Strain	Thymidine incorporation (cpm ± SEM) in response to:							
		Medium	(T,G)-A--L	(Φ,G)-A--L	(H,G)-A--L	GAT	G-A--L	(T,G)-Pro--L	PPD
a	B10.A	520 (±100)	1,300 (±500)	1,300 (±350)	1,400 (±300)	500 (±160)	750 (±250)	640 (±40)	25,600 (±1,900)
b	B10	2,800 (±300)	27,800 (±700)	15,200 (±2,200)	3,600 (±300)	22,900 (±2,100)	2,600 (±200)	2,500 (±200)	34,400 (±7,100)
b	A.BY	2,000 (±600)	28,700 (±2,300)	24,000 (±200)	3,200 (±700)	14,800 (±1,000)	1,900 (±1,200)	5,200 (±2,700)	31,100 (±5,800)
d	B10.D2	1,900 (±200)	10,900 (±700)	8,200 (±1,400)	3,100 (±500)	13,300 (±400)	2,500 (±300)	ND	32,500 (±900)
d	BALB/c	2,200 (±450)	14,400 (±700)	12,100 (±700)	3,600 (±300)	7,800 (±2,200)	3,700 (±600)	ND	48,600 (±900)
k	B10.BR	1,400 (±700)	2,300 (±300)	3,000 (±900)	ND*	2,100 (±300)	ND	ND	38,800 (±1,900)
q	SWR	700 (±300)	600 (±100)	1,400 (±400)	ND	ND	ND	1,900 (±500)	74,300 (±1,200)
s	SJL	300 (±30)	480 (±90)	500 (±160)	450 (±80)	325 (±50)	435 (±130)	490 (±70)	114,600 (±11,600)
b/a	(B10 × B10.A)F_1	1,300 (±300)	22,100 (±300)	10,100 (±800)	1,500 (±100)	8,100 (±980)	ND	ND	28,400 (±500)
i5	B10.A(5R)	1,300 (±300)	24,600 (±4,100)	11,300 (±1,800)	800 (±200)	9,200 (±1,600)	ND	1,000 (±150)	25,300 (±800)
i5	B10.A(5R)	4,700 (±600)	11,100 (±1,000)	10,700 (±800)	4,300 (±200)	9,900 (±900)	ND	ND	21,600 (±900)

PETLES from various strains of mice were harvested 3 wk after immunization with 20 μg of (T,G)-A--L in CFA and challenged in vitro with one of six different polymers or PPD. Several concentrations of each antigen were used, but only data from the dose (usually 200 μg/ml) giving maximal incorporation of a 16-h pulse of tritiated thymidine are shown. Significant stimulations over the medium control are underlined. Dotted lines indicate stimulations that were statistically significant in this experiment, but which failed to reproduce in other experiments.

* ND, not determined.

average of 40%. GAT also showed a larger degree of cross-stimulation in *H-2*^{b} mice (75–100%) than in *H-2*^{d} mice (10–50%).

The second pattern of cross-stimulation among mice immunized with (Φ,G)-A--L was shown by *H-2*^{a} and *H-2*^{k} mice. In this case, (T,G)-A--L, as well as

TABLE III

Cross-Stimulation of PETLES from Mice Immunized with (Φ,G)-A--L

H-2 type	Strain	Thymidine incorporation (cpm ± SEM) in response to:							
		Medium	(Φ,G)-A--L	(T,G)-A--L	(H,G)-A--L	GAT	G-A--L	(T,G)-Pro--L	PPD
a	B10.A	2,800 (±1,200)	26,600 (±2,500)	3,700 (±600)	3,900 (±900)	9,700 (±2,400)	2,800 (±700)	ND	81,900 (±9,300)
a	B10.A	150 (±30)	17,300 (±1,300)	450 (±200)	340 (±90)	1,200 (±60)	450 (±75)	260 (±150)	33,500 (±2,500)
b	B10	1,900 (±500)	31,900 (±100)	34,800 (±3,900)	2,300 (±900)	24,400 (±1,300)	2,500 (±950)	ND	38,000 (±6,900)
b	B10	1,100 (±500)	80,900 (±4,800)	76,500 (±12,500)	ND	ND	ND	1,300 (±300)	119,700 (±3,500)
d	B10.D2	2,300 (±800)	15,900 (±3,500)	7,400 (±400)	1,800 (±500)	3,500 (±100)	1,000 (±400)	ND	36,300 (±1,700)
d	B10.D2	700 (±100)	12,000 (±2,200)	7,400 (±1,500)	1,200 (±400)	3,900 (±200)	1,400 (±600)	1,000 (±400)	11,600 (±400)
d	BALB/c	2,300 (±900)	25,500 (±1,600)	16,700 (±3,700)	3,300 (±700)	14,400 (±300)	7,200 (±2,000)	ND	63,000 (±6,300)
k	B10.BR	700 (±200)	27,800 (±300)	800 (±200)	600 (±60)	2,300 (±460)	1,000 (±100)	2,000 (±400)	48,400 (±2,800)
q	SWR	2,100 (±1,100)	32,200 (±2,000)	900 (±300)	800 (±400)	1,000 (±200)	1,200 (±10)	ND	9,500 (±600)
s	SJL	140 (±30)	170 (±60)	160 (±35)	220 (±35)	110 (±15)	210 (±50)	240 (±110)	42,700 (±7,700)
b/a	(B10 × B10.A)F_1	1,000 (±200)	7,900 (±800)	10,300 (±600)	1,700 (±200)	5,700 (±400)	ND	ND	25,200 (±3,300)
h4	B10.A(4R)	500 (±70)	32,200 (±2,400)	1,700 (±200)	700 (±200)	3,000 (±600)	1,200 (±100)	ND	54,600 (±4,300)
h4	B10.A(4R)	4,200 (±1,200)	96,600 (±6,100)	4,800 (±600)	ND	ND	ND	ND	85,100 (±15,000)
i5	B10.A(5R)	1,200 (±200)	8,200 (±500)	13,600 (±400)	3,700 (±600)	10,900 (±1,500)	4,700 (±700)	ND	26,600 (±1,700)
i5	B10.A(5R)	4,600 (±400)	29,400 (±4,900)	26,200 (±40)	2,100 (±400)	16,300 (±3,400)	3,800 (±200)	3,500 (±300)	78,800 (±1,400)

See legend to Table II.

(H,G)-A--L, G-A--L, and (T,G)-Pro--L failed to stimulate. Only GAT elicited cross-reactive responses, and these were small and quite variable from experiment to experiment. The range of GAT cross-reactions was 0–20% with a mean of 10%. The third pattern of cross-reactivity to (Φ,G)-A--L was that shown by *H-2^q* mice which responded only to the immunogen and not to any of the other antigens tested. Finally, *H-2^s* mice did not respond to the immunogen, (Φ,G)-A--L, nor to any of the other polypeptides.

In striking contrast to the multiple cross-stimulations seen when (T,G)-A--L, and (Φ,G)-A--L were used as the immunogens, (H,G)-A--L immune PETLES showed either insignificant or only marginal cross-reactions with any of the

TABLE IV
Cross-Stimulation of PETLES from Mice Immunized with (H,G)-A--L

H-2 type	Strain	Thymidine incorporation (cpm ± SEM) in response to:							
		Medium	(H,G)-A--L	(T,G)-A--L	(Φ,G)-A--L	GAT	G-A--L	(T,G)-Pro--L	PPD
a	B10.A	400 (±140)	33,500 (±1,600)	200 (±20)	600 (±300)	1,100 (±200)	2,700 (±1,100)	ND	36,700 (±4,000)
b	B10	200 (±60)	400 (±200)	300 (±100)	300 (±200)	200 (±100)	ND	ND	50,600 (±500)
d	B10.D2	500 (±100)	900 (±40)	900 (±100)	1,200 (±130)	1,400 (±100)	1,000 (±300)	ND	38,700 (±4,600)
d	B10.D2	5,900 (±700)	7,200 (±1,900)	6,100 (±700)	3,800 (±700)	7,200 (±900)	3,200 (±700)	ND	85,500 (±9,500)
d	BALB/c	340 (±50)	1,700 (±400)	300 (±50)	380 (±150)	940 (±180)	760 (±220)	730 (±190)	35,300 (±2,300)
k	B10.BR	700 (±200)	26,500 (±2,700)	800 (±100)	1,100 (±100)	1,600 (±250)	1,400 (±200)	ND	28,900 (±1,100)
q	SWR	1,400 (±325)	1,700 (±500)	2,600 (±800)	1,100 (±450)	900 (±130)	1,400 (±500)	1,000 (±200)	69,000 (±4,000)
s	SJL	6,200 (±2,000)	6,400 (±350)	10,700 (±5,200)	5,800 (±200)	10,100 (±2,700)	7,700 (±400)	8,300 (±1,800)	110,300 (±3,500)
h4	B10.A(4R)	300 (±100)	15,400 (±1,800)	300 (±100)	500 (±250)	800 (±200)	800 (±300)	ND	53,100 (±4,500)
i5	B10.A(5R)	1,400 (±120)	1,000 (±150)	1,400 (±130)	2,200 (±700)	1,300 (±100)	1,700 (±300)	1,000 (±200)	50,600 (±300)
tl	A.TL	3,900 (±500)	33,300 (±350)	6,000 (±2,100)	5,900 (±1,000)	11,100 (±3,000)	10,800 (±2,000)	6,200 (±500)	44,900 (±400)

See legend to Table II.

other polypeptides (Table IV). This was true for all of the B10 congenic strains tested, whether they were responders (*H-2*^{a}, *H-2*^{k}) or nonresponders (*H-2*^{b}, *H-2*^{d}) to (H,G)-A--L. The responses of the recombinant strains to (H,G)-A--L, also shown in Table IV, map one *Ir* gene(s) controlling the proliferative response to this antigen to the *I-A* subregion of the mouse genome. Thus, PETLES from B10.A(4R) mice responded to (H,G)-A--L, whereas B10.A(5R) PETLES did not. Since *H-2*^{a} is the responder haplotype and the B10.A(4R) only has *H-2*^{a} alleles in the *K* and *I-A* regions, one of those two regions (or a region centromeric to *K*) must contain an *Ir-HGAL* gene(s). The responsiveness of the A.TL strain PETLES suggests that the gene lies in the *I-A* subregion. This statement is based on the fact that the *K* region and genes centromeric to it were derived from *H-2*^{s}, a nonresponder haplotype, as shown by the failure of SJL PETLES to proliferate in response to (H,G)-A--L (Table IV), while the *I-A* subregion was derived from the *H-2*^{al} responder haplotype. This map position for an *Ir-HGAL* gene(s) which controls the T-cell proliferative response is the same as the map position described for an *Ir-HGAL* gene(s) which controls the anti-(H,G)-A--L antibody response (17). As published previously for a variety of other antigens

TABLE V
Cross-Stimulation of PETLES from Mice Immunized with GAT

H-2 type	Strain	Thymidine incorporation (cpm ± SEM) in response to:							
		Medium	GAT	(T,G)-A--L	(Φ,G)-A--L	(H,G)-A--L	G-A--L	(T,G)-Pro--L	PPD
a	B10.A	400 (±100)	19,400 (±1,400)	500 (±200)	500 (±50)	250 (±30)	350 (±70)	ND	23,700 (±3,600)
b	B10	1,000 (±150)	26,700 (±2,100)	3,000 (±900)	3,900 (±600)	3,000 (±100)	1,100 (±80)	ND	23,300 (±1,200)
b	B10	2,100 (±100)	44,200 (±1,100)	2,300 (±100)	9,900 (±2,700)	1,600 (±500)	2,300 (±300)	2,100 (±200)	27,700 (±700)
d	B10.D2	650 (±70)	14,900 (±1,500)	700 (±200)	950 (±300)	300 (±10)	300 (±200)	ND	10,600 (±1,000)
d	BALB/c	3,800 (±500)	38,600 (±4,900)	7,500 (±230)	5,400 (±1,100)	5,100 (±50)	4,800 (±350)	4,200 (±500)	71,800 (±7,700)
q	SWR	6,400 (±2,600)	9,500 (±1,800)	8,800 (±1,500)	7,200 (±900)	3,800 (±900)	6,700 (±3,400)	8,300 (±1,200)	71,200 (±4,700)

See legend to Table II.

(11), this correlation suggests that the same *Ir* gene controls both immune responses.

It should be noted that PETLES from B10.D2 mice failed to respond to (H,G)-A--L, while BALB/c PETLES showed a barely significant response (Table IV). This is in contrast to the data obtained at the antibody level for $H\text{-}2^d$ mice in which at least the BALB/c strain was reported to be a moderate responder (18). It should also be noted that A.TL PETLES, in contrast to those of the B10 congenic series, showed cross-stimulations with several of the other polypeptides. Whether the A non-H-2 genetic background is responsible for this will require further study.

The most unexpected cross-reaction observed in the series of polymers studied was the stimulation of (T,G)-A--L or (Φ,G)-A--L immune PETLES by the linear random terpolymer GAT. Interestingly, however, these cross-reactions appeared to be largely unidirectional, i.e., GAT immune PETLES were either not stimulated or were stimulated only minimally by the branched-chain copolymers (Table V). PETLES from B10.D2 and B10.A mice immune to GAT were not stimulated at all by (T,G)-A--L, (Φ,G)-A--L, (H,G)-A--L, or G-A--L, even though GAT could stimulate (T,G)-A--L immune B10.D2 cells quite well and (Φ,G)-A--L immune B10.A and B10.D2 cells to a small extent. On the other hand, PETLES from B10 mice immune to GAT demonstrated a weak but reproducible cross-stimulation with (Φ,G)-A--L. Also, PETLES from BALB/c mice showed a weak cross-reaction with (T,G)-A--L. However, these cross-reactions (never greater than 18%) seemed negligible compared to the cross-stimulations observed in the opposite direction: 75% for B10 PETLES immune to (Φ,G)-A--L and 46% for BALB/c PETLES immune to (T,G)-A--L.

In many cases dose-response curves were performed in order to determine the maximum amount of each cross-reaction and the concentration of antigen required to achieve 50% of the maximal response. Figs. 1–4 show examples of

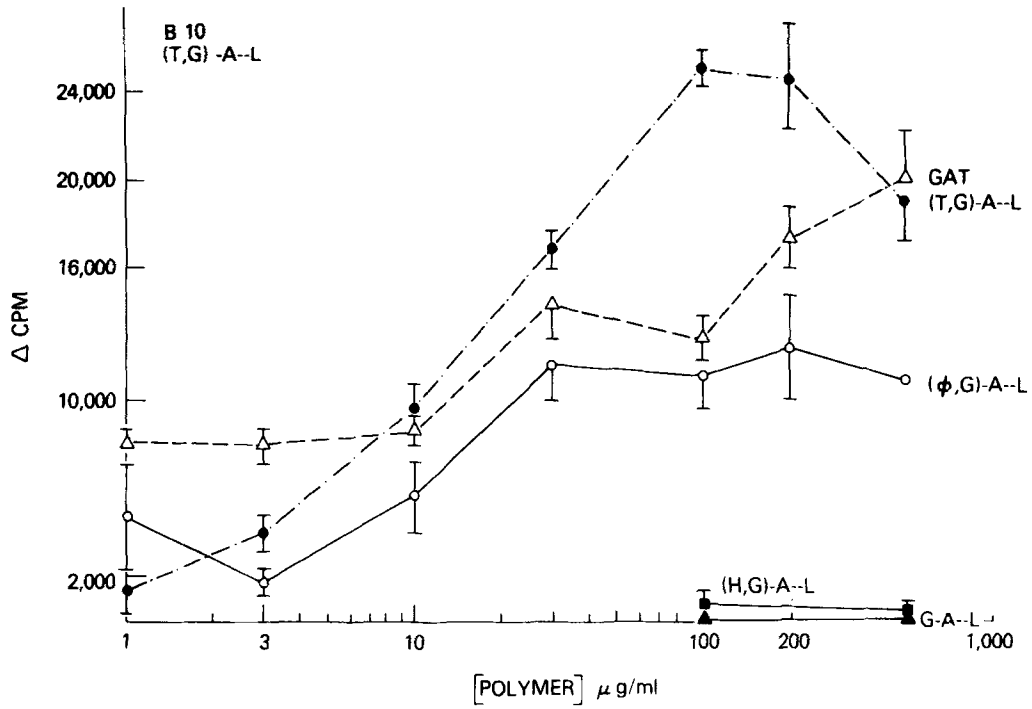

FIG. 1. C57BL/10Sn (B10) mice were immunized with 20 μg of (T,G)-A--L. 3 wk later PETLES were prepared, and 2 × 10^5 cells were cultured with various concentrations of (T,G-A--L (●), (ϕ,G)-A--L (○), GAT (△), (H,G)-A--L (■), or G-A--L (▲) for 5 days in vitro. Stimulation was assessed by measuring the incorporation of tritiated methylthymidine by cells cultured with each polymer minus the incorporation by cells cultured with medium alone (Δcpm).

such dose-response curves for PETLES from B10, B10.D2, and B10.A mice immunized to (T,G)-A--L or (Φ,G)-A--L. In general, the curves showed very shallow rises, taking three to four $\log_{10}$ increases in antigen concentration to go from initial stimulation to plateau levels of response. The maximal response usually occurred at an antigen concentration of 100–500 μg/ml. These characteristics were observed for the cross-reacting antigens as well as for the immunogen. Table VI gives a summary of the data obtained from the dose-response curves for the major cross-reacting antigens. Stimulation by GAT and (Φ,G)-A--L of PETLES from both B10 and B10.D2 mice immunized to (T,G)-A--L was 50–100% of the maximum response achieved with (T,G)-A--L. The concentration of polymer required to achieve 50% of the maximal response for that polymer was similar for both the immunogen and the cross-reacting antigens in the B10 strain and only three to fourfold higher for the cross-reacting antigens than for the immunogen in the B10.D2 strain. These results suggest a similar receptor affinity in the B10 cells for all three polymers and definitely rule out the trivial possibility that the cross-stimulations resulted from accidental contamination of the cross-reacting polymers with small amounts of the immunogen. Immunization of B10 mice with (Φ,G)-A--L gave rise to a similar pattern; the cross-reacting antigens stimulated as much proliferation as the immunogen and at

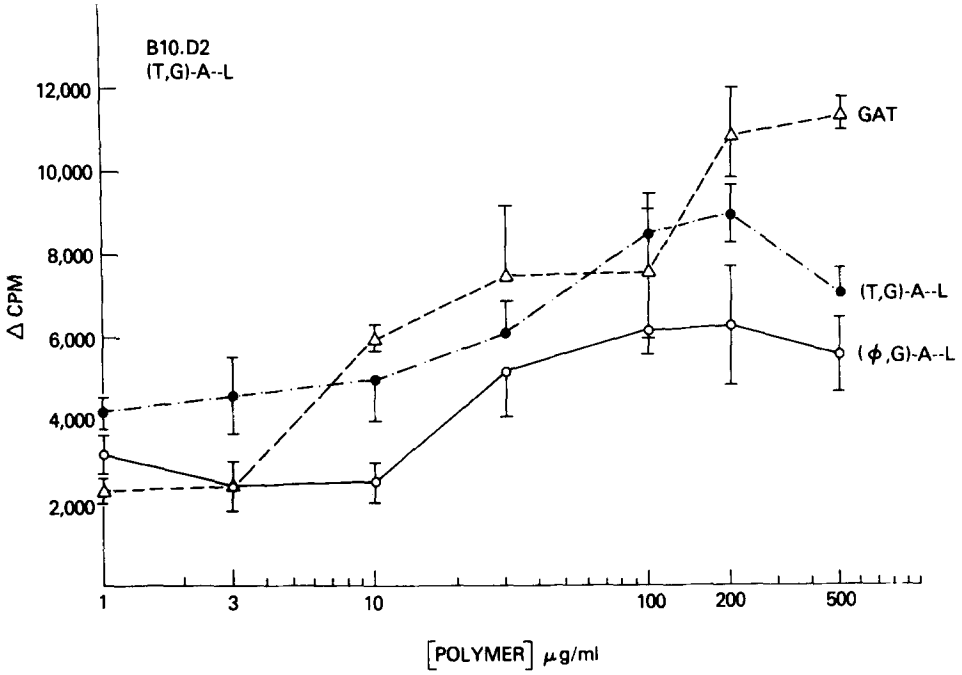

FIG. 2. B10.D2/nSn mice were immunized with 20 μg of (T,G)-A--L. 3 wk later PETLES were prepared, and 2 × 10^5 cells were cultured with various concentrations of (T,G)-A--L (●), (ϕ,G)-A--L (○), or GAT (△) for 5 days in vitro. Stimulation was assessed by measuring the incorporation of tritiated methylthymidine by cells cultured with each polymer minus the incorporation by cells cultured with medium alone (Δcpm).

similar antigen concentrations. On the other hand, immunization of B10.D2 mice with (Φ,G)-A--L led to weaker cross-reactions with (T,G)-A--L and GAT both in terms of the maximum response (10–50%) and the concentration required to achieve 50% of the maximal response (six to sevenfold higher). Cells from B10.A mice immunized with (Φ,G)-A--L showed a similar cross-reactivity to GAT as that of B10.D2 PETLES, but they failed to respond to (T,G)-A--L. The data suggest that in these cases only a portion of the immune cells have receptors that will accommodate the cross-reacting antigens and then only at higher concentrations.

The data presented in this paper indicate that the patterns of cross-reactive immune responses are controlled by genes mapping in the *K* or *I* region of the MHC. The most striking demonstration of this is the pattern seen for the B10.A recombinant strains immunized with (Φ,G)-A--L (Table III). The B10.A(5R) mouse, which has the *H-2*^{b} haplotype alleles in the *K*, *I-A*, and *I-B* regions of its MHC and the *H-2*^{a} haplotype alleles in the *I-J*, *I-E*, *I-C*, *S*, *G*, and *D* regions, showed the cross-reaction pattern of B10: namely, 50–100% stimulation of (Φ,G)-A--L immune PETLES with (T,G)-A--L and GAT. In contrast, PETLES from B10.A(4R) mice, which have the *H-2*^{a} haplotype alleles in the *K* and *I-A* regions and the *H-2*^{b} haplotype alleles for the rest of the MHC, showed the cross-reaction pattern of B10.A: namely, weak stimulation of (Φ,G)-A--L immune PETLES with GAT and barely significant or no stimulation with (T,G)-A--L.

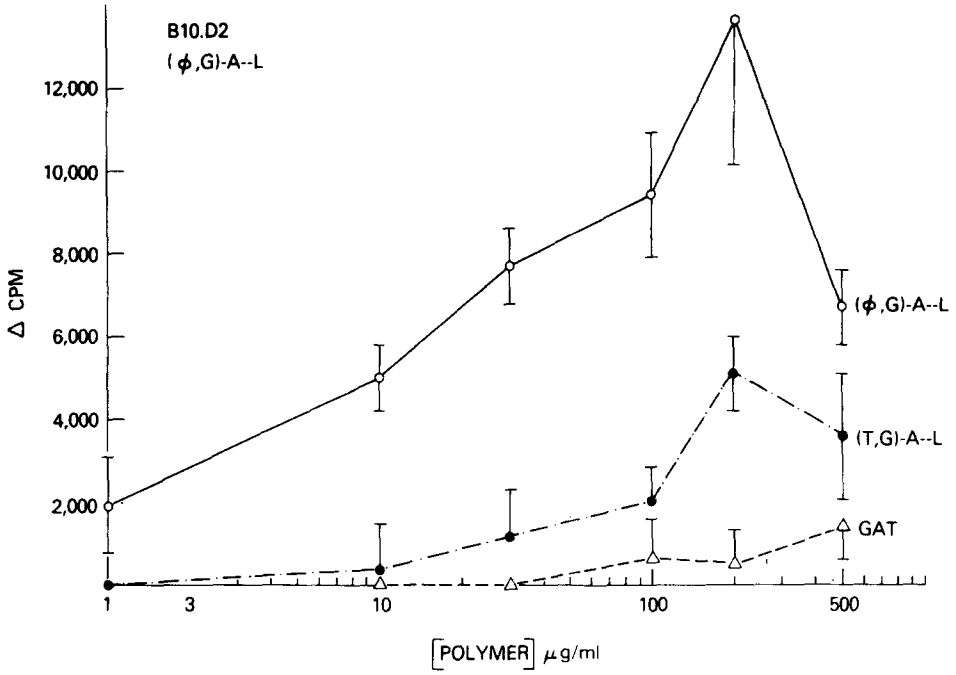

FIG. 3. B10.D2/nSn mice were immunized with 20 μg of (ϕ,G)-A--L. 3 wk later PETLES were prepared, and 2 × 10^5 cells were cultured with various concentrations of (T,G)-A--L (●), (ϕ,G)-A--L (○), or GAT (△) for 5 days in vitro. Stimulation was assessed by measuring the incorporation of tritiated methylthymidine by cells cultured with each polymer minus the incorporation by cells cultured with medium alone (Δcpm).

Thus, the genes controlling the *H-2*^{a} and *H-2*^{b} cross-reaction patterns are located in the *K* or *I-A* regions of the MHC (or possibly centromeric to the *K* region).

A similar analysis can be done to locate the genes controlling the cross-reaction pattern of B10 mice immune to (T,G)-A--L, although not as precisely (Table II). PETLES from B10.A mice immunized with (T,G)-A--L did not respond to (T,G)-A--L, GAT, or (Φ,G)-A--L. PETLES from B10 and A.BY mice immunized to (T,G)-A--L, on the other hand, did respond to (T,G)-A--L and both showed similar cross-reactions: namely, greater than 50% stimulation with GAT and (Φ,G)-A--L. These results map the genes controlling the B10 cross-reactions to the MHC. The fact that the B10.A(5R) also showed the same pattern as B10 and A.BY locates the genes to the *K*, *I-A*, or *I-B* subregions of the MHC (or the region centromeric to the *K* region).

The following general rules determining *I*-region-controlled cross-reactions are supported by the data in Tables II-V. (*a*) In order for any cross-reactions to occur, the strain being tested must respond to the immunogen: for example, PETLES from B10.A mice immunized with (T,G)-A--L did not respond to (Φ,G)-A--L, although they respond to (Φ,G)-A--L if immunized to it (Tables II and III). (*b*) The strain being tested must also be genetically capable of responding to an antigen as an immunogen in order for that antigen to elicit a cross-reaction: for example, B10.A and B10.BR mice immunized to (Φ,G)-A--L showed a weak cross-reaction to GAT, whereas SWR/J mice did not (Table III); B10.A and

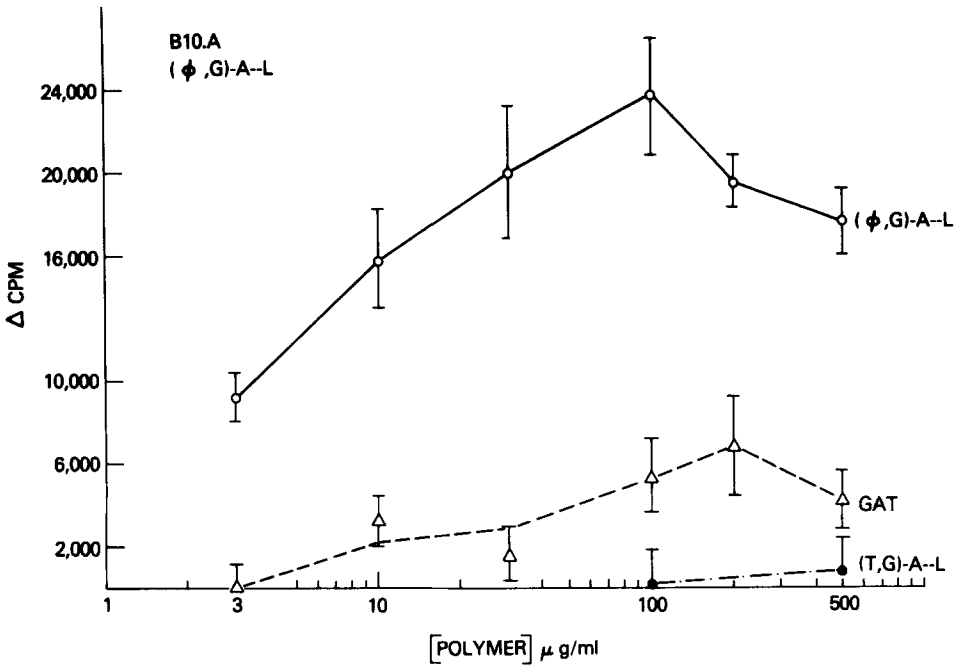

FIG. 4. B10.A/SgSn mice were immunized with 20 μg of (ϕ,G)-A--L. 3 wk later PETLES were prepared and 2 × 10^5 cells cultured with various concentrations of (T,G)-A--L (●), (ϕ,G)-A--L (○), or GAT (△) for 5 days in vitro. Stimulation was assessed by measuring the incorporation of tritiated methylthymidine by cells cultured with each polymer minus the incorporation by cells cultured with medium alone (Δcpm).

TABLE VI

Summary of Maximum Cross-Reactions and Half-Maximal Antigen Concentrations from the Dose-Response Curves

Strain	Immunogen	(T,G)-A--L		(Φ,G)-A--L		GAT	
		Percent of maximum immunogen response	[Polymer] at ½ maximum	Percent of maximum immunogen response	[Polymer] at ½ maximum	Percent of maximum immunogen response	[Polymer] at ½ maximum
			μg/ml		μg/ml		μg/ml
B10	(T,G)-A--L	100	15	50	11	80	13
B10.D2	(T,G)-A--L	100	3	70	14	127	9
B10	(Φ,G)-A--L	107	17	100	13	75	17
B10.D2	(Φ,G)-A--L	53	125	100	21	9	140
B10.A	(Φ,G)-A--L	4	≥180	100	5	29	40

Dose-response curves for (T,G)-A--L, (Φ,G)-A--L, and GAT were performed on PETLES from B10, B10.D2, or B10.A mice immune to 20 μg of (T,G)-A--L or (Φ,G)-A--L. The maximum responses of the cross-reacting polymers are expressed in the table as percentage of the maximum response for the immunogen. The concentration required of each polymer to achieve 50% of its maximal stimulation is given in micrograms per milliliter because the polymers are a heterogeneous population of molecules of differing molecular weights.

B10.BR mice are responders to GAT, while SWR/J is a nonresponder. (*c*) Responsive cross-reaction patterns appear to be dominant over nonresponsive cross-reaction patterns: for example, (B10 × B10.A)F_1 mice immunized to (Φ,G)-A--L showed strong cross-stimulations to (T,G)-A--L and GAT as did the B10 parent (Table III). However, codominant expression is not rigorously excluded by these data. Finally, points (*a*) and (*b*) are necessary to observe a cross-

reaction but not sufficient. Both (H,G)-A--L and (Φ,G)-A--L are immunogenic in B10.A mice. Yet, (Φ,G)-A--L will not cross-stimulate (H,G)-A--L immune B10.A PETLES and vice versa (Tables III and IV). This is also true for B10.A(4R) mice, where the genes controlling (Φ,G)-A--L cross-reaction patterns and (H,G)-A--L responsiveness have been located to the same portion of the MHC. This last point suggests that the fine specificity of the T-cell receptor can completely distinguish between two closely related antigens, even in strains that are genetic responders to both because they possess only the $H\text{-}2^a$ alleles of the *K* region and *I-A* subregion of the MHC.

Discussion

The fine specificity of cellular immune reactions has been studied by a number of investigators in both the guinea pig and the mouse. Whether measured by skin reactions, production of migration inhibition factor, or proliferation in vitro, the T-cell immune response could distinguish such differences as the position of a DNP residue (5), the addition or substitution of an amino acid (19, 20), or the location of a nitro group (6). In many respects the discriminatory power is similar to the fine specificity of antibody. On the other hand, T-cell specificity (or the specificity of T-cell activation) differs from that of B-cell receptors and of antibody in several respects. Among these are the carrier or conjugate specificity of responses to hapten-carrier conjugates (21), the high percentage of T cells specific for MHC gene products (22), and the differences in patterns of cross-reactivity (1–9).

In this paper, we present results that reveal certain of the factors that play an important role in regulating T-lymphocyte cross-reactions. The system studied was the cross-reactivity among branched-chain and linear synthetic polypeptides as measured by the stimulation of proliferation of T lymphocytes from primed donors. We chose this set of antigens because published studies using mouse strains of different histocompatability type indicated disparate results with regard to the existence of cross-reactivities. As suggested by one example from the recent work of Günther and Rüde in the rat (9) and as shown by us in the present studies, this discrepancy has a clear genetic basis. Our data indicate that in order for a cross-reaction to occur, the mouse strain must possess *Ir* genes allowing it to respond to both the immunogen and to the cross-reacting antigen, when the latter is used as an immunogen. Thus, the pattern of cross-reactions is regulated by MHC genes. For example, Oppenheim et al. (8) found, as did we, that T cells from C57BL mice immunized with (T,G)-A--L or (Φ,G)-A--L could be stimulated in culture by either antigen. Mice of the $H\text{-}2^b$ histocompatibility type, such as C57BL/6 and C57BL/10, possess an *Ir* gene (or genes) which allow them to respond to both polypeptides when they are used as immunogens. In contrast, T cells from B10.A mice immunized to (Φ,G)-A--L could not be stimulated by (T,G)-A--L (Table III). These mice possess *Ir* gene(s) which allow them to respond to immunization with (Φ,G)-A--L, but they are nonresponders to (T,G)-A--L.

One of the most interesting cross-reactions presented in this paper is the stimulation by the linear random terpolymer GAT of PETLES from mice immunized to the branched-chain copolymers. In B10 mice immunized with (T,G)-A--L or (Φ,G)-A--L, the cross-stimulation with GAT was 50–100%, imply-

ing the existence of the same or very similar antigenic determinant(s) in (T,G)-A--L, (Φ,G)-A--L, and GAT. For (T,G)-A--L in *H-2*^{b} mice the major antigenic determinants that elicit antibody under *Ir* gene control have been shown to be present in the defined polypeptides of the structure (T,T,G,G)-A--L (23), (T,T)-A--L (19), and (G,T)-A--L (19, 24). Since (T,G)-Pro--L and G-A--L did not stimulate PETLES from *H-2*^{b} mice immunized with (T,G)-A--L, it would suggest that the determinants recognized at the T-cell level are similar to those at the antibody level, i.e., $TTGG(A)_n$, $GT(A)_n$, and $TT(A)_n$, although this remains to be demonstrated. Thus, GAT must contain one or more of these determinants in part of its random sequence. For (Φ,G)-A--L the cross-reacting determinants in B10 mice would be $\Phi\Phi GG(A)_n$, $G\Phi(A)_n$, or $\Phi\Phi(A)_n$. This would imply that the T-cell antigen combining site in this strain can accommodate the presence or absence of the phenolic hydroxyl group.

In contrast, B10.A mice showed an entirely different pattern of cross-reactions in response to immunization with (Φ,G)-A--L. (T,G)-A--L did not cross-stimulate at all, and GAT gave at best a weak cross-reaction. Since B10.A mice lack an *Ir* gene allowing them to respond to (T,G)-A--L, it is not surprising that there is no response to the polypeptide. However, B10.A mice are responders to GAT. The ability of GAT to stimulate only meager responses in T cells from B10.A mice primed to (Φ,G)-A--L would suggest that B10.A cells react to determinants in (Φ,G)-A--L that are different from the proposed $\Phi\Phi GG(A)_n$, $G\Phi(A)_n$, and $\Phi\Phi(A)_n$ determinants that B10 cells respond to. Nonetheless, since G-A--L and (H,G)-A--L also failed to cross-stimulate, the determinants probably contain at least one phenylalanine. In fact, the determinant recognized in (Φ,G)-A--L might be the same for B10 and B10.A mice, but the receptor and/or the *Ir* gene product of B10.A might not be able to interact with the analogous tyrosine containing sequences in (T,G)-A--L and GAT because it can not accommodate the phenolic hydroxyl group.

The B10.D2 mouse appeared to be an intermediate strain between B10 and B10.A with regard to cross-reactions. When B10.D2 mice were immunized with (T,G)-A--L, both GAT and (Φ,G)-A--L gave greater than 50% cross-stimulation, although the concentration of antigen required to achieve half maximal stimulation was three to fourfold higher. The pattern is similar enough to that of the B10 strain to suggest that the determinants being recognized are those expressed by the sequences $TTGG(A)_n$, $GT(A)_n$, and/or $TT(A)_n$. In contrast, when B10.D2 mice were immunized with (Φ,G)-A--L, (T,G)-A--L showed only a partial cross-reaction (50% or less) and GAT stimulated weakly. Furthermore, the concentration of (T,G)-A--L or GAT required for half maximal stimulation was six to sevenfold higher than that required for (Φ,G)-A--L. These results suggest that the B10.D2 T cells respond principally to determinants on (Φ,G)-A--L other than those recognized by B10 T cells, although possibly not to the same determinants recognized by B10.A T cells.

Why the B10.D2 immune system, which has the capability of reacting with either set of determinants, should choose not to respond to the $TTGG(A)_n$, $GT(A)_n$, or $TT(A)_n$ determinants when challenged with (Φ,G)-A--L is not clear. What is clear is that this phenomenon of unequal or one-way cross-reactivity is controlled by MHC genes, since B10.D2 mice demonstrated it while B10 mice did not. The phenomenon was not limited to B10.D2 mice, however, as the most

striking example was found in B10 mice immunized with (T,G)-A--L or GAT. (T,G)-A--L immune B10 PETLES showed 50–100% cross-stimulation with GAT. In contrast, GAT immune B10 PETLES were barely stimulated at all by (T,G)-A--L. Thus, although GAT contains determinants similar to those found in (T,G)-A--L, when used as an immunogen in B10 mice GAT preferentially stimulates T cells which recognize other determinants on the molecule. Another example of one-way cross-reactivity involving (T,G)-A--L and (Φ,G)-A--L has been described in L.AVN rats by Günther and Rüde (9).

The data presented in this paper clearly show that the patterns of cross-stimulation are controlled by genes mapping in the *K* or *I* region of the MHC; however, they do not prove that these are the same genes that control the immune response to these antigens. This would certainly be the simplest possible interpretation. If so, it would provide another means for mapping *Ir* genes. For example, the (Φ,G)-A--L *Ir* gene(s) can not be mapped by B10.A recombinants because both B10 and B10.A mice respond to (Φ,G)-A--L. However, cross-reactions with (T,G)-A--L and GAT are different in the two strains. The failure of (T,G)-A--L to stimulate (Φ,G)-A--L immune B10.A PETLES might be attributed to the presence of the nonresponder allele of the *Ir-TGAL* gene; however the weak cross-reaction with GAT displayed by B10.A cells compared to the strong cross-reaction displayed by B10 cells cannot be attributed to a similar mechanism as both strains respond well to GAT. Since the B10.A (4R) and B10.A (5R) recombinants mapped the genetic control of the (Φ,G)-A--L cross-reactions to the *K* region or *I-A* subregion of both the $H\text{-}2^a$ and $H\text{-}2^b$ MHCs (Table III), this information can be used to infer that at least one *Ir-ΦGAL* gene maps in one of these two areas of the genome (or possibly centromeric to the *K* region). The most likely assumption is that an *Ir-ΦGAL* gene(s) maps in *I-A*, and that each allele, $Ir\text{-}\Phi GAL^a$ or $Ir\text{-}\Phi GAL^b$, has a different form of positive expression. (B10 × B10.A)F_1 mice immunized with (Φ,G)-A--L showed the B10 cross-reactive pattern. This is consistent either with dominance of the $Ir\text{-}\Phi GAL^b$ allele or with codominance of the two alleles.

Finally, the data presented here, in conjunction with the data of Lonai and McDevitt (7), indicate that the *Ir* gene(s) controlling responsiveness at the T-cell level to both (Φ,G)-A--L and (H,G)-A--L map in the *I-A* subregion of the MHC, yet no cross-reactions occur between these two antigens, which are similar in overall structure. Furthermore, the *Ir* genes controlling the response to several structurally unrelated antigens have also been definitively mapped to the *I-A* subregion, such as that for low dose ovalbumin (25), or provisionally mapped to this subregion, such as that for IgA myeloma proteins (26). These results would seem to imply that the fine specificity of the T-cell proliferative and antibody responses to all of these antigens is controlled by the genes contained in the relatively short segment of chromosome encompassed by the *I-A* subregion. Although this subregion could possibly code for a unique set of T-cell variable region genes (27), the data on shared idiotypes between T and B cells (28, 29) suggest that at least one set of T-cell variable region genes is coded for outside the MHC. Thus, if *Ir* genes achieved control of T-cell specificity by influencing or being a part of the T-cell receptor, one would have to postulate that *Ir* gene products are responsible for selecting which non-MHC variable region genes are expressed as receptors on responding clones. On the other hand, *Ir* gene products

may exert their effect in antigen-presenting cells, such as macrophages, by combining with antigen on the surface of this cell to form new determinants (complex antigenic determinants:CADs) which can or can not be recognized by the available set of T-lymphocyte receptors. If the CADs are recognized, the strain would be a responder to the antigen; if the CADs are not recognized, the strain would be a nonresponder. The data presented in this paper do not allow us to distinguish between these models.

Summary

Antibodies raised against many structurally related antigens have been shown to cross-react extensively. Manifestations of T-cell immunity, on the other hand, appear to be more restricted in their ability to be elicited by cross-reacting antigens, although examples have been reported. This paper explores the nature of the cross-reactions at the T-cell level among the branched-chain copolymers (T,G)-A--L, (Φ,G)-A--L, (H,G)-A--L, (T,G)-Pro--L, and G-A--L, as well as a related linear terpolymer, GAT, in a variety of mouse strains using the peritoneal exudate T-lymphocyte-enriched cells (PETLES) proliferation assay. (T,G)-A--L, (Φ,G)-A--L, and GAT could cross-stimulate cells immune to the other two antigens, whereas (H,G)-A--L, (T,G)-Pro--L, and G-A--L showed no cross-stimulations. The extent of the cross-reactions varied with the mouse strain and was shown to be under the control of immune response genes. It was necessary for the strain to be able to respond to both the immunogen and the cross-reacting antigen, when used as an immunogen, in order for cross-stimulation to occur; however, this was not always sufficient. Several examples of unequal or one-way cross-reactions were found. In addition, the immune responses to (H,G)-A--L and (Φ,G)-A--L showed no cross-reactions with the other antigen even though their *Ir* genes were both mapped to the *K* region or *I-A* subregion. The problem of accounting for such fine specificity of T-cell recognition in lieu of the genetic evidence demonstrating only *Ir* gene control of the response is discussed.

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