

# BIFUNCTIONAL MAJOR HISTOCOMPATIBILITY-LINKED GENETIC REGULATION OF CELL-MEDIATED LYMPHOLYSIS TO TRINITROPHENYL-MODIFIED AUTOLOGOUS LYMPHOCYTES

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In vitro sensitization with trinitrophenyl (TNP)<sup>1</sup>-modified autologous spleen cells has been shown to generate weak mixed lymphocyte reactions (MLR) (1) and strong cell-mediated lympholysis (CML) (1–3). The lysis by cytotoxic effector cells resulting from such sensitization was directed primarily against TNP-modified cell surface components controlled by the serological (*H-2K* and *H-2D*) regions of the murine major *H-2* histocompatibility complex (MHC) (3). Different levels of cytotoxic activity were generated against TNP-modified products controlled by various alleles of the *K* and *D* regions in the four C57BL/10 congenic lines of mice investigated. It is well established that the *I* region of the *H-2* complex controls immune response potential to a number of immunogens (4). On the other hand, the new antigenic determinants resulting from TNP modification which are detected by CML are controlled by the serological regions of the *H-2* complex. Therefore, the differential levels of cytotoxic reactivity detected could be attributed to *H-2*-linked *Ir* genes expressed in the responding cell population and/or to the relative immunogenicity of the new antigenic determinants created.

Phenotypic expression of *Ir* genes controlling responsiveness to many immunogens has been shown to be a dominant characteristic (4). Thus, by comparing the responses generated by lymphocytes from high and low responders with that generated by lymphocytes from an F<sub>1</sub> between high and low responder parents against modified parental and F<sub>1</sub> stimulating cells, it was possible to determine the relative contribution of immune response potential and immunogenicity in the generation of lympholysis. Evidence is presented indicating that the immunogenicity of the new antigenic determinants resulting from TNP modification of cell surface components controlled by the *d* alleles of both *H-2K* and *H-2D* is relatively weak in stimulating CML. The presence of *k* alleles in the *K*, *I-A*, and *I-B* regions also appears to be associated with the generation of an even weaker CML to TNP-modified *H-2D<sup>d</sup>* products. In contrast, the F<sub>1</sub> responding lymphocytes which express *K*, *I-A*, and *I-B* alleles of both parents generated higher responses. This latter observation is compatible with an additional contribution of an *H-2*-linked *Ir* gene.

## Materials and Methods

*Mice.* Mice used in the experiments were males, 7–9 wk of age. The B10 congenic strains were purchased from the Jackson Laboratories, Bar Harbor, Maine. The B10.A(2R), B10.A(4R), and

<sup>1</sup> Abbreviations used in this paper: CML, cell-mediated lympholysis; MHC, major histocompatibility complex; TNBS, 2,4,6-trinitrobenzene sulfonate; TNP, trinitrophenyl.

B10.A(5R) recombinant lines were raised in our mouse colony from breeders obtained either from the Jackson Laboratories or from Dr. Donald Shreffler, Dept. of Human Genetics, Univ. of Michigan, Ann Arbor, Mich. The A.TL and A.TH mice were raised in our mouse colony from breeders obtained from Dr. Donald Shreffler.

*Trinitrophenyl Modification of Spleen Cells.* Cells to be modified with TNP were prepared from the spleen of young adult mice as previously described (3). The cell pellet was suspended in a phosphate-buffered solution containing 10 mM 2,4,6-trinitrobenzene sulfonate (TNBS) (Pierce Chemical Co., Rockford, Ill.), pH 7.3 at a vol:vol ratio of cells to reagent of 1:4. The cell reagent mixture was incubated at 37°C for 10 min and then washed twice in Hank's balanced salt solution containing fetal bovine serum. Spleen cells to be used for sensitization and for targets were modified with TNBS in an identical fashion. Cells to be used as cytotoxic targets were labeled with  $\text{Na}^{51}\text{CrO}_4$  (Amersham/Searle Corp., Arlington Heights, Ill.) for 30 min at 37°C before modification with TNBS.

*In Vitro Cell Culture and Assay for Cytotoxic Effector Cells.* Splenic lymphocytes were sensitized in vitro with TNP-modified cells as previously described (1). The culture medium was supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol. After a 5-day sensitization period, effector cells were harvested, their cytotoxic reactivity determined, and the mean and standard error of the percentage of specific lympholysis was calculated as described elsewhere (1). The effector:target cell ratio used was 20:1 throughout these experiments. Nonspecific lysis of TNP-modified,  $^{51}\text{Cr}$ -labeled splenic target cells was 15–32%, which was subtracted from the total lysis to obtain the specific lympholysis.

## Results

An earlier study indicated that B10.A effector cells generated by sensitizing B10.A responding lymphocytes with TNP-modified autologous spleen cells exhibited stronger reactivity to TNP-modified *K*-end products than to modified *D*-end products (3). In contrast, B10.D2 effector cells in the TNP autologous CML system generated equal reactivity to altered *K* and *D* products (3). The results presented in Table I extend and verify this observation. B10.A effector cells lysed B10.A-TNP and B10.BR-TNP target cells but not B10.D2-TNP targets, although B10.D2 shares *I-C*, *S*, and *D* regions with B10.A. However, effector cells from B10.D2 donors sensitized with modified B10.D2 spleen cells lysed B10.A-TNP targets (which share the same *I-C*, *S*, and *D* alleles) as well as the syngeneic B10.D2-TNP targets. This indicates that the modified *d* alleles of the *D*-end serve as adequate target antigens for lympholysis. B10.A lymphocytes were sensitized against B10.D2-TNP stimulating cells in order to determine whether the failure of B10.A responding cells to generate activity to the *D*-end was due to a failure of response potential or to weak immunogenicity of the new antigenic determinants resulting from modification of *H-2D<sup>d</sup>* on the B10.A spleen cells. Sensitization of B10.A responding lymphocytes with B10.D2-TNP stimulating cells resulted in enhanced CML tested on B10.A-TNP targets. However, the same effector cells lysed TNP-modified B10.BR targets which share no known H-2 private specificities with the B10.D2 stimulating cells. This result raised the possibility that allogeneic sensitization could lead to effector cells which would nonspecifically lyse any TNP-modified target. This possibility was tested by sensitizing B10.A responding lymphocytes with unmodified B10.D2 spleen cells. The results (also shown in Table I) indicate that sensitization with unmodified allogeneic stimulating cells generates effector cells which can lyse TNP-modified target cells syngeneic with the responder. Additional data extending this point and illustrating that TNP-modified splenic targets not syngeneic with either the responding or stimulating cells can be lysed by effector

TABLE I  
 Differential Cytotoxic Activity Generated by B10.A and B10.D2 Responding Cells to  
 TNP-Modified Products of the *d* Allele of H-2D

Responding cell	Stimulating cell	Target cell	Specific lysis	TNP-modified target cell H-2 region common to stimulating cell	Responding cell H-2 region allogeneic to both stimulating and target cell	
B10.A kkkddd	B10.A-TNP kkkddd-TNP	B10.A-TNP kkkddd-TNP	$33.4 \pm 2.3$	All of H-2	None	
		B10.D2-TNP dddddd-TNP	$-3.7 \pm 4.0$	I-C, S, D	None	
		B10.BR-TNP kkkkkk-TNP	$25.8 \pm 4.0$	K, I-A, I-B	None	
	B10.D2-TNP dddddd-TNP	B10.A-TNP kkkddd-TNP	$22.7 \pm 2.2$	I-C, S, D	None	
		B10.D2-TNP dddddd-TNP	$23.3 \pm 4.1$	All of H-2	K, I-A, I-B	
		B10.BR-TNP kkkkkk-TNP	$12.3 \pm 2.8$	None	None	
	B10.D2 dddddd	B10.A-TNP kkkddd-TNP	B10.A-TNP kkkddd-TNP	$17.1 \pm 2.2$	None	None
			B10.D2-TNP dddddd-TNP	$30.4 \pm 5.1$	None	K, I-A, I-B
			B10.BR-TNP kkkkkk-TNP	$10.2 \pm 2.1$	None	None
	B10.D2 'dddddd	B10.D2-TNP dddddd-TNP	B10.A-TNP kkkddd-TNP	$12.8 \pm 2.2$	I-C, S, D	None
			B10.D2-TNP dddddd-TNP	$11.2 \pm 1.9$	All of H-2	None
			B10.BR-TNP kkkkkk-TNP	$5.0 \pm 3.0$	None	None
B10.A-TNP kkkddd-TNP		B10.A-TNP kkkddd-TNP	$44.4 \pm 0.6$	All of H-2	K, I-A, I-B	
		B10.D2-TNP dddddd-TNP	$15.4 \pm 3.5$	I-C, S, D	None	
		B10.BR-TNP kkkkkk-TNP	$33.3 \pm 3.2$	K, I-A, I-B	K, I-A, I-B	
B10.A kkkddd		B10.A-TNP kkkddd-TNP	B10.A-TNP kkkddd-TNP	$50.7 \pm 1.3$	None	K, I-A, I-B
			B10.D2-TNP dddddd-TNP	$8.1 \pm 1.0$	None	None
			B10.BR-TNP kkkkkk-TNP	$35.3 \pm 2.4$	None	K, I-A, I-B

cells generated by allogeneic sensitization are summarized in Table II. These results indicate that the question of weak immunogenicity of new antigenic determinants resulting from TNP modification of H-2 products cannot be approached by introducing allogeneic, modified stimulator cells. Furthermore, experimental protocols involving allogeneic sensitization cannot be used for investigating the specificity involved in the TNP-modified CML.

TABLE II  
*Specificity of Target Lysis by Spleen Cells Sensitized Against Modified Syngeneic or Unmodified Allogeneic Cells*

Re- spond- ing cell	Stimulating cell	Target cells					
		B10.A (kkkddd)		C57BL/10 (bbbbbb)		B10.D2 (dddddd)	
		Unmodified	-TNP	Unmodified	-TNP	Unmodified	-TNP
B10.A kkkddd	B10.A-TNP kkkddd-TNP	% ± SE -1.9 ± 1.5    36.1 ± 1.5		% ± SE 1.0 ± 0.9    7.0 ± 2.0		% ± SE -2.2 ± 1.2    7.9 ± 2.5	
	C57BL/10 bbbbbb	0.3 ± 1.4	11.9 ± 1.6	34.9 ± 3.1	37.5 ± 2.5	6.2 ± 0.9	14.6 ± 3.9
	B10.D2 dddddd	3.7 ± 1.1	11.7 ± 0.7	11.5 ± 0.9	13.9 ± 1.9	28.7 ± 1.2	38.0 ± 2.0
B10.BR kkkkkk	B10.BR-TNP kkkkkk-TNP	B10.BR (kkkkkk)		C57BL/10 (bbbbbb)		B10.D2 (dddddd)	
		Unmodified	-TNP	Unmodified	-TNP	Unmodified	-TNP
		1.1 ± 0.7	38.5 ± 1.1	0.2 ± 0.9	9.7 ± 1.5	3.0 ± 0.7	6.7 ± 1.5
	C57BL/10 bbbbbb	0.8 ± 0.9	15.2 ± 1.4	30.4 ± 1.5	37.2 ± 1.4	16.5 ± 1.0	22.6 ± 3.3
	B10.D2 dddddd	0.3 ± 1.2	23.1 ± 1.8	8.5 ± 1.0	19.8 ± 1.1	41.4 ± 2.1	38.9 ± 2.1

It was also observed in Table II that effector cells generated by sensitization of B10.A responding lymphocytes with C57BL/10 and with B10.D2 spleen cells significantly lysed to a lesser extent B10.D2 and C57BL/10 targets, respectively. Since the only known common H-2-associated specificity shared between C57BL/10 and B10.D2 and not expressed in B10.A is Ia8 (5), the possibility must be considered that the weak CML detected may have been to this *I*-region product. Another possibility is that a yet undescribed public specificity is shared between *b* and *d* alleles of the *K* region (Sachs, D., personal communication). Similar results were obtained using B10.BR responding cells (lower part of Table II). However, in this case the CML could also have been directed against the H-2D public specificities 6, 27, 28, and 29 (6).

In order to determine whether the negative or very weak CML generated by B10.A responding lymphocytes to the TNP-modified cell surface components controlled by H-2D<sup>d</sup> were due to a responding cell defect (possibly controlled by *H-2*-linked *I*<sub>r</sub> genes), responding lymphocytes from (B10.A × B10.D2)F<sub>1</sub> donors were sensitized with TNP-modified F<sub>1</sub>, B10.A, or B10.D2 stimulating cells. The effector cells generated were assayed on modified and unmodified B10.A and B10.D2 target spleen cells. The results are summarized in Table III. In Exp. 1, (B10.A × B10.D2)F<sub>1</sub> responding cells generated from 36.9 to 40.6% lympholysis toward modified H-2K<sup>k</sup> products when sensitized with either modified F<sub>1</sub> or B10.A stimulating cells. These data are comparable to the lysis obtained from effector cells generated by sensitization of B10.A splenic lymphocytes with modified syngeneic cells assayed on B10.A-TNP targets (see Table I). Effector

TABLE III  
 Dominant Genetic Expression of Cytotoxicity by (B10.A × B10.D2)F<sub>1</sub> Responding Cells to TNP-Modified Products  
 of the *d* Allele of H-2D

Responding* cell	Stimulating cell	Target cell	Specific lysis			Target cell H-2 region common to both responding cell and stimulating cell
			Exp. 1	Exp. 2	Exp. 3	
(B10.A × B10.D2)F <sub>1</sub> kkkddd dddddd	(B10.A × B10.D2)F <sub>1</sub> -TNP kkkddd-TNP dddddd-TNP	B10.A-TNP kkkddd-TNP	36.9 ± 3.5	28.5 ± 2.3	49.9 ± 5.5	All of H-2 <sup>a</sup>
		B10.D2-TNP dddddd-TNP	17.9 ± 1.5	13.4 ± 2.6	15.7 ± 3.5	All of H-2 <sup>d</sup>
	B10.A-TNP kkkddd-TNP	B10.A-TNP kkkddd-TNP	40.6 ± 4.0	35.6 ± 3.3	42.3 ± 3.7	All of H-2 <sup>a</sup>
		B10.D2-TNP dddddd-TNP	12.4 ± 2.7	15.2 ± 2.4	20.0 ± 3.9	I-C, S, D
		B10.A	1.2 ± 1.7	NT	NT	
	B10.D2-TNP dddddd-TNP	B10.A-TNP kkkddd-TNP	13.2 ± 3.2	NT	NT	I-C, S, D
		B10.D2-TNP dddddd-TNP	19.6 ± 1.9	NT	NT	All of H-2 <sup>d</sup>
		B10.D2	6.0 ± 1.6	NT	NT	

\* For comparison of lysis generated by the parental B10.A and B10.D2 responding cells with that generated by (B10.A × B10.D2)F<sub>1</sub> responding cells in the same experiment, see Table I for Exp. 1. In Exp. 2 and 3 the lysis generated by B10.A responding cells sensitized by B10.A-TNP stimulating cells were respectively 29.7 ± 3.0 and 31.8 ± 2.4 on B10.A-TNP targets and 4.9 ± 2.2 and 5.8 ± 4.3 and B10.D2 targets.

cells generated by sensitization of  $F_1$  lymphocytes with B10.A-TNP stimulating cells produced 17.4% lysis to the modified H-2D<sup>d</sup> products as assayed on B10.D2-TNP targets. This is the same level of lympholysis that was obtained when identical  $F_1$  responding lymphocytes were sensitized with B10.D2-TNP stimulating cells and assayed on B10.A-TNP target cells. The latter reaction also detects CML directed toward modified H-2D<sup>d</sup> products. These levels of reactivity generated by (B10.D2  $\times$  B10.A) $F_1$  lymphocytes directed against modified H-2D<sup>d</sup> products are similar to those obtained from B10.D2 responding cells toward the modified syngeneic H-2D<sup>d</sup> products (12.8%, Table I), but contrasts to those obtained by sensitization of B10.A responding lymphocytes with modified syngeneic cells assayed on B10.D2-TNP targets (-3.7%, Table I). Similar results are shown in Exp. 2 and 3, Table III. The negative controls assayed on unmodified parental targets indicated that no  $F_1$  antiparent CML was detected in these experiments. The different reactivities generated toward the modified H-2D<sup>d</sup> products by B10.A and B10.D2 responding lymphocytes cannot be attributed to a weaker immunogenicity of the modified H-2D<sup>d</sup> products expressed on B10.A as compared with B10.D2 stimulating cells, but appears to be due to a responder cell defect in the B10.A strain, which is expressed as a dominant trait in the  $F_1$ .

Responding lymphocytes from (B10.D2  $\times$  B10.A) $F_1$  donors generated CML toward modified H-2K<sup>k</sup> products in the range of 35–40% lysis. In contrast, identical responding cells generated CML toward modified H-2K<sup>d</sup> and H-2D<sup>d</sup> in the range of 15–20% lysis. This observation raised the possibility that TNP-modified products controlled by *d* alleles mapping in the *K* and *D* serological regions were less immunogenic than the TNP-modified *k* allele of *H-2K*. To test this possibility, the same pool of (B10.A  $\times$  B10.D2) $F_1$  responding lymphocytes were sensitized by modified  $F_1$ , B10.A, or B10.D2 stimulating cells, and the resulting effectors assayed on a common modified (B10.A  $\times$  B10.D2) $F_1$  target. The results, shown in Table IV, verify that the new antigenic determinant formed by TNP-modification of H-2K<sup>k</sup> products are more immunogenic than those formed by modification of H-2K<sup>d</sup> products. In this case, no differential contribution to immunogenicity could have been made by modified *D*-end products, since the modified H-2D<sup>d</sup> products were common in both immunogens. A comparison of the levels of cytotoxicity generated by  $F_1$ , B10.A, or B10.D2 lymphocytes against SJL alloantigens (lower part of Table IV) verified that the CML to TNP-modified autologous H-2 products is about as strong as that to alloantigens (7). Furthermore, equal responses were obtained to the SJL alloantigens with all three strains of responding cells, despite the fact that differential levels of lympholysis were obtained in the various combinations for TNP-modified autologous reactions.

In an earlier report, effector cell specificity generated in the TNP-autologous system was demonstrated in four C57BL/10 congenic strains to be directed toward modified products mapping in or near the *K* and *D* serological regions (3, 7). The results shown in Table V and VI extend the previous data to include modified autologous CML reactions using the two B10.A recombinants B10.A(4R) and B10.A(5R), as well as [B10.A(4R)  $\times$  B10.A(5R)] $F_1$ . This pair of recombinants is of particular interest in that it introduces new combinations of *K* and *D* alleles for comparison of cytotoxicity levels with previous examples.

TABLE IV  
*Differences in Immunogenicities of TNP Modified B10.A and Modified B10.D2 Cells Stimulating (B10.A × B10.D2)F<sub>1</sub>, B10.A, or B10.D2 Lymphocytes and Absence of Differential Reactivities Toward the H-2<sup>s</sup> Alloantigens*

Responding cell	Stimulating cell	Target cell	Specific lysis	
			Exp. 1	Exp. 2
			% ± SE	
(B10.A × B10.D2)F <sub>1</sub>	(B10.A × B10.D2)F <sub>1</sub> -TNP kkkkdd-TNP dddddd-TNP	(B10.A × B10.D2)F <sub>1</sub> -TNP kkkkdd-TNP dddddd-TNP	33.6 ± 4.1	31.4 ± 6.8
	B10.A-TNP kkkkdd-TNP	"	37.1 ± 2.9	26.0 ± 3.9
	B10.D2-TNP dddddd-TNP	"	10.4 ± 1.9	18.3 ± 3.0
B10.A	B10.A-TNP kkkkdd-TNP	"	31.9 ± 3.9	31.5 ± 1.7
B10.D2	B10.D2-TNP dddddd-TNP	"	11.9 ± 3.6	17.4 ± 1.6
(B10.A × B10.D2)F <sub>1</sub>	SJL ssssss	SJL ssssss	30.5 ± 4.0	NT
B10.A	"	"	30.7 ± 2.7	NT
B10.D2	"	"	33.0 ± 2.9	NT

Furthermore, the CML specificities generated can be more diverse due to the greater polymorphism of the *K* and *D* region of the (4R × 5R)F<sub>1</sub>.

Responding lymphocytes from B10.A(4R) donors sensitized with TNP-modified autologous spleen cells generated effector cells specific for modified *D*-end as well as modified *K*-end H-2 products (Table V). Both B10.BR-TNP and B10.A-TNP targets were as effective as modified syngeneic targets in detecting lympholysis. This indicates CML specificity to altered *K* or *I-A* products. The negative results obtained with A.TL-TNP targets indicates that the *K*-end specificity is directed toward altered products controlled by *K* alone or by *K* plus *I-A*. C57BL/10-TNP targets were also effective in detecting lysis by 4R effector cells. This indicates specificity toward the products of a region or regions to the right of *I-A*—which could be *H-2D*. The *I-B* region can be excluded, since the B10.A(5R)-TNP target was not lysed. The 11.9% lysis obtained with the B10.HTG target was lower than expected if the 27.1% lysis detected with B10-TNP targets were directed exclusively toward modified H-2D products. Nevertheless, the data obtained using the modified B10.HTG target indicates that a portion of the lytic specificity was directed to altered cell surface components controlled by H-2D. Responding splenic lymphocytes from B10.BR donors sensitized with modified autologous spleen cells are also included in this experiment for comparison with the 4R results (Table V). The results verify those reported previously (3, 7), and are similar in magnitude for modified H-2K<sup>k</sup> products to that obtained with 4R responding cells. It is noteworthy that the 4R responding lymphocytes generated effector cells to autologous modified H-2D products, whereas B10.BR responding lymphocytes did not.

TABLE V  
*In vitro* Induction of Cell-Mediated Lympholysis in B10.A5R and B10.A4R  
 Recombinants, and in B10.BR Spleen Cells to TNP-Modified Syngeneic Lymphocytes

Responding cell	Stimulating cell	Target cell	Specific lysis	Target cell H-2 region common to responding and stimulating cells
			% ± SE	
B10.A4R kkbbbb	B10.A4R-TNP kkbbbb-TNP	B10.A4R-TNP kkbbbb-TNP	47.1 ± 4.1	All of H-2
		B10.BR-TNP kkkkkk-TNP	45.4 ± 5.1	K, I-A
		B10.A-TNP kkkddd-TNP	31.9 ± 2.8	K, I-A
		ATL-TNP skkkkd-TNP	5.5 ± 2.6	I-A
		B10.A5R-TNP bbdddd-TNP	5.6 ± 1.7	I-B
		C57BL/10-TNP bbbbbb-TNP	27.1 ± 3.3	I-B, I-C, S, D
		B10.HTG-TNP ddddd-TNP	11.9 ± 2.4	D
		B10.D2-TNP ddddd-TNP	0.6 ± 2.8	None
		(B10.A4R × B10.A5R) F <sub>1</sub> -TNP kkbbbb-TNP bbdddd-TNP	43.4 ± 2.3	All of H-2
		B10.BR kkkkkk	B10.BR-TNP kkkkkk	B10.BR-TNP kkkkkk-TNP
B10.A-TNP kkkddd-TNP	32.3 ± 4.3			K, I-A, I-B
C3H.OH-TNP ddddd-TNP	2.8 ± 2.2			D
B10.D2-TNP ddddd-TNP	1.0 ± 0.9			None
B10-TNP bbbbbb-TNP	6.1 ± 2.7			None
B10.A5R bbbbdd	B10.A5R-TNP bbbbdd	B10.A5R-TNP bbbbdd-TNP	19.0 ± 2.2	All of H-2
		C57BL/10-TNP bbbbbb-TNP	16.6 ± 3.0	K, I-A, I-B
		B10.A4R-TNP kkbbbb-TNP	-0.11 ± 1.9	I-B
		B10.D2-TNP ddddd-TNP	13.9 ± 1.8	I-C, S, D
		B10.HTG-TNP ddddd-TNP	1.7 ± 1.5	I-C, S
		B10.BR-TNP kkkkkk-TNP	8.1 ± 2.5	None
		(B10.A4R × B10.A5R)-TNP kkbbbb-TNP bbbbdd-TNP	14.2 ± 1.2	All of H-2

TABLE VI  
*In Vitro Induction of Cell-Mediated Lympholysis in (B10.A4R × B10.A5R)F<sub>1</sub> Lymphocytes to TNP-Modified Syngeneic Spleen Cells*

Responding cell	Stimulating cell	Target cell	Specific lysis	Target cell H-2 region common to responding and stimulating cells
(B10.A4R × B10.A5R)F <sub>1</sub> kkbbbbb bbbddd	(B10.A4R × B10.A5R)F <sub>1</sub> -TNP	(B10.A4R × B10.A5R)F <sub>1</sub> -TNP kkbbbbb bbbddd	35.9 ± 3.2	All of H-2
		B10.A4R-TNP kkbbbbb	39.4 ± 2.7	All of H-2 <sup>ki</sup>
		B10.A5R-TNP bbbddd	22.7 ± 3.0	All of H-2 <sup>i</sup>
		C57BL/10-TNP bbbbbbb	19.5 ± 2.2	All of H-2 <sup>b</sup>
		B10.BR-TNP kkkkkkk	41.5 ± 1.2	K, I-A
		B10.D2-TNP ddddddd	20.2 ± 1.2	(I-C, S, D) <sup>d</sup>
		B10.HTG-TNP dddddb	15.4 ± 3.0	(I-C, S) <sup>d</sup> , D <sup>b</sup>

The CML responses generated by sensitizing B10.A(5R) with TNP-modified autologous cells are summarized in the lower part of Table V. In general, the magnitude of lytic responses were lower than that observed using 4R cells. B10.A(5R) effector cells lysed both B10-TNP and B10.D2-TNP targets as well as modified syngeneic targets, indicating that equivalent lytic responses were generated to altered products of the *K*, *I-A*, *I-B* regions and the *I-C*, *S*, *D* regions of *H-2*. That modified products of the *I-B* or *I-C*, *S* regions could have been effective targets alone was excluded by the absence of lysis on B10.A(4R)-TNP and B10.HTG-TNP targets. A weak but insignificant response was detected using B10.BR-TNP targets which share no known major *H-2* regions with the stimulating cells. The lower magnitude of lysis of B10.A(5R) effectors when compared with B10.A(4R) effectors assayed on (4R × 5R)F<sub>1</sub>-TNP targets, which express all four *K* and *D* alleles, indicates a weaker level of sensitization of the B10.A(5R) responding cells.

Since the (4R × 5R)F<sub>1</sub> lymphocytes express cell surface components controlled by all four of the *K* and *D* alleles contributed by the parental strains, TNP-modification of (4R × 5R)F<sub>1</sub> spleen cells should result in the formation of a greater diversity of altered self antigens. To test this possibility, (4R × 5R)F<sub>1</sub> splenic lymphocytes were sensitized with TNP-modified autologous spleen cells. The effector cells generated were assayed on a number of modified congenic and recombinant target spleen cells (Table VI). TNP-modified F<sub>1</sub> and both the modified B10.A(4R) and B10.A(5R) target cells were lysed by F<sub>1</sub> effector cells. However, the extent of lysis of the B10.A(5R) targets was not as high as that obtained with the F<sub>1</sub> and B10.A(4R) targets, although it was equal to the lysis

produced by B10.A(5R) effectors (Table V). The lysis of B10.BR-TNP, B10.D2-TNP, and B10.HTG-TNP by (4R × 5R)F<sub>1</sub> effector cells detected specificities to modified self components controlled by H-2K<sup>k</sup>, H-2D<sup>d</sup>, and H-2D<sup>b</sup>, respectively. The extent of lysis obtained by the F<sub>1</sub> effector cells for each specificity was similar to that observed by the parental strain expressing the specificity detected on a given target. These results demonstrate that a greater number of distinct new antigenic specificities are created as a result of TNP modification of H-2 products of F<sub>1</sub> lymphocytes (at least three) than of inbred parental lymphocytes (only two).

### Discussion

Since the recent independent discoveries that viruses (8–12), and chemicals (1–3) can alter cell surface products controlled by the serological regions of the *H-2* complex to create new cell-bound antigens, a potentially relevant autologous function has been ascribed for the structural antigens controlled by the MHC (3). Hence, lymphocytic choriomeningitis (8–10), ectromelia (11), and vaccinia (12) viruses, and trinitrobenzene sulfonate (1, 3, 7) modified cells stimulate syngeneic (or autologous) lymphocytes to generate thymus-derived effector cells capable of lysing only target cells which are modified by the same agent and which express common *K* or *D* serological regions with the modified stimulator cells.

It was previously observed that effector cells generated by TNP-autologous sensitization of B10.A lymphocytes did not appreciably lyse TNP-modified splenic targets expressing common *D*-end antigens, although they did effectively lyse modified common *K*-end targets (3, 7). In contrast, B10.D2, which expresses the same *I-C*, *S*, and *D*, but different *K*, *I-A*, and *I-B* alleles responded to both TNP-modified *K* and *D* products (3, 7). Thus, there was a differential effect on the response to TNP-modified H-2D<sup>d</sup>, which was not directly associated with the region controlling the antigen itself, but was influenced by alleles in the "left half" of *H-2* (*K*, *I-A*, *I-B*). This could have been due to: (a) an *H-2*-linked *I<sub>r</sub>* gene controlling response potential and mapping in one of the known *I* regions; or (b) an influence of the new antigenic determinants created by modification of *K*-end products on the immunogenicity of modified components controlled by the *D*-end.

By sensitizing B10.A lymphocytes with B10.D2-TNP stimulating cells, attempts were made to test the relative immunogenicity of TNP-modified H-2D<sup>d</sup> products without the possible effects of altered antigens controlled by H-2K<sup>k</sup>. As shown in Tables I and II, however, semiallogeneic or allogeneic CML reactions with unmodified stimulating cells generated effectors which lysed to a variable extent (8.1–23.1%) any TNP-modified target spleen cell. This observation illustrates the importance of using only syngeneic mixtures of responder and stimulating cells in the sensitization phase of the CML. Thus, experiments designed to test "TNP-hapten-specific" CML by this protocol (13) can lead to lysis of "hapten-modified" targets, which, however, is unrelated to the presence of the "hapten" on the stimulating cell. The mechanism of this nonspecific lysis is unknown.

The results summarized in Table III demonstrate that F<sub>1</sub> responding lympho-

cytes can generate equivalent CML responses specific for modified H-2D<sup>d</sup> products when sensitized either with B10.A or B10.D2 stimulating cells modified with TNP. The levels of lympholysis detected ranged from 12.4 to 20.0% using F<sub>1</sub> effectors, which were indistinguishable from the levels of lysis produced by B10.D2 effectors toward modified D-end products (12.8–19.2%) (3, 7), but higher than that by B10.A (–3.7–5.2%). These results indicate that the new cellular antigens resulting from TNP modification of H-2D<sup>d</sup> products are expressed to the same extent on B10.A and B10.D2 spleen cells. Furthermore, the lack of a CML response in the B10.A to this new antigenic specificity appears to be due to a responding cell defect which is associated with genes mapping in the *K*, *I-A*, or *I-B* regions of the MHC. This effect may be attributable to an *Ir* gene, which is expressed as a dominant trait by a *d* allele in the relevant *I* or *K* region.

A genetic defect in cellular immunity controlled by the MHC can be visualized either as a control by that region on the repertoire of T-cell receptors specific for new cellular antigens, i.e., on the available clones, or as a defect at the level of the cooperation of two cell populations known to act synergistically in the generation of cytotoxic effector cells (14, 15). The expression of the genetic defect at the level of cell-to-cell interaction could be analogous to that suggested for the mechanism of action of *Ir* genes controlling antibody production to thymus-dependent antigens (16, 17). A distinction between these two alternatives has not yet been realized.

Genetic control of T-cell-mediated immunity in the mouse has been recently reported for stimulation of thymidine incorporation (18, 19) and delayed hypersensitivity to the multichain synthetic polypeptide polyL(Tyr,Glu)-polyDLAla--polyLys<sup>2</sup> which is under *H-2*-linked genetic control (20), cell-mediated lympholysis to P-815 mastocytoma (21), and for delayed hypersensitivity to contact sensitization with picryl chloride (22). In contrast to what is shown in the present report, responsiveness to picryl chloride was recessive. Furthermore, Shultz and Bailey presented evidence suggesting that distinct genes mapping at either end of *H-2* influenced responsiveness (22). Similar effects of genes at both ends of the MHC has been demonstrated for responsiveness to autoimmune thyroiditis (23). Thus, in addition to a gene mapping in the *K*-end of *H-2*, other loci located at the *D*-end or outside of *H-2* were observed to modify responsiveness in autoimmune thyroiditis (23).

These two independent observations that distinct genes mapping in the *K*- and *D*-ends of the MHC appear to influence *H-2*-linked immune responsiveness can be accounted for by assuming that conventional *Ir* genes mapping in the known *I*-regions are responsible for the *K*-end influence or responsiveness. The *D*-end association could be accounted for by at least two mechanisms. First, it is possible that a separate region mapping near the *D* region of the murine MHC also expresses *Ir* genes. Alternatively, it is possible that the *D*-end products are involved in the formation of new antigenic determinants resulting from the modification of self-antigens relevant for the generation of autoimmune reactions. The results presented in this report (Tables IV, V, and VI) suggest that

<sup>2</sup> Davis, Stephen, Gene M. Shearer, Edna Mozes, and Michael Sela. 1975. Genetic control of the murine cell-mediated immune response in vivo. II. H-2 linked responsiveness to the synthetic polypeptide poly(Tyr,Glu)-poly(DLAla)--poly(Lys). Manuscript submitted for publication.

different levels of immunogenicity are generated by TNP modification of distinct alleles of the *K*- and/or *D*-region products of the MHC. Within the C57BL/10 background, more effective generation of effectors was achieved with sensitization of (B10.A × B10.D2) $F_1$  responding cells by modified  $K^k$  products than by modified  $K^d$  or  $D^d$  products (Table IV). A similar intermediate level of lympholysis was generated toward the H-2D<sup>d</sup> modified products by the B10.A(5R) responder, which introduces *b* alleles in the *K*, *I-A*, and *I-B* regions (Table V). Unlike the examples of selective reactivity toward the modified  $K^k$  products of B10.A and B10.BR (3), a strong CML both to modified H-D and H-2K products can be obtained, as shown in Table V with B10.A(4R). Since differences between these congenic and recombinant strains involved both the immunogen (modified *b* allele of H-2D) and *I-A*, *I-B*, *I-C*, and *S* region alleles, the relative importance of the two potential influences on the generation of response toward modified H-2D products is being studied.

The data summarized in Table VI indicate that TNP-modification of [B10.A(4R) × B10.A(5R)] $F_1$  spleen cells results in the formation of at least three distinct new antigenic specificities, i.e., modified products of H-2K<sup>k</sup>, H-2D<sup>d</sup>, and H-2D<sup>b</sup>. Thus, the  $F_1$  cells, which express greater polymorphism than the parental cells, also generate more new specificities upon modification of cell surface components. Polymorphism at the MHC has been assumed to have a selective advantage in nature (24). Since products of the serological regions of the *H-2* complex appear to serve as cell surface components which can be modified chemically (1, 3, 7) or by viruses (8–11) to form new cellular antigenic specificities, it has been postulated that H-2 polymorphism would provide a greater number of cell surface structures susceptible to alteration and formation of new antigens (3, 25). Such a mechanism could be advantageous for surveillance against viral induced neoplasia, but disadvantages in autoimmunity.

Since modified H-2K and modified H-2D products are involved in the formation of new cellular antigens and since the responses to these modified self antigens appear to be controlled by *H-2*-linked *Ir*-like genes, it may be predicted for those cases in which autoimmune-like phenomena are involved in mouse (1–3, 8–12, 13) and in man (26), that some degree of regulation of immunity will be effected by *Ir* region genes as well as by genes mapping in or near both serologically defined regions, which control the cell surface relevant for altered self.

### Summary

Murine thymus-derived lymphocytes can be sensitized *in vitro* to trinitrophenyl (TNP)-modified autologous spleen cells (1, 2). Cytotoxic effector cells were generated which were specific for TNP-modified target cells expressing the same *H-2K* and *H-2D* serological regions as the modified stimulator cells (3, 7). Spleen cells from two C57BL/10 congenic strains of mice sharing common *I-C*, *S*, and *D* regions, but differing at *K*, *I-A*, and *I-B* regions, generated different levels of lytic responses to the shared modified H-2D<sup>d</sup> products upon sensitization with autologous TNP-modified cells. Lymphocytes from an  $F_1$  between responder and nonresponder strain generated a level of cytotoxicity toward the H-2D<sup>d</sup> modified specificity which was of the same order of magnitude as that

obtained with the high responder, irrespective of whether F<sub>1</sub> or either parental strain of modified stimulator cell was used. These results suggest that the modification of H-2D<sup>d</sup> products resulted in formation of new antigenic determinants in both parental strains. However, the difference observed in responsiveness appeared to be due to a gene or genes mapping in the *K*, *I-A*, or *I-B* region which influenced the ability of the responding lymphocytes to react to these modified H-2D<sup>d</sup> products. Responsiveness was expressed as a dominant trait in the F<sub>1</sub>.

It also appeared that within the C57BL/10 background differences in immunogenicity were detected among the various new antigenic determinants formed by TNP modification, depending on the alleles expressed in the *K* and *D* serological regions. Modified H-2K<sup>k</sup> products were more immunogenic than modified H-2K<sup>d</sup> or modified H-2D<sup>d</sup> products, when used as stimulators of (B10.A × B10.D2)F<sub>1</sub> responding lymphocytes. Furthermore, these F<sub>1</sub> lymphocytes generated a broader spectrum of responses to modified autologous components than did either parental strain. The possibility is raised that *H-2* polymorphism may have a selective advantage both in terms of the library of *Ir* genes available for responding to and in the formation of a greater spectrum of new cell-bound antigenic specificities in those systems in which autologous H-2 modified products serve as immunogens. The results of this report imply bifunctional *H-2*-linked regulation of a T-cell-mediated immunity in the response potential to and in the formation of new modified self antigens.

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