ON THE ROLE OF THE *H-2* HISTOCOMPATIBILITY COMPLEX IN DETERMINING THE SPECIFICITY OF CYTOTOXIC EFFECTOR CELLS SENSITIZED AGAINST SYNGENEIC TRINITROPHENYL-MODIFIED TARGETS*

By JAMES FORMAN

(From The University of Texas, Department of Microbiology, Southwestern Medical School, Dallas, Texas 75233)

Interaction of spleen cells with trinitrobenzene sulfonic acid (TNBS)[1] results in the generation of new antigenic determinants (NAD) on the cell surface (1). The trinitrophenyl (TNP)-modified cells are capable of sensitizing syngeneic spleen cells in vitro so that effector T cells are generated that display a specific cytotoxic effect against only TNP-modified target cells. Further, when allogeneic TNP-modified *H-2* target cells are substituted for syngeneic targets, no cytolysis is observed (1). Therefore, the *H-2* complex plays a role in determining the specificity of the cytotoxic effect in this syngeneic system of cell-mediated lympholysis (CML).

There are two interpretations to explain this data. The first is that TNBS modifies *H-2* antigens, or products controlled by the *H-2* complex, so as to create a NAD which is specific for the *H-2*-product modified. Thus, killer T cells sensitized against syngeneic $H\text{-}2^k$-TNP-modified stimulators will kill only $H\text{-}2^k$-TNP targets and not $H\text{-}2^b$ TNP-modified targets since the NAD created on the two different carriers would be different. The second interpretation is that the specificity of the T cell generated in this system is against several TNP-modified cell surface proteins, which would suggest a wide range of cross-reactivity against all TNP-modified target cells. However, the *H-2* complex would restrict or control specificity by requiring identity between effector and target cell at some region of the *H-2* complex in order for cell-mediated lysis to occur. This would be analogous to the findings of Katz et al. (2), who have shown that for an optimal immune response, T and B cells must share *H-2* genes.

The data presented in this report demonstrate that the first interpretation is correct; i.e., the specificity of the effector cell is directed against altered self *H-2* antigens. Further, the products modified by the reaction with TNBS, that are the target structures in these reactions, are not controlled by the *I* region of the *H-2* complex, but rather by the *K* region, although minor involvement of the *D* region cannot be excluded.

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; CML, cell-mediated lympholysis; Con A, concanavalin A; FCS, fetal calf serum; LCM, lymphocytic choroimenenigitic virus; NAD, new antigenic determinants; PBS, phosphate-buffered saline; TNBS, trinitrobenzene sulfonic acid.

Materials and Methods

Animals. Mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and maintained in our animal colonies at the University of Texas Southwestern Medical School, Dallas, Texas. Some of the strains used in the mapping studies were made available through the generosity of Dr. Jan Klein, University of Texas Southwestern Medical School.

Tumors. EL4 (H-2^b) tumor cells were maintained in the ascites form by weekly passage to C57BL/6 mice. The *H-2*^{d} tumor line is maintained in vitro, and was the gift of Dr. David Grausz, Department of Biology, University of California at San Diego, La Jolla, Calif. This tumor was typed serologically in our laboratory.

Isotopes. ^{51}Cr was obtained from Amersham/Searle Corp., Arlington Heights, Ill.

Radiation. Cells were irradiated with a ^{137}Cs source at a rate of 115 rads/min.

Preparation and Labeling of Target Cells for the Cytotoxicity Assay. Mice were killed by cervical dislocation, their spleens excised, and a cell suspension made in a balanced salt solution (BSS). After washing, the cells were suspended at a concentration of 4.0×10^6 nucleated cells/ml in RPMI 1640 (Grand Island Biological Corp., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 3×10^{-5} M 2-mercaptoethanol. 4 ml of this suspension were added to individual Falcon flasks (no. 3013, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and placed on a rocking platform at 37°C. After 2 days, concanavalin A (Con A) (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp, Cleveland, Ohio) was added at a concentration of 5 μg/ml. 3 days later the cells were harvested (usually from two flasks) and centrifuged together in a 17 x 100 mm plastic tube. The cell pellet was resuspended in 0.2 ml of RPMI 1640 with 1% FCS and 200 μCi of [^{51}Cr]sodium chromate (Amersham/Searle Corp.) was added in a vol of 0.2 ml. The cells were incubated at 37°C for 1.5 h and then washed three times in BSS. After the third wash, the cells were resuspended in 1.0 ml of phosphate-buffered saline (PBS) or PBS containing 1.0 mM of TNBS (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) and placed at 37°C for 10 min. The cells were then washed twice in BSS containing 10% FCS, and incubated for 1.5 h at 37°C in RPMI with FCS. The cells were washed twice more and adjusted to a concentration of 10^5 viable cells/ml. 0.1 ml of this target cell suspension was added to Petri dishes containing the effector cells. Tumor cells were labeled by harvesting the cells from either animals or flasks, washing them two times in BSS, and then incubating 5×10^6 cells with ^{51}Cr in RPMI 1640 as described above for the Con A lymphoblasts.

Generation of Effector Cells for the CML Assay. Effector cells were generated by coculturing responder cells along with stimulator cells in 35 mm plastic Petri dishes in the same media as described for the culture of target cells. 5 million responder cells were added in a total vol of 1.0 ml to each dish along with 1×10^6 stimulator cells in a vol of 0.1 ml, and each group consisted of three replicates. The cells were cultured in a CO_2 atmosphere for 5 days as previously described (3), and at this time 10^4 target cells in a vol of 0.1 ml were then added to the dishes to assay for cytotoxic activity. After 4 h, the contents of the dishes were harvested with a plastic policeman and transferred to 12 x 75 mm tubes, centrifuged, and half of the supernatant was removed.

Data Processing. The formula used to calculate percent release of isotope is:

$$\% \text{ release } {}^{51}\text{Cr} = \frac{(\text{cpm supernate} - \text{bk}) \times 2}{(\text{cpm supernate} + \text{pellet}) - 2 \times \text{bk.}} \times 100,$$

where bk is background in counts per minute.

Inhibition assay of CML activity. Con A lymphoblasts were prepared in the same manner as the target cells for the CML assay except that they were not labeled with ^{51}Cr. The cells were adjusted to 10 times the concentration required for the assay and 0.1-ml vol of these inhibitors were added to the effector cell mixtures at the same time as the target cells.

Modification of Stimulator Cells with TNBS. To modify the stimulator cells with TNBS, approximately 30×10^6 spleen cells were incubated in PBS containing 1.0 mM TNBS. After 10 min at 37°C, the cells were washed twice in BSS containing 10% FCS and then adjusted to a concentration of 10×10^6 cells/ml. 0.1 ml of this suspension was added to individual Petri dishes as the source of stimulator cells.

Results

Ability of TNP-Sensitized Spleen Cells to Generate a Specific Cytotoxic Effect against TNP-Modified Target Cells of Different H-2 Haplotypes. Shearer (1) reported that TNP-modified cells sensitize syngeneic splenic T cells so that they display a specific cytotoxic effect against only *H-2*-compatible TNP-modified target cells. The experimental data presented in Table I confirm these results. Thus, B10.A ($H\text{-}2^a$) cells sensitized to B10.A TNP-modified stimulators are

TABLE I

Specificity of Sensitized Effector Cells against TNP-Modified Targets of a Different H-2 Haplotype

Line	Responder (genotype)	Stimulator	Target	^{51}Cr release ± SEM	Net release*
				%	%
1	B10.A ($H\text{-}2^a$)	B10.A	B10.A	40.8 ± 2.2	−2.1
	B10.A ($H\text{-}2^a$)	B10.A-TNP	B10.A	38.7 ± 3.4	
2	B10.A	B10.A	B10.A-TNP	39.8 ± 1.9	35.7
	B10.A	B10.A-TNP	B10.A-TNP	75.5 ± 4.8	
3	B10.A	B10.A	B10-TNP	36.1 ± 3.4	8.9
	B10.A	B10.A-TNP	B10-TNP	45.0 ± 2.9	
4	B10 ($H\text{-}2^b$)	B10	B10	30.7 ± 1.2	3.1
	B10 ($H\text{-}2^b$)	B10-TNP	B10	33.8 ± 2.9	
5	B10	B10	B10-TNP	36.3 ± 2.1	11.3
	B10	B10-TNP	B10-TNP	47.6 ± 2.1	
6	B10	B10	B10.A-TNP	41.7 ± 2.7	3.7
	B10	B10-TNP	B10.A-TNP	45.4 ± 3.9	
7	B10.BR ($H\text{-}2^k$)	B10.BR	B10.BR-TNP	41.0 ± 2.0	28.7
	B10.BR ($H\text{-}2^k$)	B10.BR-TNP	B10.BR-TNP	69.7 ± 1.1	
8	B10.BR ($H\text{-}2^k$)	B10.BR	B10.D2-TNP	36.7 ± 1.1	1.4
	B10.BR ($H\text{-}2^k$)	B10.BR-TNP	B10.D2-TNP	38.1 ± 0.7	
9	B10.D2 ($H\text{-}2^d$)	B10.D2	B10.D2-TNP	30.4 ± 0.6	7.6
	B10.D2 ($H\text{-}2^d$)	B10.D2-TNP	B10.D2-TNP	38.0 ± 1.4	
10	B10.D2 ($H\text{-}2^d$)	B10.D2	B10.BR-TNP	46.6 ± 1.4	−0.9
	B10.D2 ($H\text{-}2^d$)	B10.D2-TNP	B10.BR-TNP	45.7 ± 1.5	

*Net release, % ^{51}Cr release from target cells in the presence of effector cells sensitized to TNP-modified stimulators − % ^{51}Cr release from target cells in the presence of effector cells sensitized to unmodified stimulators (nonimmune).

strongly cytotoxic against only TNP-modified B10.A targets and not to *H-2*-congeneic C57BL/10 ($H\text{-}2^b$), abbreviated B10, targets (line 2 vs. line 3). Similarly, B10 cells sensitized to B10-TNP stimulators kill B10-TNP target cells but not B10.A-TNP targets (line 5 vs. line 6). Further, B10.BR ($H\text{-}2^k$) anti-B10.BR-TNP effector cells are cytotoxic to B10.BR-TNP but not B10.D2-TNP ($H\text{-}2^d$) targets (line 7 vs. line 8). The same was found in the reverse direction (line 9 vs. line 10).

In several experiments we have observed a low level of effector cell-induced cytotoxicity against *H-2*-unrelated TNP-modified targets (e.g. Table I, line 3). We interpret this as cross-reactivity between haplotypes of TNP-modified products controlled by the *H-2* complex.

Ability of TNP-Modified Parental Cells to Sensitize Semisyngeneic Responders to Kill TNP-Modified Parental Targets. While the *H-2* complex restricts the specificity of the cytotoxic effect, the previous experiment does not distinguish whether the specificity of the killer T cell is directed against an altered self *H-2* antigen; or alternatively, if the effector and target cell must share a region of the *H-2* complex in order for an optimal cytotoxic effect to occur. Therefore, F_1 hybrid cells sensitized to irradiated and TNP-modified parental cells were tested for their cytotoxic potential against the TNP-modified stimulator cell as well as the other parental cell that was also TNP modified. If the specificity of the effector cell was against an altered self H-2 antigen, only the stimulator cell should be lysed, while both parental targets should be killed if there is a requirement for sharing of *H-2* genes between the effector and target cell. In Table II is presented the results of one of eight such experiments, all of which have shown similar findings. (C57BL/6 × DBA-2) ($H\text{-}2^b/H\text{-}2^d$), abbreviated BDF_1, cells sensitized to DBA/2 ($H\text{-}2^d$) TNP-modified stimulators are cytotoxic against DBA/2-TNP or $H\text{-}2^d$ tumor target cells, but not against B6 ($H\text{-}2^b$) or EL4 ($H\text{-}2^b$) targets. When BDF_1 cells were cocultured with B6-TNP stimulators, the cytotoxic effect generated was directed against B6-TNP or EL4-TNP targets, but not against DBA/2-TNP or the $H\text{-}2^d$ TNP tumor line. Therefore, sensitization of F_1 hybrid cells with irradiated TNP-modified parental cells results in the generation of cytotoxic effector cells that are specific only for the parental cell to which they are sensitized, and not against TNP-modified targets of the other parent, even though in the latter case the effector and target cell share the major histocompatibility complex. This indicates that the specificity of the effector cell in this system is for a modified parental cell surface structure, rather than the specificity of the cytotoxic effect being controlled by a requirement for sharing of a portion of the major histocompatibility complex between the effector and target cell.

Ability of Unlabeled TNP-Modified Parental Cells to Block the Cytotoxic Effect of F_1 Hybrid Cells against TNP-Modified Parental Targets. As further proof that the specificity of the receptor on the killer T cell is directed against an altered self cell surface product, we sensitized F_1 hybrid cells to parental TNP-modified cells and then attempted to block the specific cytotoxic effect by adding, along with labeled specific targets, unlabeled TNP-modified Con A lymphoblasts that either were *H-2* compatible with the parental sensitizing strain, or *H-2* incompatible with the sensitizing strain, but *H-2* compatible with the other parental *H-2* haplotype of the F_1. If the receptors on the killer cells have

TABLE II

Specificity of the Cytotoxic Effect of F_1 Hybrid Spleen Cells Sensitized to Irradiated and TNP-Modified Parental Stimulators

Responder (genotype)	Stimulator (genotype)	^{51}Cr release ± SEM from targets (genotype)							
		DBA/2 (*H-2*^{d})	DBA/2-TNP (*H-2*^{d})	(H-2^d tumor (H-2^d)‡	H-2^d tumor-TNP (H-2^d)	B6(*H-2*^{b})	B6-TNP (*H-2*^{b})	EL4(*H-2*^{b})	EL4-TNP (*H-2*^{b})
		%		%	%	%	%	%	%
(B6 × DBA/2)F$_1$ (*H-2*^{b}/*H-2*^{d})	DBA/2-TNP (*H-2*^{d})	40.7 ± 0.8	54.1 ± 2.5	40.6 ± 5.6	79.5 ± 0.5	39.3 ± 2.9	38.4 ± 0.6	36.2 ± 4.9	37.8 ± 0.8
		(13.4)*		(38.9)		(−0.9)		(1.6)	
(B6 × DBA/2)F$_1$ (*H-2*^{b}/*H-2*^{d})	B6-TNP (*H-2*^{b})	34.7 ± 0.4	40.3 ± 1.2	49.1 ± 2.2	55.9 ± 7.2	36.4 ± 3.0	49.4 ± 1.0	44.4 ± 4.8	63.6 ± 4.0
		(5.6)		(6.8)		(13.0)		(19.2)	

* Net release, % ^{51}Cr from TNP-modified target cells − % ^{51}Cr release from unmodified target cells.

‡ Phenotype.

a broad specificity against several cell surface TNP-modified proteins, and the role of the *H-2* complex in restricting the specificity of the effector cell is by requiring that the killer and the target cell share a region of the *H-2* complex in order for optimal target cell lysis to occur, then the addition of *H-2*-allogeneic unlabeled TNP-modified cells to the cytotoxic assay should block the cytotoxic effect.

The data in Fig. 1 demonstrates that the (C3H × DBA/2)F_1 ($H\text{-}2^k/H\text{-}2^d$) hybrid cells sensitized to C3H ($H\text{-}2^k$) TNP-modified stimulators are cytotoxic to these targets. The reaction is almost completely inhibited by a 50- to 100-fold

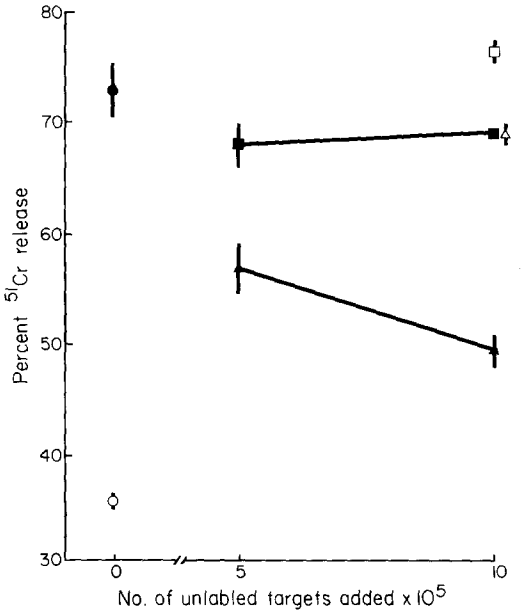

FIG. 1. Percent of $^{51}Cr \pm$ SEM from 10^4 TNP-modified C3H Con A lymphoblasts in the presence of (C3H × DBA/2)F_1 spleen cells sensitized against irradiated C3H-TNP stimulators. Various numbers of unlabeled TNP-modified Con A lymphoblasts from B10.BR (▲), B10.D2 (■), or unmodified unlabeled Con A lymphoblasts from B10.BR (△), or B10.D2 (□), strain mice were added along with the labeled targets at the start of the CML assay. (●), immune release in the absence of unlabeled targets; and (○), nonimmune (control) release in the absence of unlabeled targets.

excess of unlabeled B10.BR-TNP targets ($H\text{-}2^k$) that are *H-2* compatible with the C3H stimulators. However, B10.D2 ($H\text{-}2^d$) TNP cold targets, that share their *H-2* haplotype with the F_1 hybrid cells but not the C3H stimulators, do not block. Unmodified cold targets at a 100-fold excess also show no blocking activity in this system. Thus, in agreement with other previous experiments, this data shows that the specificity of the effector cell is directed against an altered self H-2 antigen.

Ability of Effector Cells Sensitized against TNP-Modified Allogeneic Targets to Kill TNP-Modified Syngeneic Targets. Another experimental approach to show that sharing of an *H-2*-controlled cooperating factor between effector and target cells is not required for killing of TNP-modified targets was to sensitize

cells with allogeneic TNP-modified stimulators and test to see if these cells are cytotoxic against TNP-modified syngeneic targets. B10.D2 ($H\text{-}2^d$) cells, sensitized to TNP-modified B10.BR ($H\text{-}2^k$) stimulators, were cytotoxic to these targets but did not kill B10.D2-TNP targets (Table III; row 2, columns 4 and 2). The same result was observed when B10.BR cells, sensitized to B10.D2-TNP stimulators, were tested against B10.BR-TNP targets (Table III; row 3, column 2). Thus, even though the effector and target cell are identical, TNP-modified target cells are not killed, which further indicates that sensitization by TNP-modified cells involves the presentation of an altered self H-2 antigenic determinant.

TABLE III

Ability of Effector Cells Sensitized against TNP-Modified H-2 Allogeneic Stimulators to Display a Cytotoxic Effect against TNP-Modified Targets Syngeneic with the Effector Cells

			% ^{51}Cr Release ± SEM			
			Stimulator:			
Row	Responder	Target	B10.D2 (1*)	B10.D2-TNP (2)	B10.BR (3)	B10.BR-TNP (4)
1	B10.D2	B10.D2-TNP	35.4 ± 1.4‡	49.1 ± 1.9 (13.7)§	45.0 ± 3.3 (9.6)	45.1 ± 0.6 (9.7)
2	B10.D2	B10.BR-TNP	34.4 ± 0.7‡	41.3 ± 2.0 (6.9)	79.7 ± 0.8 (45.3)	76.6 ± 2.3 (42.2)
3	B10.BR	B10.BR-TNP	48.7 ± 1.5 (10.2)	45.4 ± 1.2 (6.9)	38.5 ± 2.3‡	76.2 ± 2.8 (37.7)
4	B10.BR	B10.D2-TNP	69.6 ± 2.5 (34.6)	72.2 ± 2.5 (37.2)	35.0 ± 1.7‡	43.2 ± 1.4 (8.2)

* Column number/see text.

‡ Nonimmune control groups.

§ Net release, % ^{51}Cr release from targets in the presence of immune cells − % ^{51}Cr release from targets in the presence of nonimmune cells (see footnote ‡).

Ability of F_1 Hybrid Spleen Cells Sensitized to Parental TNP-Modified Cells to Display a Cytotoxic Effect against TNP-Modified F_1 Targets. To demonstrate that products controlled by the *H-2* complex are altered in a similar manner on parental as well as F_1 hybrid cells, we sensitized F_1 cells against parental TNP stimulators and then tested the effector cells against TNP-modified parental and F_1 hybrid targets. The data in Table IV show that $H\text{-}2^k$-controlled products are altered in a similar manner on both parental and F_1 cells because (AKR × DBA-2)F_1, abbreviated AKD2F_1 cells, ($H\text{-}2^k/H\text{-}2^d$) sensitized to AKR-TNP-irradiated stimulators, are cytotoxic to these targets as well as AKD2F_1-TNP targets. As found in other experiments, DBA/2-TNP ($H\text{-}2^d$) targets are not lysed.

Ability of F_1 Hybrid Cells to Display a Cytotoxic Effect against TNP-Modified Parental Cells as well as other Targets that Share either the K or D End of the

TABLE IV

Ability of F_1 Hybrid Spleen Cells Sensitized to Parental TNP-Modified Cells to Display a Cytotoxic Effect against TNP-Modified F_1 Target Cells

Responder	Stimulator	Target	^{51}Cr release ± SEM	Net release*
			%	%
(AKR × DBA/2)F_1	AKR	AKR-TNP	42.3 ± 5.5	34.1
(AKR × DBA/2)F_1	AKR-TNP	AKR-TNP	76.4 ± 3.0	
(AKR × DBA/2)F_1	AKR	DBA/2-TNP	44.7 ± 1.9	0.9
(AKR × DBA/2)F_1	AKR-TNP	DBA/2-TNP	45.6 ± 1.0	
(AKR × DBA/2)F_1	AKR	(AKR × DBA/2)F_1-TNP	30.6 ± 3.4	30.8
(AKR × DBA/2)F_1	AKR-TNP	(AKR × DBA/2)F_1-TNP	61.4 ± 0.5	

* See Table I for explanation.

H-2 Complex with the Stimulator. *H-2*-recombinant strains were tested in order to determine whether the *K* or *D* end of the *H-2* complex controlled the gene products altered by the TNBS. AKD2F_1 cells ($H\text{-}2^k/H\text{-}2^d$), sensitized to AKR ($H\text{-}2^k$) TNP-modified cells were cytotoxic against AKR-TNP targets, but not against DBA/2-TNP ($H\text{-}2^d$) targets (Table V). When tested against an *H-2*-unrelated target, B10 ($H\text{-}2^b$), a weak effect was observed, similar to that shown in the experiment presented in Table I. When TNP-modified A/J ($H\text{-}2^a$) cells, a strain which is an *H-2* recombinant that shares the *K* end of the *H-2* complex with $H\text{-}2^k$ (4) were tested as targets, a strong cytotoxic effect was observed. Thus, the data shows that identity at the *K* end of the *H-2* complex with the sensitizing strain will lead to CML in this system, similar to that observed by Shearer (1).

When DBA/2-TNP stimulators were used, DBA/2-TNP targets were killed while AKR-TNP targets were not. Again, there was a weak reactivity against B10-TNP targets. A/J-TNP targets, that in this case share the *D* end of the *H-2* complex with the DBA/2 ($H\text{-}2^d$) haplotype were also killed, but to a much lesser extent than when AKR-TNP cells were the stimulators. To demonstrate that TNP-modified B10 cells can be killed, we sensitized B10 spleen cells against B10-TNP targets and showed that such targets were lysed.

In Table VI the protocol was the same as for the experiment shown in Table V, except that we used *H-2*-congenic lines for target cells in order to determine if the specificity was in fact controlled by the *H-2*-gene complex. The results here confirm the data of the experiment presented in Table V, and show that the requirement for positive CML in this system is controlled by the *H-2* complex. It was also noted that when DBA/2-TNP cells were the stimulators, the cytotoxic effect was much less than when AKR-TNP cells were the stimulators, a result that has been frequently observed in other experiments as well. Thus, the fact that B10.A-TNP targets were not killed by the AKD2F_1 anti-DBA/2 TNP effectors in this experiment may have been due to the weak cytotoxic effect that was generated.

TABLE V

Ability of F_1 Hybrid Spleen Cells, Sensitized to Irradiated and TNP-Modified Parental Cells, to Display a Cytotoxic Effect against TNP-Modified Targets that are H-2 Related to the Stimulator

Responder (genotype)	Stimulator (genotype)	^{51}Cr release ± SEM			
		Target (genotype):			
		AKR-TNP (H-2^k)	DBA/2-TNP (H-2^d)	B10-TNP (H-2^b)	A/J-TNP (H-2^a)
		%	%	%	%
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	AKR (H-2^k)	35.9 ± 1.4	39.3 ± 3.6	46.0 ± 0.8	45.8 ± 0.9
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	AKR-TNP	69.3 ± 1.5 (33.4)*	38.3 ± 0.8 (−1.0)	53.6 ± 1.2 (7.6)	74.6 ± 4.9 (28.8)
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	DBA/2 (H-2^d)	40.3 ± 7.9	43.0 ± 1.6	43.2 ± 2.6	49.6 ± 3.6
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	DBA/2-TNP	39.3 ± 1.3 (−1.0)	58.9 ± 2.4 (15.9)	48.5 ± 2.6 (5.4)	59.4 ± 2.3 (9.8)
B10 (H-2^b)	B10 (H-2^b)			45.6 ± 1.7	
B10 (H-2^b)	B10-TNP			63.0 ± 0.9 (17.4)	

* Net release, see Table I for explanation.

TABLE VI

Ability of F_1 Hybrid Spleen Cells, Sensitized to Irradiated and TNP-Modified Parental Cells, to Display a Cytotoxic Effect against TNP-Modified H-2 Congenic Targets

Responder (genotype)	Stimulator (genotype)	^{51}Cr release ± SEM			
		Target (genotype):			
		B10.BR-TNP (H-2^k)	B10.D2-TNP (H-2^d)	B10.TNP (H-2^b)	B10.A-TNP (H-2^a)
		%	%	%	%
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	AKR (H-2^k)	37.0 ± 1.5	32.5 ± 2.6	39.1 ± 0.8	42.7 ± 2.3
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	AKR-TNP	53.1 ± 0.6 (16.1)*	32.0 ± 0.6 (−0.5)	39.4 ± 2.0 (0.3)	60.5 ± 2.1 (17.8)
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	DBA/2 (H-2^d)	36.4 ± 2.9	34.1 ± 1.9	39.5 ± 1.6	43.6 ± 2.6
(AKR × DBA/2)F_1 (H-2^k/H-2^d)	DBA-TNP	37.6 ± 2.6 (1.2)	40.8 ± 1.6 (6.7)	40.6 ± 2.2 (1.1)	42.8 ± 0.9 (−0.8)

* Net release, see Table I for explanation.

Region of the H-2 Complex that Controls the Cell Surface Product being Modified by TNBS. While our previous data show that both the *K* and *D* end of the *H-2* complex control the expression of the altered cell surface antigens, it does not distinguish the role of the individual *K*, *D*, and *I* regions. Therefore, experiments were done using *H-2*-recombinant strains to resolve the role of these individual regions. In Table VII a representative experiment is presented where B10.BR (*kkkk*) (gene symbols refer to the *K*, *I*, *S*, and *D* regions of the *H-2*

TABLE VII

Role of H-2 Regions in Determining Cytotoxicity against TNP-Modified Targets

Responder (genotype)	Stimulator (genotype)	^{51}Cr release ± SEM							
		Target cell (genotype):							
		B10.BR (*kkkk*)* ‡	B10.BR-TNP (*kkkk*)	B10.AQR (*qkdd*)	B10.AQR-TNP (*qkdd*)	B10.A (*kkdd*)	B10.A-TNP (*kkdd*)	C3H.OH (*dddk*)	C3H.OH-TNP (*dddk*)
		%	%	%	%	%	%	%	%
B10.BR (*kkkk*)	B10.BR-TNP (*kkkk*)	34.8 ± 3.8	71.6 ± 0.5 (36.8)	40.9 ± 4.0	36.0 ± 0.4 (−4.9)	27.0 ± 3.7	67.7 ± 2.2 (40.7)	27.9 ± 0.4	40.7 ± 2.1 (12.8)

* Gene symbols refer to the *K*, *I*, *S*, and *D* regions of the *H-2* complex.

‡ Underlined gene symbol denotes sharing of region between stimulator and target cell.

complex) effector cells, sensitized to B10.BR-TNP-modified targets were cytotoxic to B10.BR, B10.A (*kkdd*), AND C3H.OH (*dddk*), but not B10. AQR (*qkdd*) TNP-modified targets. In Table VIII all of the mapping data, using several other recombinant strains, are summarized. The results show that if a target shares the *K* and *I* region of the *H-2* complex with the stimulator, CML results, while if the *I* region only is shared, the result is negative. When only the *D* region of the *H-2* complex is shared with the specific target, an isotope release of greater than 10% was observed in only half of the experiments. Further, in these cases the cytotoxic effect was much less than that observed against the specific target cells. We conclude that if the *D* region of the *H-2* complex is involved, it is to a much lesser degree than that of the *H-2K* region.

Discussion

There is a large body of evidence demonstrating that cytotoxic effector T cells are generated both in vivo and in vitro in *H-2*-allogeneic combinations and the cytotoxic effect that is observed is specific for either targets of the sensitizing strain or *H-2*-recombinant strains that share either their *K* or *D* end with the sensitizing strain (5, 6). In fact, the strongest CML reponses are demonstrated in allogeneic combinations, while generally it has been difficult to demonstrate syngeneic cytotoxicity; e.g., tumor immune systems (7, 8).

The evidence in this study as well as other reports, where the cytotoxic effect has been directed against determinants other than *H-2* antigens, indicates that for the expression of T-cell-mediated cytotoxicity, there is a restriction in that the effector and target cell must share the major histocompatibility complex. Thus, I observed that spleen cells, sensitized to TNP-modified syngeneic splenic stimulators, display a cytotoxic effect against only TNP-modified targets that are *H-2* compatible with the stimulator. Similar results have been found by Shearer (1) and by Koren et al. (9) using a different hapten. In a viral system, it has been demonstrated that the host response to lymphocytic choriomenigitic virus (LCM) in vivo results in the generation of effector cells that are cytotoxic in vitro against only *H-2*-compatible LCM-infected targets (10). In another model, Ilfeld et al. (11) have shown that spleen cells sensitized to syngeneic fibroblasts in vitro generate cytotoxic effector cells against syngeneic or *H-2*-compatible fibroblasts whereas *H-2*-incompatible fibroblasts are not killed.

TABLE VIII

Summary of CML Reactivity of Effector Cells against TNP-Modified H-2-Recombinant Target Cells

TNP-modified target cell (genotype)*	Cytotoxic effect of responder antistimulator cells (denotes *H-2* region shared by target cell and stimulator [or effector] cell)		
	B10.BR anti-B10.BR-TNP‡	B10.A anti-B10.A-TNP§	B10 anti-B10-TNP‖
B10.BR (*kkkk*)	+¶ (*K I S D*)**	+ (*K I - -*)	NT
B10.D2 (*dddd*)	NT‡‡	-§§ (*- I S D*)	NT
B10.A (*kkdd*)	+ (*K I - -*)	+ (*K I S D*)	- (- - - -)
B10 (*bbbb*)	NT	- (- - - -)	+ (*K I S D*)
B10.AQR (*qkdd*)	- (*- I - -*)	NT	NT
C3H.OH (*ddkk*)	+ (*- - - D*)	NT	NT
B10.S (7R) (*sssd*)	NT	- (*- - - D*)	NT
B10.A (4R) (*kkbb*)	NT	+ (*K I - -*)	+ (*- I S D*)
B10.BYR (*qkdb*)	NT	- (*- I S -*)	- (*- - - D*)

*Refers to *K*, *I*, *S*, and *D* regions of *H-2* complex.

‡ One experiment.

§ Three experiments.

‖ One experiment.

¶ + indicates net release of $^{51}Cr \geq 10\%$.

** Indicates *H-2* regions shared by stimulator (or effector) cell and target.

‡‡ Not tested.

§§ Net release $< 10\%$.

While the role of the *H-2* complex in restricting the specificity of the cytotoxic effect in these experiments could be explained by a cooperation model which would require effector and target cell to share some *H-2*-gene products in order for an optimal cell-to-cell interaction, the data in this study show that this is not the case for the TNP-modification system; rather, the specificity of the effector cell is directed against an altered self cell surface product controlled by the *H-2* complex. Thus, F_1 hybrid cells sensitized to a TNP-modified parental strain cell displayed a cytotoxic effect against only that TNP-modified parental target and not against TNP-modified cells from the other parent even though one haplotype of the effector cell in this latter case shared the *H-2* complex with the target. Further, F_1 hybrid cells sensitized to parental TNP-modified cells were blocked from killing these parental targets by unlabeled TNP-modified targets *H-2* compatible with the parental strain used for sensitization, but not with cold TNP-modified targets that were *H-2* syngeneic with the other parental strain. This finding shows that the specificity of the receptor on the killer cell is specific for a particular TNP-modified *H-2*-controlled-gene product. Moreover, I observed that cells of one strain, sensitized to TNP-modified *H-2*-allogeneic strain cells, did not kill TNP-modified targets that were syngeneic to the effector cells, which should have occurred if the cooperation model were correct, since the effector and target cell in this case are *H-2* identical.

Another possible interpretation of this data is that there is allelic exclusion of a cooperating factor controlled by the *H-2* complex. Thus, only F_1 cells expressing a product in common with one of the modified parental cells would be sensitized, and these

sensitized cells would not be able to interact with modified cells from the other parental haplotype. Since there is no evidence for allelic exclusion in the *H-2* complex where both gene products are expressed in a codominant fashion (12) this interpretation is unlikely.

By employing *H-2*-recombinant mouse strains, we ascertained that a cytotoxic effect was observed against TNP-modified target cells that shared their *K* region with the stimulator strain, since those targets that shared only the *I* region were not killed. Strains that shared only their *D* region with the stimulator were lysed in the assay, but the amount of lysis was much less that if the *K* region were shared, and I conclude, therefore, that if the *D* region is involved in this phenomenon, then it is to a much lesser extent than the *K* region. Thus, the findings here contrast with the role of the *H-2* complex in the immune response to antigens under *Ir*-gene control, which requires sharing of the I region between responder T and B cells for an optimal response (13). Similar results have been observed in another system by Blanden et al. (14), in which they showed that cytotoxic T cells sensitized against LCM-infected cells must share the *K* or *D*, but not *I*, region of the *H-2* complex with the target cell in order for lysis to occur. Further, it was demonstrated (15) that LCM sensitized F_1 ($H\text{-}2^k/H\text{-}2^b$) cells transferred in vivo to $H\text{-}2^k$ recipients will retain cytotoxic activity against $H\text{-}2^k$ LCM-infected targets in vitro, while transfer to recipients bearing other *H-2* haplotypes will not.

The findings in this study demonstrate that syngeneic killing is analogous to classical allogeneic cytotoxicity, since the effector cells in this syngeneic system are in reality sensitized to *H-2*-gene products (in this case a modified self H-2 antigen), and further suggest that some *H-2*-gene products may play an obligatory role in bringing about target cell lysis by effector T cells. Speculations on the possible mechanisms underlying this type of cell-mediated cytotoxicity are diagrammatically illustrated in Fig. 2. Here it is postulated that *H-2*-gene products are linked to an enzyme(s) on the cell surface, or have intrinsic enzymatic activity that readily allows the destruction of the target cell when interaction occurs with the receptor on a cytotoxic T cell (Fig. 2 A and B). Alternatively, the interaction of this gene product with the receptor on the T cell

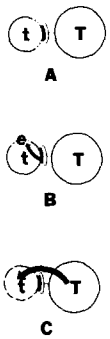

FIG. 2. (▬), *H-2*-controlled product; *T*, cytotoxic T cell; and *t*, target cell. Interaction of the receptor on a cytotoxic T cell with an *H-2*-controlled product leads to A, activation of intrinsic *H-2* enzymatic activity, or B, activation of an enzyme (*e*) coupled on the cell surface to the *H-2* product. The activated enzyme causes the formation of lytic lesions in the cell membrane. C, interaction of the receptor on a cytotoxic T cell with an *H-2*-controlled product leads to the transmission of a substance from the killer cell to the target cell cytoplasm. The substance acts as an intracellular toxin causing target cell death.

could result in the transmission of an active fragment from the killer cell into the target cell cytoplasm which inhibits macromolecular synthesis, analogous to the action of diphtheria toxin (Fig. 2 C) (16). Here, the altered target cell permeability that is observed would be a secondary effect. If this model is correct, then in the TNP system one would expect many clones of T cells to be sensitized to several different TNP-modified cell surface proteins, but the only modified determinant that would result in effective or optimal target cell lysis by interaction with a reactive T cell is that associated with an H-2 product. If H-2 antigens are required on the target cell for the expression of T-cell-mediated lysis against cell surface antigens in general, then phenotypic H-2-negative cells should be resistant to T-cell-mediated cytolysis. We have reported such a finding using a 129 strain primitive teratocarcinoma cell line, F9, that is H-2 negative both by serological and CML analysis (17, 18). When we modified this F9 tumor line with TNP, we found that it is not killed by 129 anti-129 TNP effector cells while 129-TNP-modified spleen cells were (18).

There is some structural similarity (19) and considerable cross-reactivity at the antibody level between products of different *H-2* haplotypes, as well as between products of the *K* and *D* regions (20, 21). We have observed that the majority of iodinatable cell surface protein is derivatized by TNP in this coupling procedure (E. S. Vitetta, unpublished observations). Therefore, it is difficult at this time to understand the basis for the specificity of this response. One explanation is that the serologically characterized *H-2K* glycoproteins are not the products modified, but rather another product, different between haplotypes and closely linked to *H-2K*, is the modified target antigen for this reaction. While there is some evidence in support of this interpretation (22–24), the absence of recombinants in mouse or man that separate these two postulated products makes this possibility unlikely.

It is presently not possible to distinguish whether the reaction of TNBS on the *H-2*-gene product itself is the critical step in creating the NAD, or whether another cell surface protein, that is also TNP modified, then associates with H-2 antigens in a complex to create the NAD. In other systems there is evidence to suggest a physical association on the cell surface of H-2 products with viral-specified antigens. For example, Aoki (25) has observed that viral budding particles from Gross virus-induced leukemias are associated with H-2 antigens in $H\text{-}2^k$-infected cells, but not in $H\text{-}2^b$-infected cells. Hecht and Summers (26) have observed H-2 activity in vesicular stomatitis virus particles released from L cells. Fujimoto et al. (27) have found a physical association of histocompatibility antigens with tumor-associated antigens in the sera of mice bearing a spontaneous lymphoma. Rossi et al. (28) found that while DBA/2 bone marrow cells were not rejected by semisyngeneic recipients, Friend leukemia virus-treated bone marrow cells were. They interpreted this finding to an enhancement of the expression of hybrid histocompatibility genes in these parental cells which allow the F_1 hybrid to exhibit "hybrid resistance" (29). However, creation of a NAD on the parental cell consisting of self H-2 antigens and Friend leukemia virus is another possibility.

In summary, I have provided evidence to show that modification of cells with TNBS results in altered cell surface antigens controlled principally by the *K* region of the *H-2* complex, and the specificity of the killer cell generated in in vitro culture systems is directed against this altered *H-2*-gene product. I suggest

that *H-2K-* and *D*-controlled antigens may play an obligatory role in cell-mediated lysis by T cells.

Summary

Spleen cells cultured with syngeneic trinitrophenyl (TNP)-modified stimulator cells display a cytotoxic effect against syngeneic TNP-modified targets, but not against modified targets from unrelated *H-2* haplotypes. Targets that share the *K* and *I* region of the *H-2* complex with the stimulator (or effector) cell are lysed to the same extent as the specific targets, while targets that share the I region only are not. When only the *D* region is shared, a weak cytotoxic effect is observed. Therefore, the stimulator (or effector) and target cell must share the *K* or *D* but not the *I* region of the *H-2* complex in order for optimal cytotoxicity to occur.

Spleen cells sensitized to irradiated TNP-modified *H-2*-allogeneic cells are cytotoxic to these specific targets, but not against TNP-modified targets syngeneic with the effector cells. Coculture of F_1 hybrid cells with irradiated TNP-modified parental cells results in a cytotoxic effect against only those specific parental cells and not TNP-modified cells from the other parent. The cytotoxic effect of the F_1 effector cells in the cell-mediated lympholysis test is blocked by the addition of unlabeled TNP-modified targets that are *H-2* syngeneic with the sensitizing parental strain, but not *H-2* syngeneic with the other parental strain. These data demonstrate that the specificity of the effector cell in this syngeneic cytotoxicity system is directed against altered self *H-2*-controlled-gene products, rather than a requirement for sharing of histocompatibility genes between effector and target cell in order for lysis to occur. The role of *H-2* antigens in determining the sensitivity of a target cell to T-cell-mediated lysis is discussed.

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References

1. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* **4**:527.
2. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral responses to hapten protein conjugates. *J. Exp. Med.* **137**:1405.
3. Forman, J., and G. Möller. 1973. Generation of cytotoxic lymphocytes in mixed lymphocyte reactions. I. Specificity of the effector cells. *J. Exp. Med.* **138**:672.
4. Snell, G. D., P. Smith, and F. Gabrielson. 1953. Analysis of the histocompatibility 2 locus in the mouse. *J. Natl. Cancer Inst.* **14**:457.
5. Alter, B. J., D. J. Schendel, M. L. Bach, F. H. Bach, J. Klein, and J. H. Stimpfling. 1973. Cell-mediated lympholysis. Importance of serologically defined *H-2* regions. *J. Exp. Med.* **137**:1303.
6. Nabholz, M., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rynbeck, and D.

Shreffler. 1974. Cell mediated cell lysis *in vitro*: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* **4**:378.

7. Hellström, I., and K. E. Hellström. 1969. Studies on cellular immunity and its serum-mediated inhibition in Maloney-virus-induced mouse sarcomas. *Int. J. Cancer.* **4**:587.
8. Wagner, H., and M. Rollinghoff. 1973. Cell-mediated immunity *in vitro* against syngeneic mouse plasma tumor cells. *Nat. New Biol.* **241**:53.
9. Koren, H. S., J. R. Wunderlich, and J. K. Inman. *In vitro* development of cytotoxic T-cells specific for hapten associated antigens on syngeneic cells. *Transplant Proc.* In press.
10. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.).* **248**:701.
11. Ilfeld, D., C. Carnaud, and E. Klein. Cytotoxicity of autosensitized lymphocytes restricted to *H-2K*-end identical targets. *Immunogenetics.* In press.
12. Bjarring, B., and G. Klein. 1968. Antigenic characterization of heterozygous lymphomas after immunoselection *in vivo*. *J. Natl. Cancer Inst.* **41**:1411.
13. Katz, D. H., M. Graves, M. E. Dorf, H. Dimuzio, and B. Benacerraf. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J. Exp. Med.* **141**:263.
14. Blanden, R. V., P. C. Doherty, M. B. C. Dunlop, I. D. Gardner, and R. M. Zinkernagel. 1975. Genes required for cytotoxicity against virus infected target cells in *K* and *D* regions of *H-2* complex. *Nature (Lond.).* **254**:269.
15. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature (Lond.).* **251**:547.
16. Gill, D. M., A. M. Pappenheimer, Jr., and T. Uchida. 1973. Diphtheria toxin, protein synthesis, and the cell. *Fed. Proc.* **32**:1508.
17. Artzt, K., and F. Jacob. 1974. Absence of serologically detectable *H-2* on primitive teratocarcinoma cells in culture. *Transplantation (Baltimore).* **17**:633.
18. Forman, J., and E. S. Vitetta. Absence of H-2 antigens capable of reacting with cytotoxic T cells on a teratoma line expressing a *T* locus antigen. *Proc. Natl. Acad. Sci. U. S. A.* In press.
19. Brown, J. L., K. Kato, J. Silver, and S. C. Nathenson. 1974. Notable diversity in peptide composition of murine H-2K and H-2D alloantigens. *Biochemistry.* **13**:3174.
20. Klein, J., and Shreffler, D. C. 1971. The *H-2* model for histocompatibility systems. *Transplant Rev.* **6**:3.
21. Murphy, D. B., and D. C. Shreffler. 1975. Cross reactivity between H-2K and H-2D products. I. Evidence for extensive and reciprocal serological cross-reactivity. *J. Exp. Med.* **141**:374.
22. Edidin, M., and C. S. Henney. 1973. The effect of capping H-2 antigens on the susceptibility of target cells to humoral and T-cell-mediated lysis. *Nat. New Biol.* **246**:47.
23. Forman, J., and J. Klein. 1975. Analysis of *H-2* mutants: evidence for multiple CML target specificities controlled by the *H-2K*^{b} gene. *Immunogenetics.* **1**:469.
24. Berke, G., and B. D. Amos. 1973. Cytotoxic lymphocytes in the absence of detectable antibody. *Nat. New Biol.* **242**:237.
25. Aoki, T. 1971. Surface antigens of murine leukemia cells and murine leukemia viruses. *Transplant Proc.* **3**:1195.

26. Hecht, T. T., and D. F. Summers. 1972. Effect of vesicular stomatitis virus infection on the histocompatibility antigen of L cells. *J. Virol.* **10**:578.
27. Fujimoto, S., C. H. Chen, E. Sabbadini, and A. C. Sehon. 1973. Association of tumor and histocompatibility antigens in sera of lymphoma-bearing mice. *J. Immunol.* **111**:578.
28. Rossi, G. B., G. Cudkowicz, and C. Friend. 1971. Requirement of live Friend leukemia virus for enhanced expression of *hybrid histocompatibility* genes. *Proc. Soc. Exp. Biol. Med.* **138**:783.
29. Cudkowicz, G., and J. H. Stimpfling. 1964. Deficient growth of C57BL marrow cells transplanted in F_1 hybrid mice. *Immunology.* **7**:291.