CELL-MEDIATED LYMPHOLYSIS OF TRINITROPHENYL-MODIFIED AUTOLOGOUS LYMPHOCYTES

Effector Cell Specificity to Modified Cell Surface Components Controlled by the *H-2K* and *H-2D* Serological Regions of the Murine Major Histocompatibility Complex

By GENE M. SHEARER, TERRY G. REHN, AND CAROL A. GARBARINO

(From the Immunology Branch, National Cancer Institute, Bethesda, Maryland 20014)

The major histocompatibility complex (MHC)[1] has been shown to play an important and probably essential role in the immune systems of a number of animal species ranging from mouse to man (1–3). The murine MHC known as the *H-2* complex has been divided into four major regions, and include two serological regions known as *H-2K* and *H-2D* or *K* and *D*, which are separated by two other regions designed *I* and *S* (4). The *K* and *D* regions determine the strong serologically detectable transplantation antigens of the mouse, which seem to be important as the target antigens for T-cell-mediated lympholysis (5–10). The *S* region is positioned adjacent to and to the left of the *D* region with respect to the centromere. It determines the levels of a serum alpha globulin known as Ss as well as a sex-linked allotypic variant of Ss designated Slp (11). Genes mapping within the *I* region designated *Ir* or immune response genes, are located between *K* and *S*, and control immune response potential to a number of synthetic and natural immunogens (for review see 12–14). Recent *Ir*-gene (15–17) and Ia-antigen (18–21) studies in B10.A recombinant mouse strains have led to the division of the *I* region into three regions designated *I-A*, *I-B*, and *I-C* (22). At the present time, therefore, the *H-2* complex is clearly divisible into six regions: *K*, *I-A*, *I-B*, *I-C*, *S*, and *D*.

Some of the immunological functions attributed to MHC genes have been demonstrated by employing allogeneic grafting or cell combinations between incompatible partners. Although such procedures are necessary for detecting alloantigens, it is unlikely that these immunological phenomena as well as the antigenic differences they reflect were naturally selected in evolution for the purpose of allogeneic immune reactions.

The results presented in this report demonstrate that murine T-cell-mediated cytotoxic effector lymphocytes generated in vitro by sensitization to trinitrophenyl (TNP)-modified autologous spleen cells (23) are specific for TNP-modified target cells expressing *K* and/or *D* serological specificities identical to those of the modified stimulating cells. Furthermore, the differential response patterns observed to TNP-modified *K*- and *D*-region products by responding lymphocytes of mice expressing *k* alleles in *K* and *I* regions suggest that *H-2*-linked *Ir* genes

[1] *Abbreviations used in this paper:* BSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; SD, serologically defined; TNBS, 2,4,6-trinitrobenzene sulfonate; TNP, trinitrophenyl.

may be controlling response potential to new cellular antigenic determinants resulting from TNP modification of the *K* and *D* serological specificities. These findings necessitate a fresh examination of certain functional aspects of the genes associated with the MHC by involving them in an autologous model. A syngeneic functional role for the cell surface products controlled by the *K* and *D* regions is considered.

Materials and Methods

Mice. All mice used in the experiments were males, 7–9 wk of age. The B10 congenic strains were purchased from Jackson Laboratories, Bar Harbor, Maine. The B10.A(2R), B10.A(4R), and B10.A(5R) recombinant lines were raised in our mouse colony from breeders obtained either from Jackson Laboratories or from Dr. Donald Shreffler, Department of Human Genetics, University 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. Other inbred strains were obtained from the NIH Animal Production facility, Bethesda, Md.

TNP Modification of Spleen Cells. Cells to be modified with TNP were prepared from the spleen of young adult mice by gentle washing with blunt forceps, aspirating through a syringe in Hanks' balanced salt solution (BSS), and filtering through nylon mesh to remove clumps and debris. Erythrocytes were lysed by a 2 min exposure of the cell suspension to ammonium chloride lysing buffer, followed by washing in BSS. 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 volume:volume 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 BSS 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 [^{51}Cr]Na_2CrO_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 (23) by the Mishell and Dutton procedure (24). After a 5 day incubation period, effector cells were harvested and their cytotoxic reactivity was determined by a modification of the method of Canty and Wunderlich (25). The mean and standard error of the percentage of specific cytotoxicity was calculated as described elsewhere (26).

Blocking of Effector Phase with Nonradioactive, TNP-Modified Target Cells. Nonradioactive splenic target cells sharing *K*- and/or *D*-end specificities with cells of the stimulating phase were preincubated with B10.BR effector lymphocytes for 30 min before addition of ^{51}Cr-labeled, TNP-modified target cells in the 4-h cytotoxic phase. The ratios of unlabeled to ^{51}Cr-labeled target cells were 5:1, 10:1, and 20:1. The unlabeled targets were maintained in the effector-^{51}Cr-labeled target cell mixture throughout the effector phase.

Results

Effector Cell Specificity Using TNP-Modified Spleen Target Cells. An earlier report demonstrated that in vitro induction of T-cell-mediated lympholysis to TNP-modified autologous spleen cells resulted in the generation of effector lymphocytes which lysed TNP-modified syngeneic target cells, but not TNP-modified *H-2*-nonrelated congenic targets (23). However, modified target cells which shared *H-2K*-end specificities with the responder and stimulating cells were lysed. These results and the subsequent preliminary reports (27, 28) suggested that a part of the effector cell specificity was directed against TNP-altered cell surface components controlled by genes mapping within the *H-2* complex. In order to verify the initial observations and to map within *H-2* the genes controlling the cellular components modified by TNP, splenic lymphocytes from B10.A, B10.BR, B10.D2, and C57BL/10 donors were sensitized in vitro with TNP-modified autologous spleen cells. The effector cells generated 5 days later

were tested on unmodified and TNP-modified syngeneic target spleen cells, as well as on a number of modified congenic, allogeneic, and recombinant splenic targets which share certain intra-*H-2* alleles with the responding and stimulating cells. In order to minimize any possible antigenic differences that might exist between TNP-modified stimulating and target cells, these experiments employed TNP-modified normal spleen cells as targets rather than *H-2*-matched tumor targets or syngeneic, mitogen-induced splenic blasts. The spontaneous ^{51}Cr release from TNP-modified spleen cell targets in the 4-h lytic phase ranged between 21 and 43%. Results representative of these effector cell specificity experiments are summarized in the first four tables of this report. Depending on the availability of target cell donors each combination was verified 2—20 times. The first three columns of these tables indicate the strains of the responding, stimulating, and target cells, respectively. The *H-2* alleles for the various regions of *H-2* are shown beneath each strain. The fourth column gives the mean percent of specific cytotoxicity with standard errors of the means. The last column indicates the *H-2* region common between the target cells and the responding and stimulating cells.

Results obtained by testing effector cells generated from cultures of B10.A-responding and B10.A-TNP-stimulating cells on the different targets are summarized in Table I. B10.A effector cells did not lyse unmodified B10.A targets, whereas they did lyse TNP-modified B10.A targets. This verifies that modification of the syngeneic target cell is necessary in order to obtain cytotoxicity. Modified B10.A(2R), which shares all known *H-2* regions except *D*; B10.BR which shares *K*, *I-A*, and *I-B*; and B10.A(4R) which shares *K* and *I-A* with B10.A were all effective targets for detecting lympholysis by the B10.A effectors. These results indicate that at least part of the specificity of the B10.A effector cells is directed against TNP-modified cell surface components controlled by *K* or *K* plus *I-A*. In contrast, TNP-modified B10.D2 and B10.A(5R) which share *I-C*, *S* and *D*; A.TL which shares *I-A*, *I-B*, and *D*; C57BL/10 which share no known *H-2* regions; and B10.HTG and C3H.OH which share *I-C* and *S* with the responding and stimulating cells were all ineffective targets. These findings indicate that the cytotoxicity of the B10.A effectors was not directed against the *D* end and was exclusively specific for the *K* end.

Effector cells generated from cultures of B10.BR lymphocytes sensitized with B10.BR-TNP spleen cells were also tested on a series of TNP-modified targets. This strain shares *K*-end *H-2* alleles (i.e., *K*, *I-A*, and *I-B*) with B10.A, but differs in the *D*-end alleles (*I-C*, *S*, and D). Representative results, using targets identical to those used to test B10.A effector cells are summarized in Table II. Modification of syngeneic targets was necessary in order to obtain cytotoxicity. B10.A-TNP and B10.A(2R)-TNP targets, which share *K*-end alleles with B10.BR, were lysed as effectively as syngeneic B10.BR-TNP targets. C3H.OH-TNP targets which share only the *H-2D*-region allele with the responding and stimulating cells were not lysed. A.TL-TNP targets, which appear to share all *H-2* regions with B10.BR except the *K* and *D* serological regions, were ineffective targets. The other three strains shown which share no known *H-2* alleles with B10.BR were not lysed. These results indicate that B10.BR lymphocytes sensitized with TNP-modified syngeneic spleen cells respond to the TNP-modi-

TABLE I

In Vitro Induction of Cytotoxicity of B10.A Spleen Cells to TNP-Modified Autologous Spleen Cells Assayed with TNP-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis ± SE	Target cell *H-2* region common to responding and stimulating cells
			%	
B10.A kkkddd	B10.A-TNP kkkddd	B10.A kkkddd	−3.2 ± 2.0	All of *H-2*
" "	" "	B10.A-TNP kkkddd	28.6 ± 3.9	All of *H-2*
" "	" "	B10.A(2R)-TNP kkkddb	34.3 ± 6.4	*K, I-A, I-B, I-C, S*
" "	" "	B10.BR-TNP kkkkkk	31.5 ± 2.5	*K, I-A, I-B*
" "	" "	B10.A(4R)-TNP kkbbbb	37.3 ± 5.0	*K, I-A*
" "	" "	B10.D2-TNP dddddd	3.9 ± 2.0	*I-C, S, D*
" "	" "	B10.A(5R) bbbddd	3.0 ± 1.4	*I-C, S, D*
" "	" "	A.TL-TNP skkkkd	3.9 ± 4.8	*I-A, I-B, D*
" "	" "	C57BL/10-TNP bbbbbb	3.1 ± 2.5	None
" "	" "	B10.HTG-TNP dddddb	2.4 ± 3.0	*I-C, S*
" "	" "	C3H.OH-TNP dddddk	1.9 ± 3.1	*I-C, S*

Effector:target cell ratio, 20:1

fied *K* or *K* plus *I-A* regions and not to the modified *D* allele. Thus, the pattern of specificity observed for the B10.BR effector cells is identical to that noted for B10.A effectors (Table I), i.e. lymphocytes from both strains responded to *K*-end modification (which are identical), but not to their respective (and different) modified *D* alleles.

In contrast to B10.BR, the B10.D2 strain shares the *D* end (*I-C, S, D,*) with B10.A. It was of interest to test the specificity of B10.D2 effector cells generated in the TNP-modified autologous system, particularly since B10.A effector cells did not respond to TNP-modified *H-2D d*-allele products. The results, shown in

TABLE II

In Vitro Induction of Cytotoxicity of B10.BR Spleen Cells to TNP-Modified Autologous Spleen Cells Assayed with TNP-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis ± SE	Target cell *H-2* region common to responding and stimulating cells
			%	
B10.BR kkkkkk	B10.BR-TNP kkkkkk	B10.BR kkkkkk	-4.0 ± 1.9	All of *H-2*
" "	" "	B10.BR-TNP kkkkkk	33.2 ± 3.0	All of *H-2*
" "	" "	B10.A-TNP kkkddd	31.5 ± 2.5	*K*, *I-A*, *I-B*
" "	" "	B10.A(2R)-TNP kkkddb	26.9 ± 2.2	*K*, *I-A*, *I-B*
" "	" "	C3H.OH-TNP dddddk	2.1 ± 2.1	*D*
" "	" "	A.TL-TNP skkkkd	-2.2 ± 2.2	*I-A*, *I-B*, *I-C*, *S*
" "	" "	A.TH-TNP sssssd	-0.4 ± 1.3	None
" "	" "	SJL-TNP ssssss	-0.7 ± 1.2	None
" "	" "	B10.D2-TNP dddddd	-2.2 ± 2.4	None

Effector:target cell ratio, 20:1

Table III, again indicate that TNP modification of the syngeneic targets is necessary to obtain lysis. The various TNP-modified targets lysed by the B10.D2 effector cells were B10.D2, B10.A, B10.A(5R), A.TL, and C3H.OH. The modified targets in which significant lysis was not obtained include B10.A(2R), B10.BR, and B10.A(4R). In contrast to the results obtained with B10.A and B10.BR, B10.D2 lymphocytes responded to TNP-modified *D* serological region, and in fact, to the same modified *D*-end allele to which B10.A did not respond (compare results in Tables I and III using A.TL-TNP targets). This difference in response to the TNP-modified *H-2D d*-allele by B10.A and B10.D2 raises the possibility of *H-2*-linked *Ir* genes (see Discussion). The B10.D2 effector cells also responded to one or more TNP-modified *H-2* alleles other than *H-2D*, as shown by the 14.7% lysis obtained with C3H.OH-TNP targets. Disregarding specificity, the B10.D2 effectors generally gave lower cytotoxic levels than did the B10.A and B10.BR effector cells. Modified targets which share no known regions of *H-2* with cells

of the sensitizing phase show no significant lysis, e.g., B10.BR-TNP and B10.A(4R)-TNP, or reduced lysis as in the case of C57BL/10-TNP. The latter targets were lysed 11.0 ± 2.9% by B10.D2 effectors, which is of questionable significance.

The C57BL/10 strain, which does not share any known *H-2* alleles with the congenic lines tested above, was also tested for effector cell specificity. The results of representative experiments are summarized in Table IV. The TNP-modified targets that detected high levels of effector lympholysis were C57BL/10, B10.A(5R), B10.A(4R), and B10.A(2R), as well as the noncongenic but *H-2*-matched C3H.SW. The positive results obtained using B10.A(5R)-TNP targets map some of the specificity to modified *K*-end products, whereas the 24.5 and

TABLE III

In Vitro Induction of Cytotoxicity of B10.D2 Spleen Cells to TNP-Modified Syngeneic Spleen Cells Assayed with TNP-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis ± SE	Target cell *H-2* region common to responding and stimulating cells
			%	
B10.D2 dddddd	B10.D2-TNP dddddd	B10.D2 dddddd	−2.4 ± 2.8	All of *H-2*
" "	" "	B10.D2-TNP dddddd	19.2 ± 2.4	All of *H-2*
" "	" "	B10.A-TNP kkkddd	17.6 ± 3.6	*I-C*, *S*, *D*
" "	" "	B10.A(5R)-TNP bbbddd	12.8 ± 2.1	*I-C*, *S*, *D*
" "	" "	B10.A(2R)-TNP kkkddb	0.8 ± 1.6	*I-C*, *S*
" "	" "	A.TL-TNP skkkkd	15.6 ± 3.7	*D*
" "	" "	C3H.OH-TNP dddddk	14.7 ± 1.2	*K*, *I-A*, *I-B*, *I-C*, *S*
" "	" "	B10.BR-TNP kkkkkk	2.4 ± 0.6	None
" "	" "	C57BL/10-TNP bbbbbb	11.0 ± 2.9	None
" "	" "	B10.A(4R)-TNP kkbbbb	5.0 ± 3.1	None

Effector:target cell ratio, 20:1

TABLE IV
In Vitro Induction of Cytotoxicity of C57BL/10 Spleen Cells to TNP-Modified Autologous Spleen Cells Assayed with TNP-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis ± SE	Target cell *H-2* region common to responding and stimulating cells
			%	
C57BL/10 bbbbbb	C57BL/10-TNP bbbbbb	C57BL/10 bbbbbb	1.7 ± 2.1	All of *H-2*
"	"	C57BL/10-TNP bbbbbb	34.3 ± 2.7	All of *H-2*
"	"	B10.A(5R)-TNP bbbddd	23.9 ± 2.7	*K, I-A, I-B*
"	"	B10.A(4R)-TNP kkbbbb	24.5 ± 1.9	*I-C, S, D*
"	"	B10.A(2R)-TNP kkkddb	16.9 ± 1.5	*D*
"	"	B10.BR-TNP kkkkkk	7.5 ± 2.4	None
"	"	B10.D2-TNP dddddd	4.4 ± 1.6	None
"	"	B10.A-TNP kkkddd	9.5 ± 2.2	None
"	"	C3H.SW-TNP bbbbbb	15.1 ± 1.1	All of *H-2*

Effector:target cell ratio, 20:1

16.9% lysis detected with B10.A(4R)-TNP and B10.A(2R)-TNP targets, respectively, map another specificity component to the *D* end and to modified cell surface components controlled by the *b* allele of *H-2D*. In contrast to B10.A and B10.BR effector cells which did not significantly lyse modified targets not sharing known *H-2* alleles with cells of the sensitization phase, but similar to the weak B10.D2 effector lysis of C57BL/10-TNP targets, C57BL/10 effectors did lyse to a low, variable and possibly significant extent modified targets which do not share known *H-2* alleles. However, some Ia antigens, as well as certain serological specificities are shared between the C57BL/10 cells of the stimulating phase and these modified targets (i.e., B10.BR-TNP and B10.A-TNP) (4).

Effector Cell Specificity Studies Using TNP-Modified Tumor Target Cells. In the preceding section effector cell cytotoxicity was observed only if the spleen target cells were TNP modified and expressed the same *K* and/or *D* regions as the stimulating and/or responding cells. It could be argued that tumor

cells are more sensitive targets than splenic lymphocytes and that the peculiar *H-2*-associated specificity observed may be unique for splenic targets but not for the more sensitive tumor targets. It might even be possible to detect hapten-specific (TNP) cytotoxicity using modified tumor target cells. To test these possibilities B10.A, B10.BR, B10.D2, and C57BL/10 splenic lymphocytes were sensitized with TNP-modified autologous spleen cells and the effector cells generated 5 days later were assayed on unmodified and TNP-modified tumor targets expressing the same and different *H-2* haplotypes as the responding and stimulating cells. The results of a typical experiment are shown in Table V. Unmodified target cells were not lysed by the effectors. TNP-modified tumor target cells expressing the same *H-2* as the responding and stimulating cells were the most effective target cells, and without exception, yielded higher cytotoxic levels than did modified, non-*H-2* compatible tumor targets. However, in contrast to the results shown in Tables I–IV using modified spleen cells as targets, a significant though lesser amount of cytotoxicity was detected when modified tumor cells not *H-2* matched with the cultured cell mixture were used as targets.

TABLE V
In Vitro Induction of Cytotoxicity of B10.A, B10.BR, B10.D2 and C57BL/10 Spleen Cells to TNP-Modified Autologous Spleen Cells Assayed with TNP-Modified H-2-Matched and Unmatched Tumor Target Cells

Responder cells	Stimulator cells	Target cells	Specific lysis ± SE	Target cell *H-2* haplotype
			%	
B10.A (kkkddd)	B10.A-TNP (kkkddd)	RDM4	2.2 ± 2.2	*k*
" "	" "	RDM4-TNP	36.0 ± 1.5	*k*
" "	" "	P815-TNP	4.3 ± 0.5	*d*
" "	" "	LSTRA-TNP	6.1 ± 0.5	*d*
" "	" "	EL4-TNP	12.9 ± 0.4	*b*
B10.BR (kkkkkk)	B10.BR-TNP (kkkkkk)	RDM4	1.4 ± 0.9	*k*
" "	" "	RDM4-TNP	54.3 ± 3.7	*k*
" "	" "	P815-TNP	6.2 ± 1.2	*d*
" "	" "	LSTRA-TNP	5.8 ± 1.4	*d*
" "	" "	EL4-TNP	20.3 ± 3.9	*b*
B10.D2 (dddddd)	B10.D2-TNP (dddddd)	P815	5.0 ± 1.2	*d*
" "	" "	LSTRA	5.0 ± 1.6	*d*
" "	" "	P815-TNP	31.9 ± 3.5	*d*
" "	" "	LSTRA-TNP	41.0 ± 1.3	*d*
" "	" "	RDM4-TNP	26.0 ± 2.4	*k*
" "	" "	EL4-TNP	25.7 ± 1.4	*b*
C57BL/10 (bbbbbb)	C57BL/10-TNP (bbbbbb)	EL4	1.6 ± 1.5	*b*
" "	" "	EL4-TNP	40.7 ± 5.4	*b*
" "	" "	P815-TNP	4.5 ± 1.1	*d*
" "	" "	LSTRA-TNP	3.2 ± 1.6	*d*
" "	" "	RDM4-TNP	21.1 ± 2.9	*k*

Effector:target cell ratio, 20:1

Cytotoxic levels of 12.9, 20.3, and 29.7% were obtained, respectively, when EL4-TNP targets were used to assay cultures of B10.A-, B10.BR-, and B10.D2-responding and modified stimulating cells. Likewise, RDM4-TNP was an effective target for detecting cytotoxicity of effectors generated in B10.D2 and C57BL/10 cultures. It is not surprising that RDM4-TNP is an effective target for B10.A cultures, since B10.A expresses *K* alleles at *K*, *I-A*, and *I-B*.

However, an exception to the *H-2*-associated cross-reactivity of modified tumor cells was observed for the two $H\text{-}2^d$ tumor lines used. Both P815-TNP and LSTRA-TNP were effective targets only when assayed with effectors generated from B10.D2 donors. Thus, TNP modified $H\text{-}2^d$ tumor cell targets behaved more like the modified spleen targets. Furthermore, P815-TNP and LSTRA-TNP did not detect cytotoxicity of effector cells generated from B10.A-responding and B10.A-TNP-stimulating cells. (B10.A spleen and $H\text{-}2^d$ tumor share *D*-end serological specificities.) This observation is also remarkably similar to the specificity observed with splenic targets but contrasts with the other modified tumor targets. Finally, the *H-2*-associated specificity restrictions noted with the $H\text{-}2^d$ tumors applies only to $H\text{-}2^d$ target cells and not to $H\text{-}2^d$ effectors. The B10.D2 effectors lysed both RDM4-TNP and EL4-TNP targets.

Verification of Exclusive TNP-Modified K-end Specificity of B10.BR Effector Cells by Blocking with Modified Nonradioactive Targets. As an independent verification of preferential specificity of B10.BR lymphocytes toward TNP-modified *K*-end components, nonradioactive TNP-modified cells were added to the ^{51}Cr-labeled spleen target and effector cell mixture for the duration of the 4-h effector phase. The nonradioactive, modified cells added were: (*a*) B10.BR-TNP, which do not discriminate between the *K* and *D* ends, since they are syngeneic with responding and target cells; or (*b*) C3H.OH-TNP which should compete for the modified *k* allele at the *D* end only, providing B10.BR lymphocytes are capable of responding to TNP-modified autologous *D* region. Results of a typical experiment are shown in Table VI. Addition of unlabeled, unmodified cells

TABLE VI
Effect of Adding TNP-Modified, Nonradioactive Target Cells to a Mixture of B10.BR Effector Cells and ^{51}Cr-Labeled, TNP-Modified B10.BR Target Spleen Cells

Nonradioactive target cells added to effector phase ($\times 10^5$)	Lysis ± SE		Reduction in cytotoxicity
	Unmodified	TNP modified	
	%	%	%
2.5 B10.BR	19.5 ± 3.7	14.0 ± 2.1	28.2
5.0 B10.BR	29.1 ± 3.9	10.0 ± 1.6	66.5
10.0 B10.BR	27.5 ± 1.1	8.0 ± 3.3	70.9
2.5 C3H.OH	22.2 ± 1.2	24.3 ± 3.0	−9.5
5.0 C3H.OH	22.3 ± 2.8	22.1 ± 2.6	0.9
10.0 C3H.OH	20.1 ± 2.4	23.2 ± 3.1	−15.4

The numbers of effector and ^{51}Cr-labeled target cells mixed in the effector phase were 1×10^6 and 5×10^4, respectively, i.e., 20:1 ratio. Cytotoxic levels of B10.BR effector cells assayed on B10.BR-TNP and C3H.OH-TNP target cells without any nonradioactive targets were 39.4 and 8.5%, respectively.

tended to reduce the cytotoxic levels from 39.4 to between 19.5 and 29.1%. This reduction could be due to a crowding effect of the extra cells added to the cytotoxic phase. Nonradioactive B10.BR-TNP had an additional effect on reducing cytotoxic levels. This reduction was a direct function of the number of nonradioactive B10.BR cells added. In contrast, the addition of unlabeled C3H.OH-TNP cells to the effector phase had no specific effect on reducing the cytotoxic level below control values. These results verify that the specificity of the B10.BR effector lymphocytes is directed toward an entity other than TNP-modified cell surface components controlled by the *K* allele of the *H-2D*.

Demonstration that the H-2K and/or D Homology is Required between Modified Targets and Modified Stimulating Cells. In preceding sections of this report *H-2K*- and/or D-associated homology between TNP-modified stimulating cells responding lymphocytes was repeatedly demonstrated. None of the results involving spleen cell targets discriminated among the possibilities that the strict intra-*H-2* homology observed is required: (*a*) between modified target cells and responding lymphocytes; (*b*) between modified target and modified stimulating cells; or (*c*) among all three of these cell types. In order to determine whether these requirements of specificity are between target and responder cells or modified target and modified stimulator cells, (C57BL/10 × B10.A)F_1 lymphocytes were sensitized with TNP-modified B10.A or with TNP-modified C57BL/10-stimulating cells. The effector cells generated from these cultures were then assayed on TNP-modified target spleen cells from both parents. If homology were required between target and responding cells, then effectors generated by sensitization of F_1 cells with either parent should lyse target cells from either parent providing that both stimulating and target cells were modified. If, however, *K*- and *D*-region homology were required between modified stimulator and modified target cells, then lysis of modified parental targets would occur only when the responding lymphocytes were sensitized with modified spleen cells from an identical parent. As shown in Table VII, cytotoxicity was obtained only in those cases in which the modified target and stimulating cells were identical. These results, therefore, verify the initial interpretation (23) that this phenomenon involves antigenic recognition of and response to TNP-altered *H-2*-controlled

TABLE VII
In Vitro Induction of Cytotoxicity of (B10 × B10.A)F_1 Spleen Cells to TNP-Modified B10 or B10.A Spleen Cells Assayed with TNP-Modified B10 or B10.A Spleen Cells

Responding cells	Stimulating cells	Target cells	Specific lysis ± SE	Target cell homology with:	
				Responding cells	Stimulating cells
			%		
(B10 × B10.A)F_1	B10.A-TNP	B10.A-TNP	26.0 ± 2.2	Yes	Yes
"	B10.A-TNP	B10-TNP	5.5 ± 1.8	Yes	No
"	B10-TNP	B10-TNP	38.1 ± 3.4	Yes	Yes
"	B10-TNP	B10.A-TNP	4.6 ± 2.7	Yes	No

Effector:target cell ratio, 20:1

cell surface components, which are subsequently required as targets for the stimulated clone(s) of effector cells generated.

Discussion

The data shown throughout this report (with the exception of certain tumor cell targets, Table V) repeatedly indicate that the cytotoxic specificity of the thymus-derived effector cells is directed toward altered cell surface components which map within the murine MHC. By sensitizing splenic lymphocytes with TNP-modified autologous spleen cells and testing for lympholysis on modified splenic targets differing from the responding and stimulating cells within *H-2*, it was possible to map the particular MHC region responsible for the specificity to the *K* end for B10.A and B10.BR, and to both the *K* and *D* ends for B10.D2 and C57BL/10. Preliminary reports of results similar to a portion of those shown here have been presented recently (27, 28).

Effector cells generated by sensitization of B10.A and B10.BR lymphocytes with the respective TNP-modified autologous spleen cells lysed all modified splenic targets which expressed the *k* allele in the *K* and *I-A* regions (Tables I and II). The failure of both of these effectors to lyse A.TL-TNP targets indicated that modified cell surface components controlled by the *k* allele of the *K* region are a necessary part of this specificity. Since no recombinants were available which express the *k* allele in the *K* region but not in *I-A*, it was not possible to establish whether the specificity involved modified components controlled by *K* alone or *K* plus *I-A*. The failure of B10.A effector cells to lyse B10.D2-TNP and B10.A(5R)-TNP targets indicated that the specificity of these lymphocytes is directed primarily toward the *K* end, and that modified products of *I-C*, *S*, and *D* regions are not recognized by B10.A-responding cells. The failure of B10.A to respond to modified products of *D* and of *I-C* and *S* are verified separately using the A.TL-TNP and the B10.HTG-TNP and C3H.OH-TNP targets. Similarly, the fact that B10.BR effector cells did not lyse C3H.OH-TNP targets, which shares only the *D* region, nor three other targets, which share no known *H-2* regions with B10.BR, indicate that these responding lymphocytes are exclusively specific for modified products of the *K* end of *H-2*. This was further verified by the failure of unlabeled, TNP-modified C3H.OH cells to reduce the cytotoxic levels of the B10.BR effector phase (Table IV).

Restriction of reactivity to altered *K*-end products, however, is not necessarily a characterisitic of all mouse strains. Both B10.D2 and C57BL/10 responded as well to their TNP-modified *D* region *d* and *b* alleles as to their respective modified *K* ends. B10.D2 effector cells lysed both TNP-modified targets which share only the *D* region (A.TL-TNP, Table III) with the sensitizing cells; and C3H.OH-TNP which are syngeneic with B10.D2 at all *H-2* regions except *D*. This indicates that B10.D2 lymphocytes respond to autologous TNP-modified *D*-region products and to at least a second TNP-modified autologous specificity mapping to the left of *H-2D*, which could be controlled by *H-2K* (although this was not proven). Similarly, C57BL/10 effector cells generated by sensitization with modified autologous cells lysed B10.A(4R)-TNP and B10.A(2R)-TNP targets, the latter of which localizes the cytotoxic specificity to modified products of *H-2D*; as well as B10.A(5R)-TNP, which maps a second modified specificity controlled by the *b*

allele(s) and the *K* end of *H-2*. Similar observations have been made for cytotoxic specificity against lymphocytic choriomeningitis virus-infected target cells (29, 30). Another observation made in the B10.D2 and C57BL/10 strains but not seen in the B10.A and B10.BR strains was the weak, variable, and statistically questionable reactivity observed to some TNP-modified splenic targets which do not share known *H-2* regions with the responding and stimulating cells. If these weak responses are real, they may represent reactivities toward TNP-modified Ia antigens and/or serological specificities shared between the targets and cells of the stimulating phase.

It is noteworthy that the strict *H-2*-dependent cytotoxic specificity observed with modified spleen target cells was not seen when TNP-modified tumor cells were used as targets. Modified tumor cells (with the exception of $H\text{-}2^d$ tumors) not *H-2* matched with the cells of the stimulating phase were effective targets, although not as good as modified *H-2*-matched tumor cells. This finding indicates that the tumor targets detected modified *H-2*-controlled specificity, as well as nonhistocompatibility-associated specificity. The latter specificity could be directed toward the TNP hapten exclusively, and might not be detected using the less sensitive spleen targets. However, such an interpretation would not account for the failure of two $H\text{-}2^d$ tumor targets to be lysed by non-*H-2*-matched effectors (see Table V). The nature of the additional specificity component detected with $H\text{-}2^k$ and $H\text{-}2^b$ TNP-modified tumor target cells remains to be elucidated. The discrepancy reported here and previously (23) in the ability of B10.D2 effectors to lyse EL4-TNP targets may be due to changes in culture conditions such as the addition of 2-mercaptoethanol. Since tumor cell targets are often used for allogeneic T-cell-mediated cytotoxic assays, this comparison of modified tumor and spleen cells illustrates the importance of using splenic rather than tumor targets for demonstrating cytotoxic reactivity to *H-2*-controlled chemically modified cellular components.

The dependence of lympholysis in this system on *H-2* compatibility between the TNP-modified splenic target cells and the cells of the stimulating phase is noteworthy. At least two explanations can be given to account for this intra-*H-2* dependency: (*a*) effector-target cell interaction involving some *H-2*-controlled surface structure; or (*b*) sensitization of the relevant lymphoid clones to TNP-modified, *H-2*-controlled autologous cell surface components, leading to the generation of effector cells specific for and capable of lysing only targets expressing the same modified *H-2*-controlled antigenic specificities. The results shown in Table VII indicate that the second alternative is the correct one, since cytotoxicity was obtained only when the modified targets were syngeneic with the modified stimulating cells. Thus, this phenomenon is "autoimmune-like" in that pre-existing clones of responding lymphocytes react against modified autologous *H-2*-controlled self-components. Nevertheless, TNP is necessary in order to obtain cytolysis, as shown in the first two lines of Tables I–IV. It appears that the type of modifying agent is also important in determining the antigenic specificity of the recognition unit (T. G. Rehn et al., unpublished observations and reference 31), although it has not yet been established whether the modifying agent is an integral part of the antigen or whether it simply serves as modifier. A similar interpretation has been made for the *H-2*-dependent T-cell-mediated cytotoxic-

ity to lymphocytic choriomeningitis virus-infected target cells (32). It is evident from Table VII that there are at least two distinct clones of responding lymphocytes from the F_1 donors, one recognizing TNP-modified *H-2*^{b} components and the other TNP-modified *H-2*^{a} specificities.

The immunogenic entities resulting from TNP modification appear to involve "modified-self" and may represent altered histocompatibility antigens. If new cellular antigens are created by modification of the *H-2* serological specificities, the remarkable intra-*H-2* specificity observed raises the possibility that the major new determinants formed are restricted to private specificities, since some public specificities are shared between certain of the effector and target cells tested (4). Alternatively, it is possible that new antigens are formed by both modified private and public specificities. If so, then the mouse strains investigated are mainly responsive to the new cellular antigens resulting from TNP alteration of private specificites, but not to those involving modification of public specificities, possibly due to lack of expression of the proper *Ir* genes. Whatever the cell surface changes resulting from TNP modification are, they do not detectably affect expression of the *H-2* alloantigens involved in either the sensitization or effector phases of the in vitro cytotoxic response (28). It has not been excluded that *H-2*-dependent modification of cell surface alters *H-2* products which heretofore have not been detected.

It is noteworthy that the specificity of B10.A effector lymphocytes was directed exclusively toward modified products controlled by the *K* end, but not to products of the *H-2D* region, whereas B10.D2-responding cells sensitized with TNP-modified autologous cells generated effectors specific for modified *K*-end products and for modified *D*-region products (compare lines 6 and 4 of Tables I and III, respectively). Since B10.A and B10.D2 mice share the *H-2D* region, this comparison indicates that the lack of reactivity of B10.A splenic lymphocytes to TNP-modified cell surface components controlled by the *d* allele of *H-2D* was not due to a failure of the creation of a new modified autologous antigen. Furthermore, these observations indicate that formation of a new antigenic specificity is not sufficient in all instances to generate a cytotoxic response. However, by replacing the *k* alleles at the *K*, *I-A*, and *I-B* regions (B10.A) with the corresponding *d* alleles (B10.D2), i.e. at intra-*H-2* regions not detectably involved in this particular specificity, a cytotoxic response was obtained. It is noteworthy that the region(s) responsible for reactivity does not include *H-2D*, which is associated with the TNP-modified antigen, but does include the *I-A* and *I-B* regions, which have been shown to control immune responsiveness to a number of immunogens (1, 14). Thus, it is probable, but not yet proven that the failure of B10.A-responding lymphocytes to generate an in vitro cytotoxic response to TNP-modified products controlled by the *d* allele of the *D* region is due to the lack of expression of the proper *H-2*-linked *Ir* gene(s), which maps in the *K* end. The gene(s), however, appears to be expressed in the B10.D2 strain, which differs from B10.A at *I-A* and *I-B*, as well as at *K*. If *Ir* genes are involved in controlling sensitization to TNP-modified autologous cells, this model offers some unique properties which include the demonstration: (*a*) of an *Ir*-gene-controlled primary immune response in vitro; (*b*) of *Ir*-controlled T-cell-mediated lympholysis which implies an *Ir*-gene defect exclusively attributable to a functional thymus-derived cell population;

and (*c*) that the MHC can be shown to be involved both in the response potential to and in the formation of new cellular antigenic determinants.

The TNP modification of cells in these experiments is likely to induce many conformational cell surface alterations which need not necessarily be associated with the MHC, particularly not with what appear primarily to be private *H-2* serological specificities. How, then, might we account for this restriction of specificity? Restriction of cellular immunity to TNP-modified autologous products of the serologically defined regions can be accounted for in at least three nonmutually exclusive ways. First, the immune system might be genetically capable of responding to many altered self-antigens resulting from modification of *H-2* products, but the restriction could be due to the formation of a very limited number of new antigenic determinants resulting from the modification. Second, it is possible that many new antigenic determinants are formed as a result of TNP modification, but the restriction on reactivity may result from a limited number of *H-2*-linked *Ir* genes, which define and limit the number of distinct and specific clones in the immunological pool of an inbred mouse strain. Finally, it could be argued that the serologically defined (SD) regions of the MHC control alloantigenic specificities which are important as cytotoxic effector cell targets (5), and that the same restrictions apply to TNP-modified autologous SD-controlled products. At the present time it can be said that at least one precedent appears to exist in this system, which is compatible with the second interpretation. Splenic lymphocytes do not respond to TNP-modified autologous products controlled by the *d* allele of *H-2D*, whereas B10.D2 responding lymphocytes which express different *I* alleles and therefore different *Ir* genes do.

What is the significance of control by the MHC of both the formation of new antigenic specificities and the response potential to these specificities? It may be that bifunctional histocompatibility linked control of the immune response involving *Ir* genes as well as new antigens resulting from modified histocompatibility gene products is more general than that demonstrated for TNP-modified autologous antigens. Such a model could account for multigenic control of autoimmune thyroiditis (33) and viral-induced leukemia in mice (34–36), as well as HL-A-associated disease susceptibility in humans (3).

The possible functional significance of histocompatibility antigens has been reviewed elsewhere (37). Snell has considered that histocompatibility antigens may serve as receptor sites for the attachment of viruses to cell surfaces (38). In this model the receptor site was postulated to be essential for infection. The selective advantage of such a model has been questioned, however, since heterozygotes would be most susceptible to infectious virus (37). However, it should be pointed out that the heterozygote would be at an advantage if the possibility is now considered that the histocompatibility controlled serological specificities serve not as receptors for viral infection (and therefore to the agent's disadvantage), but rather as easily modifiable autologous cell surface structures which are particularly suited for cell-mediated immune reactions against the infection. In support of this, lymphocytes from heterozygous F_1 donors have a broader range of reactive potential to TNP-modified autologous components than do the cells from either homozygous parent (A.-M. Schmitt-Verhulst and G. M. Shearer, unpublished observations). Susceptibility could result if the proper

serological specificities were not expressed to form immunogenic complexes with the infectious agents on the cell surface, and/or if the relevant *H-2*-linked *Ir* genes were not expressed. Such a hypothetical mechanism could serve in surveillance against viral-induced autologous tumors, since it might remove viral-transformed potentially neoplastic cells, and, to the individuals disadvantage, in autoimmunity.

Based on the results of this report and the above discussion, the following working hypothesis is proposed: (*a*) It is probable that the cell surface antigens controlled by the MHC have some evolutionary and possibly current immunological significance. (*b*) It is improbable that this significance is for allograft reactivity, since the system evolved in an autologous state, although fetal-maternal interactions may have been important (37). (*c*) One autologous function (based on the results presented here) is that major histocompatibility controlled serological specificities can serve as cell surface components which are chemically modifiable, by virus infection or possibly by other means (e.g., autoimmune complexes) to create new cell-bound antigens which are particularly suitable for inducing cell-mediated immunity. (*d*) Whether or not immune reactivity will result from such alterations depends; (i) on the expression of immune response genes which map in the same genetic region, and/or (ii) on the nature of these new cell-bound antigens. Studies are in progress to test this hypothesis in vitro and in vivo using a number of chemical-modifying agents and in experimentally induced autoimmunity.

Summary

Splenic lymphocytes from four C57BL/10 congenic resistant mouse strains were sensitized in vitro with trinitrophenyl (TNP)-modified autologous spleen cells. The effector cells generated were incubated with ^{51}Cr-labeled unmodified or TNP-modified spleen or tumor target cells, and the percentage of specific lympholysis determined. The results obtained using syngeneic-, congenic-, recombinant-, and allogeneic-modified target cells indicated that TNP modification of the target cells was a necessary but insufficient requirement for lympholysis. Intra-*H-2* homology either between modified stimulating cells and modified target cells or between responding lymphocytes and modified target cells was also important in the specificity for lysis. Homology at the *K* serological region or at *K* plus *I-A* in the B10.A and B10.BR strains, and at either the *D* serological region or at some other region (possibly *K*) in the B10.D2 and C57BL/10 strains were shown to be necessary in order to detect lympholysis. Experiments using (B10.A × C57BL/10)F_1 responding lymphocytes sensitized and assayed with TNP-modified parental cells indicated that the homology required for lympholysis was between modified stimulating and modified target cells. The possibility is raised that histocompatibility antigens may serve in the autologous system as cell surface components which are modified by viruses or autoimmune complexes to form cell-bound modified-self antigens, which are particularly suited for cell-mediated immune reactions. Evidence is presented suggesting that *H-2*-linked *Ir* genes are expressed in the TNP-modified autologous cytotoxic system. These findings imply that the major histocompatibility complex can be functionally involved both in the response potential to and in the formation of new antigenic determinants involving modified-self components.

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