

H-2-LINKED GENETIC CONTROL OF MURINE
T-CELL-MEDIATED LYMPHOLYSIS TO AUTOLOGOUS
CELLS MODIFIED WITH LOW CONCENTRATIONS OF
TRINITROBENZENE SULFONATE

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The murine major histocompatibility complex (MHC or H-2)¹ has been shown to control the in vitro generation of cytotoxic T-effector cells to trinitrophenyl-modified syngeneic cells (TNP-self) at two distinct but interacting levels (1-3). First, the cytotoxic effectors generated against TNP-self were shown to exhibit preferential lysis of targets matched with the responding and stimulating cells at the K or D regions of the MHC (4, 5). Second, the cytotoxic responses of spleen cells from mice expressing the *k* haplotype at the K region and at part, or all, of the I region were found to be poor genetic responders to TNP-self at H-2D, irrespective of whether the haplotype at D was *d* (B10.A) (1, 2), *b* (B10.A[4R]), or *k* (B10.BR) (3, 6). Thus, for the T-cell-mediated lympholysis (CML) responses, K and D region products appear to affect the specificity of self recognition in association with TNP, whereas K and I region products influence, at least in this system, the response potential (presumed to operate via immune response [*Ir*] genes) for self-recognition of D-end-coded products. Other H-2 restricted viral-specific (7-12) and sex-linked antigen-specific (13) CML systems have been recently described in which H-2 products appear to be involved in *Ir*-gene control.

Earlier studies indicated that *Ir* genes which control antibody responses could be most clearly demonstrated for complex multideterminant immunogens such as proteins at low immunizing doses (14, 15). Under limiting antigen doses, immune responses appeared to be triggered by only the more immunogenic determinants, and the *Ir* genes, controlling responses to those determinants, were detected in the absence of an immune response to other determinants (not under the control of the same *Ir* genes). It is known that the CML response to TNP-self by both mouse (4, 16, 17) and human (18, 19) leukocytes is complex, and involves a number of clones of effector cells. Distinct clones have been identified with self specificity associated with the serologically detectable MHC region products (4, 5, 18), with I region products (20, 21), and with widely shared species-restricted self products (18, 19). In the present

¹ *Abbreviations used in this paper:* CML, cell-mediated lympholysis; *Ir*, immune response; MHC, major histocompatibility complex; MSA, mouse serum albumin; PHA, phytohemagglutinin; TNBS, trinitrobenzene sulfonate; TNP-, trinitrophenyl-.

study, experiments were performed using mouse spleen cells modified with lower concentrations of trinitrobenzene sulfonate (TNBS) than commonly used to determine whether strain-dependent differences in CML responses could be detected, and if so, whether they would be linked to the H-2 complex. This report compares the CML responses of spleen cells from various mouse strains to syngeneic cells modified with low concentrations of TNBS, and discusses the potential relevance of *Ir*-gene effects and self recognition at low immunogen doses.

Materials and Methods

Mice. All mice used were males, 7–12 wks old. The C3H/HeJ, C3H.SW, and all the C57BL/10 congenic mouse strains were purchased from The Jackson Laboratory, Bar Harbor, Maine. The BALB.K and BALB.B strains were kindly provided by Dr. D. H. Sachs, Immunology Branch, National Cancer Institute. The BALB/c and B10.A (5R) strains were obtained from the Animal Production Unit, National Cancer Institute.

Cell Cultures. Mouse spleen cells (7×10^6) were cultured with 3×10^6 irradiated (2,000 rads) TNBS-modified autologous spleen cells for 5 d as previously described (4, 22). Modification of stimulating, blocking, and target cells was performed by incubation of cells at 37°C for 10 min, as described (4, 22), with various concentrations of TNBS (0.05–10.0 mM) diluted in phosphate-buffered saline.

Preparation of TNP-conjugated bovine serum albumin and bovine gamma globulin, and the stimulation of spleen cell cultures with these TNP-conjugated proteins have been described in an earlier report (22).

The 4-h ^{51}Cr -release assays and the nonradiolabeled cold target blocking assays were performed as reported elsewhere (4, 22). The target cells used were mouse spleen cells, stimulated 48 h before assay with 1 $\mu\text{g}/\text{ml}$ phytohemagglutinin PHA, except where otherwise indicated. The blocking cells used were freshly harvested mouse spleen cells.

The percent lysis is calculated as previously described (22) and standard errors of the mean have been excluded from the table and the figures for simplicity, because they were usually <5%.

Immunofluorescence and Flow Microfluorometry. Fluoresceinated anti-TNP antibodies were prepared, B10.BR and C57BL/10 (B10) spleen cells were stained and fixed, and flow microfluorometry was performed as described in a previous report (23) using a FACS II flow microfluorometer (Becton, Dickinson Electronics Laboratory, Mountain View, Calif.).

Results

Strain-dependent Differences of Cytotoxic Responses to Autologous Cells Modified with Low Concentrations of TNBS. Spleen cells from B10.BR ($H-2^k$) and B10 ($H-2^b$) mice were compared for their ability to generate cytotoxic responses to TNP-self when stimulated in vitro with autologous spleen cells modified with different concentrations of TNBS (e.g., 10, 0.3, and 0.1 mM). The results of a representative experiment are summarized graphically in Fig. 1. Strong cytotoxic activity was observed by B10.BR effectors, irrespective of the TNBS concentration used for modification. At the effector:target cell ratios shown, no differences were detected in lytic activity of B10.BR effectors generated with 10, 0.3, or 0.1 mM TNBS modification. This is partly a result of the fact that the effector cell titration curve is still on the plateau for B10.BR in this range (see other figures). In contrast to the B10.BR results, lytic activity of B10 spleen cells was detected in cultures stimulated with TNP-self at 10 mM TNBS, but not at 0.3 or 0.1 mM TNBS. Furthermore, although comparable lysis was observed at an effector:target cell ratio of 40:1 for B10.BR and B10 effectors stimulated with 10 mM TNBS, B10 effector cell activity titrated out more rapidly than that of the B10.BR effectors. At least fourfold more B10 than B10.BR effectors were required to obtain 50% lysis.

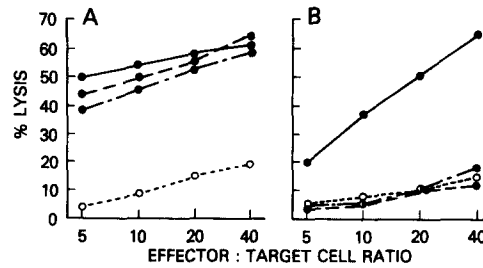


FIG. 1. Cell-mediated cytotoxicity to TNP-self at four effector:target cell ratios using B10.BR (A) or B10 (B) effector cells generated by sensitization with autologous spleen cells modified with 10 mM (●—●), 0.3 mM (●---●), or 0.1 mM (●----●) TNBS, or with unmodified (○--○) autologous cells. Effectors were assayed on syngeneic PHA-stimulated spleen blasts modified with 10 mM TNBS.

These results indicate that H-2-linked strain-dependent differences exist between the B10.BR and B10 effectors to TNP-self.

To make a more thorough comparison of the cytotoxic responses of B10.BR and B10 spleen cells to TNP-self modified with different TNBS concentrations, B10.BR- and B10-stimulating spleen cells were modified with twofold dilutions of TNBS ranging from 2.0 to 0.031 mM, and added to cultures of B10.BR- and B10-responding cells, respectively. The lytic activity of the effectors generated was assayed on B10.BR-TNBS and B10-TNBS targets modified with 10 mM TNBS, and is summarized in Fig. 2. B10.BR effector cell activity was strong and about equal by stimulation in the range between 2.0 and 0.25 mM TNBS. A slight decrease was noted at 0.12 and 0.062 mM, and still lower activity was detected at 0.031 mM, although stimulators treated with all concentrations of TNBS were immunogenic. In contrast, B10 effector cell activity was much more dependent on the TNBS concentration used for stimulating cell modification. Modification with 1.0 mM TNBS generated effector activity approximately equivalent to that observed with B10.BR spleen cells stimulated with 0.031 mM TNBS. For comparison, the effectors generated were also assayed on the respective TNBS-modified H-2 nonmatched targets. The results summarized in Fig. 2C and 2D illustrate that the B10.BR effectors lysed B10-TNBS targets better than B10 effectors lysed B10.BR-TNBS targets, and that the stimulatory differences detected in these two strains on their respective modified H-2 matched targets are also reflected on modified H-2 unmatched target cells.

Strain Differences Are Independent of the Degree of TNP Conjugation of Individual Stimulating Cells. The possibility exists that the differences observed in the CTL responses of B10.BR and B10 spleen cells could have resulted from the conjugation of different amounts of TNP to the cell-surface proteins of B10.BR and B10 spleen cells. To test this possibility, B10.BR and B10 spleen cells were modified with different concentrations of TNBS, and then incubated with affinity-purified sheep anti-TNP IgG antibodies conjugated with fluorescein isothiocyanate (23). The modified and stained cells were compared for their relative anti-TNP fluorescence intensity by flow microfluorometry using the FACS II flow microfluorometer (23). As summarized in Table I, the mean fluorescence data obtained from the FACS II profiles indicate that quantitatively equivalent amounts of anti-TNP antibody were bound to the modified cells of the two strains for any given concentration of TNBS, which is consistent with equal numbers of TNP groups conjugated to the cell surfaces of B10.BR and B10

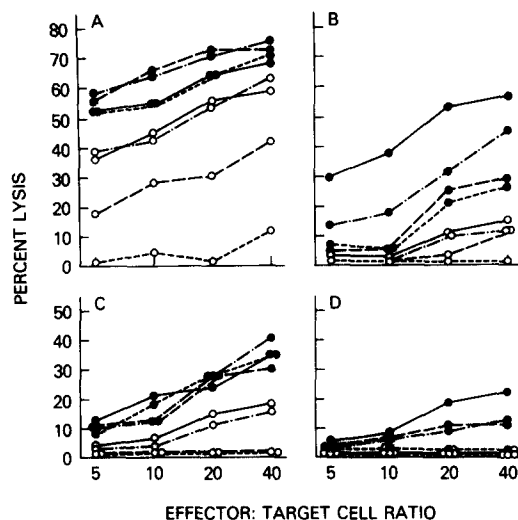


FIG. 2. Cell-mediated cytotoxicity to TNP-self at four effector:target cell ratios using B10.BR (A, C) or B10 (B, D) effector cells generated by sensitization with autologous spleen cells modified with 2 mM (●—●), 1 mM (●---●), 0.5 mM (○—○), 0.25 mM (●----●), 0.125 mM (○—○), or 0.062 mM (○---○) or 0.031 (○—○) TNBS, or with unmodified (○----○) autologous cells. Effectors were assayed on B10.BR (A, D) or B10 (B, C) PHA-stimulated spleen blasts modified with 10 mM TNBS.

spleen cells. Therefore, the differences observed between the cytotoxic responses of B10.BR and B10 spleen cells to TNP-self using low concentrations of TNBS do not appear to be a result of different amounts of TNP on the stimulating cells of these two strains, and can be attributed to some immunogenetic parameter(s).

Strain Differences to Low Doses of TNP by Stimulation with TNP-Conjugated Proteins or Low Dose TNBS-modified Glass-Adherent Cells. It has been previously shown that TNP-specific, H-2-restricted effector cells can be generated by stimulating cultures of mouse spleen cells with TNP-conjugated proteins (22). Such stimulation might introduce limiting amounts of TNP in an immunogenic form on the cell surface, which could result in activation of B10.BR but not B10 effector cell clones. Cultures of B10.BR and B10 spleen cells were stimulated with 100 $\mu\text{g}/\text{ml}$ of: (a) TNP-conjugated bovine gamma globulin (TNP-BGG); (b) BGG; (c) TNP-conjugated bovine serum albumin (TNP-BSA); or (d) BGG. B10.BR and B10 effector cell activity was assessed 5 d later on 10 mM TNBS-modified $H-2^k$ (RDM-4) and $H-2^b$ (EL-4) tumor targets, respectively. The results, illustrated in Fig. 3, indicate that B10.BR spleen cells generated cytotoxic activity when stimulated with 10 mM TNBS-modified syngeneic cells or with either TNP-BGG or TNP-BSA. In contrast, B10 spleen cells did not generate cytotoxic activity above control values when stimulated with TNP-BGG or TNP-BSA, although these spleen cells did generate a strong response when stimulated with syngeneic cells modified with 10 mM TNBS. The absence of reactivity in the B10 strain to 100 $\mu\text{g}/\text{ml}$ of TNP-BGG or TNP-BSA parallels the observation that the amount of TNP bound to the cell surface corresponds to a TNP modification of from 0.1 to 1.0 mM TNBS (22).

Glass-adherent spleen cells modified with 10 mM TNBS have been shown to be $\cong 10$ times more efficient on a per cell basis in stimulating the generation of TNP-

TABLE I
*Comparison of Quantitative FITC Anti-TNP Binding to TNBS-Modified
 B10.BR and C57BL/10 Spleen Cells as Measured by Flow
 Microfluorometry**

TNBS concentration used to modify spleen cells	Mean fluorescence‡ of TNBS-modified spleen cells from	
	B10.BR	C57BL/10
<i>mM</i>		
Unmodified	21	22
1.0	3,756	4,024
0.3	1,602	1,628
0.1	1,200	1,148
0.03	245	227

* Determined by FACS II.

‡ Fluorescence values shown have been corrected for gain of 8, and are expressed in arbitrary units of fluorescence.

specific cytotoxic effector cells than modified unfractionated spleen cells (24). To determine whether B10 glass-adherent cells modified with low concentrations of TNBS could stimulate B10 responding cells more efficiently, unfractionated spleen cells from B10.BR and B10 donors were sensitized in vitro with unfractionated or glass-adherent syngeneic spleen cells modified with 10, 1.0, 0.1, or 0.01 mM TNBS. The effector cells generated 5 d later were assayed on *H-2^k* (RDM-4) or *H-2^b* (EL-4) tumor target cells modified with 10 mM TNBS. The results are summarized in Fig. 4. B10.BR spleen cells responded to syngeneic unfractionated spleen cells modified with any of these four TNBS concentrations, and to glass-adherent cells modified with all but the lowest concentration of TNBS. In contrast, B10 spleen cells responded to unfractionated syngeneic cells modified with only 10 mM TNBS and to glass-adherent cells modified with 10 or 1.0 mM, but not those modified with 0.1 or 0.01 mM TNBS.

Strain Differences Can Be Demonstrated by Reducing the Stimulator Cell Number Modified at 10 mM TNBS. The results presented thus far have not established whether the differences between the cytotoxic responses of B10.BR and B10 spleen cells would be reflected in the concentration of TNP groups on individual-modified stimulating cells or in the total amount of TNP added to the cultures. Were the response differences dependent on the TNBS concentration on individual stimulating cells, i.e., on the extent of cell surface modification, cultures of B10.BR and B10 spleen cells should not exhibit the response differences that were observed in Figs. 1 and 2 when the cultures were stimulated with different numbers of syngeneic cells modified with 10 mM TNBS. The results of Fig. 5 illustrate that B10.BR spleen cells respond better for any given number of syngeneic cells modified with 10 mM TNBS than do B10 spleen cells. However, the results also indicate that titration of the total TNP concentration in the cultures by diluting out the cell number (at a fixed concentration of TNP per cell) provides a more critical reduction in immunogenic dose for B10.BR-responding cells than by reducing the TNBS (at a constant stimulator cell number) (compare Figs. 5 and 2). This could indicate that presentation of TNP groups on a relatively large number of stimulating cells is a more efficient means of stimulation than

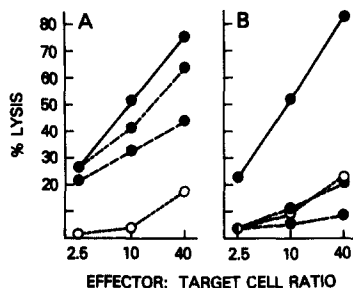


FIG. 3. Cell-mediated cytotoxicity to TNP-self at three effector:target cell ratios using B10.BR (A) or B10 (B) effector cells generated by sensitization with: autologous spleen cells modified with 10 mM TNBS (●—●); 100 μg TNP-BGG (●- -●); 100 μg TNP-BSA (●- ·-●); or unmodified autologous cells (○- - -○). Effectors were assayed on syngeneic PHA-stimulated spleen blasts modified with 10 mM TNBS.

presentation of the same number of TNP groups on a lower number of stimulator cells.

Demonstration of Strain Differences in Target Cells Modified with Low Concentrations of TNBS. The results presented above indicate that differences between the CTL responses of B10.BR and B10 cells to low doses of TNP-self can be demonstrated during sensitization, i.e., at the responder and/or stimulator cell level. To determine whether such TNP dose-dependent differences can also be detected between these two strains at the lytic phase, B10.BR and B10 spleen cells were sensitized to TNP-self modified with 10, 1.0, or 0.1 mM TNBS. The effectors generated were assayed 5 d later on syngeneic PHA-stimulated blast targets modified with 10, 1, or 0.1 mM TNBS at an effector:target cell ratio of 40:1 to detect maximum lysis. The results, shown in Table II, indicate that B10.BR effector-cell activity was generated to TNP-self at 10, 1, or 0.1 mM TNBS, and that the lytic activity could be detected on TNP-self targets modified with 10, 1, 0.1 mM TNBS. In contrast, B10-effector cell activity was generated to TNP-self only at 10 mM. This lytic activity was detected on TNP-self targets modified with 10 and 1 mM TNBS, but not on TNP-self modified with 0.1 mM TNBS. These results suggest that modification of B10 spleen cells with 0.1 mM TNBS fails to form the necessary target antigen(s) or antigenic complex required for lysis by B10 effectors.

The above finding is confirmed and extended by the results of a similar experiment summarized in Fig. 6, which is assayed on TNP-self and TNP-allogeneic (for cross-reactive lysis) targets at several effector:target cell ratios. B10.BR effectors generated by sensitization to TNP-self at 10, 1, or 0.1 mM TNBS lysed TNP-self targets modified with 10, 1, or 0.1 mM TNBS. B10 effectors generated by sensitization to TNP-self at 10 or 1 mM TNBS lysed TNP-self targets modified with 10 or 1 mM TNBS, but not those modified with 0.1 mM TNBS. Furthermore, no B10 effector activity could be detected by sensitization with 0.1 mM TNBS and assaying on TNP-self targets modified with 10 or 1 mM TNBS. With respect to cross-reactive lysis, little or no lysis was detected on modified B10.BR targets by B10 effectors except when both the B10 stimulators and the B10.BR targets were modified with 10 mM TNBS. B10 targets modified with 10 or 1 mM, but not 0.1 mM TNBS, were weakly lysed by BR effectors generated by sensitization of TNP-self treated with 10 or 1 mM but not with 0.1 mM TNBS. These results indicate that B10 spleen cells do not generate effectors when

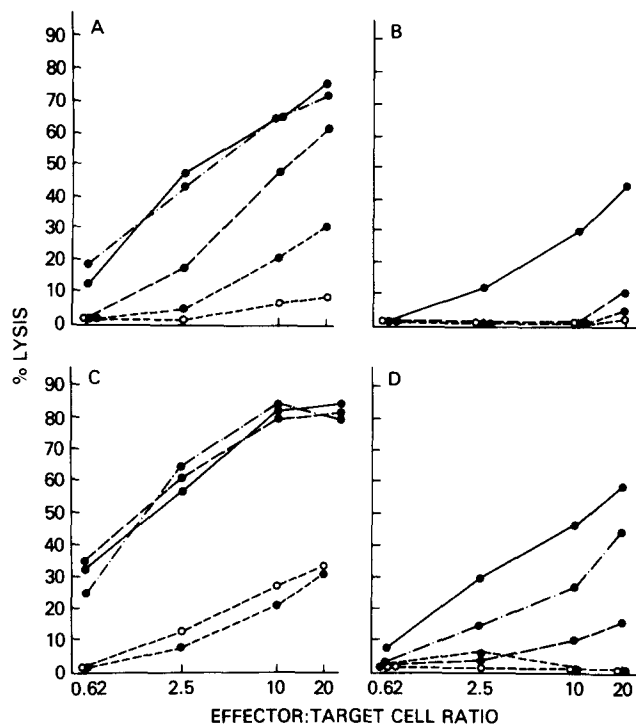


FIG. 4. Cell-mediated cytotoxicity to TNP-self at four effector:target cell ratios using B10.BR (A, C) or B10 (B, D) effector cells generated by sensitization with autologous unfractionated spleen cells (3×10^6 /well) (A, B), or splenic glass-adherent cells (0.3×10^6 /well) (C, D), modified with 10 mM (●—●), 1 mM (●---●), 0.1 mM (●—○), or 0.01 mM (●---○) TNBS, or with unmodified autologous cells (○—○). Effectors were assayed on RDM-4 ($H-2^k$) or EL-4 ($H-2^b$) lymphoid tumor target cells modified with 10 mM TNBS.

stimulated with low doses of TNP-self which lyse B10.BR targets modified with low concentrations of TNBS, and that B10.BR effectors generated with low doses of TNP-self do not lyse B10 targets modified with low concentrations of TNBS, despite the fact that the low-dose B10.BR effectors and targets are adequate for detecting cytotoxic activity on modified self targets.

The Strain Differences in Cytotoxic Response to Syngeneic Cells Modified with Low Concentrations of TNBS Are H-2 Linked. The observation that the congenic resistant B10.BR and B10 strains which share the B10 background and differ only at H-2 were the respective responder and nonresponder to low concentrations of TNP was consistent with the differences being controlled by genes which are linked to the H-2 complex. To verify this, the cytotoxic responses to TNP-self by other congenic $H-2^k$ and $H-2^b$ strains on the C3H and BALB genetic backgrounds were compared. Irrespective of the non-H-2 background, spleen cells from $H-2^k$ mice responded well to syngeneic cells modified with 10 or 0.1 mM TNBS (Fig. 7). In contrast, cells from $H-2^b$ mice responded moderately to syngeneic cells modified with 10 mM, but not to those modified with 0.1 mM TNBS.

To provide more information on the cytotoxic responses of other B10 congenic strains to low concentrations of TNP-self, spleen cells from B10.A and B10.D2 mice were compared with those from B10.BR to syngeneic cells modified with 10, 1.0, or

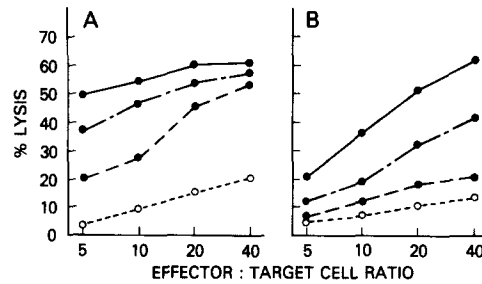


FIG. 5. Cell-mediated cytotoxicity to TNP-self at four effector:target cell ratios using B10.BR (A) or B10 (B) effector cells generated by sensitization with 3×10^6 (●—●), 3×10^5 (●---●), or 1×10^5 (●- -●) autologous spleen cells all modified with 10 mM TNBS, or with 3×10^6 unmodified autologous spleen cells (○- -○). Effectors were assayed on syngeneic PHA-stimulated spleen blasts modified with 10 mM TNBS.

0.1 mM TNBS (Fig. 8). B10.A spleen cells generated strong cytotoxic activity when stimulated with syngeneic cells modified with any of the three concentrations of TNBS (similar to B10.BR). B10.D2 spleen cells generated moderate to strong responses to syngeneic cells modified with 10 or 1.0 mM TNBS, but only marginal responses to cells modified with 0.1 mM TNBS (similar to B10). Thus, $H-2^k$ and $H-2^a$ but not $H-2^b$ and $H-2^d$ strains appear to generate strong cytotoxic activity to low concentrations of TNP-self.

The H-2 Associated Differences in Cytotoxic Response to Syngeneic Cells Modified with Low Concentrations of TNBS Are Expressed by (B10 × B10.BR)F₁ Responding and Stimulating Cells. To determine whether the TNP dose-related differences between B10.BR and B10 spleen cells could be detected at the stimulator cell level without necessarily involving responding lymphocytes, (B10 × B10.BR)F₁ responding cells were sensitized with B10, B10.BR, or (B10 × B10.BR)F₁ stimulating cells modified with 10, 1.0, or 0.1 mM TNBS. The effector cells generated were assayed 5 d later on 10 mM TNBS-modified, parental or F₁, PHA-stimulated target cells. The results, summarized in Fig. 9, indicate that F₁-responding cells do not respond better than B10 cells to low doses of $H-2^b$ TNP-self, irrespective of whether the modified stimulators or targets were of B10 or F₁ origin (compare Fig. 9 B, C, and E with Fig. 9 A and B). The results of Fig. 9 F and G indicate that the F₁ behaves as a responder when stimulated with low doses of TNBS-modified $H-2^k$ parental cells and assayed on TNBS-modified F₁ or $H-2^k$ parental targets. The results of Fig. 9 H and I indicate the levels of TNP cross-reactive lysis detected by B10.BR and B10 effectors generated by sensitization to 0.1 mM TNP-self.

Discussion

The present study demonstrates the existence of H-2-linked genetic differences in the ability of mouse spleen cells to generate cytotoxic effector cell activity specific for TNP-self targets, by limiting the amount of TNBS used to modify the stimulating or target cells or by using limiting numbers of TNP-self stimulator cells. $H-2^a$ and $H-2^k$ strains are high responders to TNP-self, whereas $H-2^b$ and $H-2^d$ strains are low responders; (H-2^b × H-2^k)F₁ mice are high responders to $H-2^k$ -TNP but lower responders to $H-2^b$ -TNP. The observed genetic differences in responses to TNP-self could result from a relative defect in the low responder strains during the sensitization

TABLE II
Comparison of Cytotoxic Activity Generated by B10.BR and C57BL/10 Spleen Cells Stimulated with TNP-Self Modified with 10, 1, or 0.1 mM TNBS, and Assayed on TNP-Self Targets Modified with 10, 1, or 0.1 mM TNBS

Responding-, stimulating-, and target-cell strain	Concentration of TNBS used to modify stimulating cells	Lysis on synergic target cell modified with TNBS at:		
		10 mM	1 mM	0.1 mM
		%	%	%
B10.BR	10 mM	77.5	87.5	55.9
	1 mM	76.3	91.9	61.2
	0.1 mM	59.8	100.0	48.0
	---	10.1	0.7	-2.8
C57BL/10	10 mM	48.4	26.8	-6.0
	1 mM	5.5	0.1	-9.3
	0.1 mM	-10.7	-15.6	-9.3
	---	-5.6	-15.3	-9.3

Effector cell activity was assayed at effector:target cell ratio of 40:1 on 48-h PHA-stimulated syngeneic spleen blasts.

phase (involving the responder, stimulator, helper, or presenting cells) or during the lytic phase of the response (involving the effector or target cells). Defects expressed at the stimulator and/or target cell levels could conceivably be a result of the failure of cells from low responder mice modified with limiting doses of TNBS to form immunogenic self-TNP complexes. Such would be the case if low doses of TNP specifically failed to modify the relevant low responder haplotype H-2 structures required for self recognition either because low responder cells were generally less susceptible to modification by TNP or because low responder cells expressed fewer self-recognition determinants than high responder cells. Alternatively, the defect might result from an inability of low responder cells to respond to competently presented TNP-modified self antigens. This might result from decreased affinity or decreased frequency of self-TNP specific cytotoxic precursor cells in low responder strains, or could result from a generally lower cytotoxic activity of low responder effector clones.

The microfluorometry data shown in Table I indicate that the observed genetic differences are not a result of detectable differences in the total amount of TNP conjugated to the surfaces of B10 and B10.BR spleen cells at any given TNBS concentration. Furthermore, the fact that these strain-dependent differences could be observed by varying the total amount of TNP in the culture, either by sensitizing the responding cells by using a constant number of stimulator cells modified with varying concentrations of TNBS or by using a constant relatively high concentration of TNBS (10 mM) for modification and varying the stimulator cell number, indicates that the density of TNP group per cells in the range studied was not the only important factor. A 10-fold decrease in the TNBS concentration used for modifying the stimulating cells resulted in a more drastic reduction in the level of CTL generated than a 10-fold dilution in the number of stimulating cells (modified with 10 mM TNBS). However, this result does not exclude the possibility that fewer relevant determinants are modified on *H-2^b* than on *H-2^k* cells. Nevertheless, the finding that the strain-

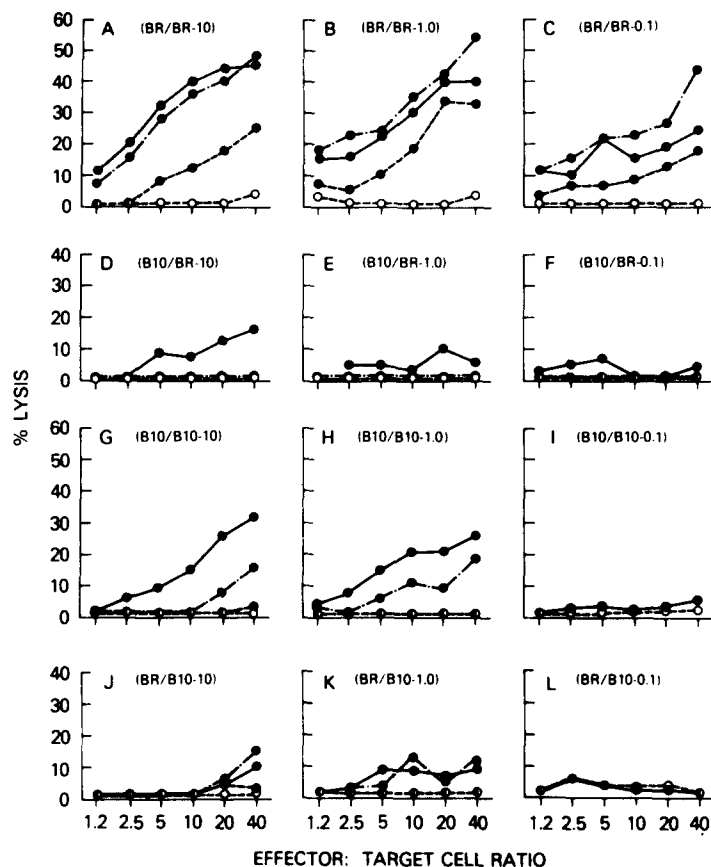


FIG. 6. Cell-mediated cytotoxicity to TNP-self at six effector:target cell ratios using B10.BR (A, B, C, J, K, L) or B10 (D, E, F, G, H, I) effector cells generated by sensitization with autologous spleen cells modified with 10 mM (●—●), 1 mM (●- -●), or 0.1 mM (○- -○) TNBS, or with unmodified (○- -○) autologous cells. Effectors were assayed on B10.BR (A, B, C, D, E, F) or B10 (G, H, I, J, K, L) PHA-stimulated spleen blasts modified with 10 mM (A, D, G, J), 1 mM (B, E, H, K), or 0.1 mM (C, F, I, L) TNBS. (/) indicates the effectors/targets-molarity of TNBS used for target modification.

dependent differences can be detected by using a lower number of stimulating cells modified with a fixed concentration of TNBS is consistent with the density of TNP molecules on the cell surface being less limiting than some other determinant in the low responder haplotype. It is not known whether there are quantitative differences in the amount of H-2 molecules relevant for recognition by CTL per cell on *H-2^b* and *H-2^k* spleen cells. If no overall difference exists, it is still possible that self recognition structures relevant for TNP recognition constitute a minor component of the total K and D antigens. These components could differ significantly in *H-2^b* and *H-2^k* mice. It should be noted that Forman and coworkers (25-28) have made an extensive study of the amount of TNP conjugated to cell-surface proteins as a function of TNBS concentrations. Their investigations indicated that a direct correlation could be demonstrated between the ability of modified syngeneic murine cells to serve as stimulators or targets in the H-2-restricted TNP-specific CML and the degree of conjugation of TNP groups to H-2 antigens (26).

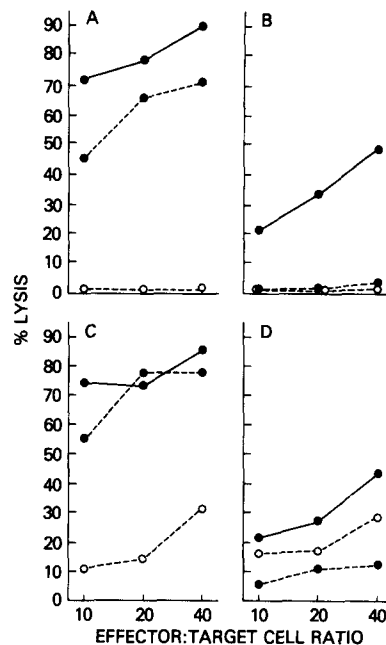


FIG. 7. Cell-mediated cytotoxicity to TNP-self at three effector:target cell ratios using C3H/HeN (A), C3H.SW (B), BALB.K (C), or BALB.B (D) effector cells generated by sensitization with autologous spleen cells modified with 10 mM (●—●) or 0.1 mM (●- -●) TNBS, or with unmodified (○- -○) autologous cells. Effectors were assayed on C3H/HeN (A, C) or DBA/2 (B, D).

Our results indicate that a defect in $H-2^b$ spleen cells can be detected by lowering the TNBS concentration used to modify the target cells. $H-2^b$ but not $H-2^k$ effector cells activated by 10 mM TNP-self lysed 0.1 mM TNBS-modified syngeneic target cells, which demonstrates a target cell defect, or an inability of $H-2^b$ lymphocytes to react with syngeneic stimulator or target cells modified with 0.1 mM TNBS. The results obtained with cross-reactive lysis on TNBS-modified H-2-nonmatched targets are also compatible with a defect at the target cell level which could be independent of effector cell generation, because B10.BR effectors lysed B10.BR but not B10 targets modified with low concentrations of TNBS. However, the TNP cross-reactive data also suggest that a defect probably exists at the responding and/or stimulating cell level, because B10 effectors generated by stimulation with low doses of TNP-self did not lyse B10.BR targets modified with 10 mM TNBS, although the same targets were lysed by BR effectors generated with low doses of TNP-self. If a defect exists in $H-2^b$ -stimulating cells, it is not reversed by using the most potent stimulating cell population known (24), because the use of TNBS-modified, splenic-adherent stimulating cells resulted in the same genetic differences observed when unfractionated spleen cells were used as stimulators.

$I-r$ -like genetic effects have been demonstrated for H-2-restricted (1-3) and cross-reactive (21, 29) cytotoxic responses to TNP-self, as well as for restricted cytotoxic responses to virus-infected cells (7-12) and cells expressing the HY antigen (13). Responses of (B10 × B10.BR) F_1 spleen cells stimulated by B10.BR-, B10-, or F_1 -stimulating cells modified with low concentrations of TNBS were investigated by

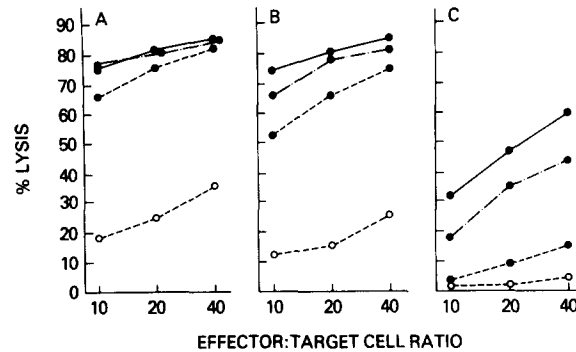


FIG. 8. Cell-mediated cytotoxicity to TNP-self at three effector:target cell ratios using B10.BR (A), B10.A (B), or B10.D2 (C) effector cells generated by sensitization with autologous spleen cells modified with 10 mM (●—●), 1 mM (●- -●), or 0.1 mM (● · · · ●) TNBS, or with unmodified (○- -○) autologous cells. Effectors were assayed on syngeneic PHA-stimulated spleen blasts modified with 10 mM TNBS.

comparing the cytotoxic activity of $H-2^k$, $H-2^b$ and $(H-2^b \times H-2^k)F_1$ spleen cells and that of F_1 -responding cells sensitized with modified parental or F_1 -stimulating cells to determine whether introduction of: (a) the $H-2^k$ haplotype would increase response potential to low doses of TNP- $H-2^b$, or (b) the $H-2^b$ haplotype would decrease response potential to low doses of TNP- $H-2^k$. The results indicate that F_1 spleen cells were not better responders to $H-2^b$ -stimulating cells modified with low doses of TNBS than were the unresponsive $H-2^b$ -parental-responding cells, although the same pool of F_1 cells generated strong cytotoxic activity when stimulated with low doses of TNBS-modified $H-2^k$ cells. Consequently, if a responding cell defect exists, these results demonstrate that neither introduction of the $H-2^k$ haplotype nor differentiation in the F_1 thymus results in correction of nonresponsiveness to low doses of $H-2^b$ -TNP-self. This is similar to the *Ir* gene-controlled antibody response to (T, G)-A--L, in which F_1 T cells can initiate a response through interaction with responder accessory cells, but not by interaction with nonresponder accessory cells (30). Because the present study does not clearly demonstrate a responding cell defect, we cannot address the question of whether such a defect would be expressed as differences in: (a) responding cell frequency; (b) affinity of responding cells; or (c) cytotoxic activity of effectors from $H-2^b$ and $H-2^k$ mice. However, the observation that F_1 -responding cells generated strong cytotoxic activity to low doses of $H-2^k$ -TNP-self but not to $H-2^b$ -TNP-self indicates that the differences in the reactivity between B10.BR and B10 cells was not a result of nonspecific, generally lower response potential to low doses of TNP-self, because low responsiveness was haplotype-specific in the F_1 .

It is noteworthy that the cross-reactive lysis detected by effectors generated by TNP-self and assayed on TNP-modified allogeneic targets was lower in magnitude but similar in pattern to that observed for different doses of TNP-self assayed on modified self targets. The finding that cross-reactive lysis was stronger for B10.BR than for B10 effectors contrasts with those of Billings et al. (21, 29) who found that spleen cells from B10.BR and B10 mice were the respective low and high responders in cross-reactive lysis to TNP. The reasons for the differences between our results and those of Billings et al. are not known.

Earlier studies have shown that the *in vitro* generation of cytotoxic effector cells to

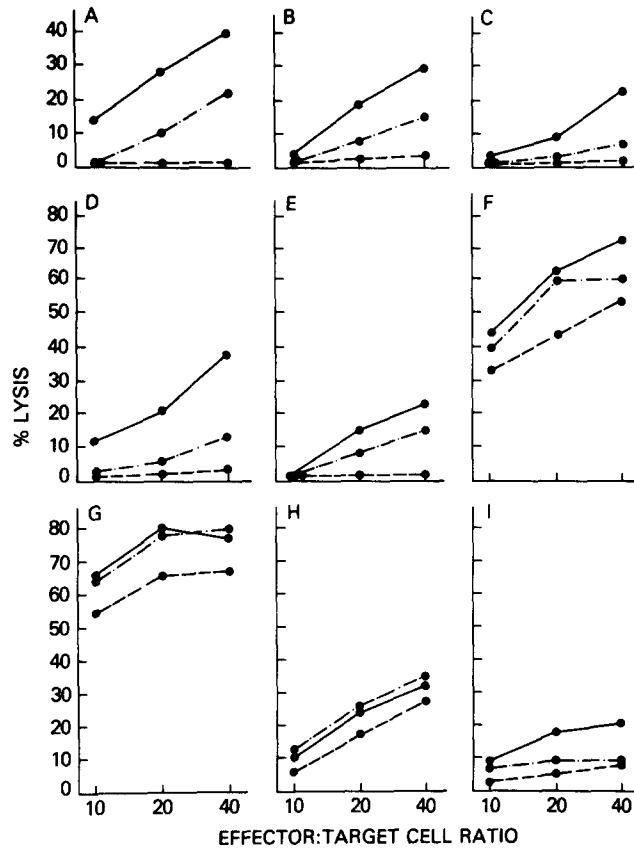


FIG. 9. Cell-mediated cytotoxicity to TNP-self at three effector:target cell ratios using B10.BR, B10, or (B10 \times B10.BR) F_1 effector cells generated by sensitization of F_1 or parental spleen cells modified with 10 mM (●—●), 1 mM (●- -●), or 0.1 mM (● · · ●) TNBS. The lytic values from cultures sensitized with unmodified cells were $<2\%$ on all targets assayed in this experiment, and have been omitted from the graphs. Effectors were assayed on F_1 or parental PHA-stimulated spleen blasts modified with 10 mM TNBS. (Responders/Stimulators/Targets) for each graph are indicated as follows: A, (B10/B10-TNP/B10-TNP); B, (F_1 /B10-TNP/B10-TNP); C, (F_1 / F_1 -TNP/B10-TNP); D, (B10/B10-TNP/ F_1 -TNP); E, F_1 /B10-TNP/ F_1 -TNP); F, (F_1 /B10.BR-TNP/ F_1 -TNP); G, (B10.BR/B10.BR-TNP/B10.BR-TNP); H, (B10.BR/B10.BR-TNP/B10-TNP); I, (B10/B10-TNP/B10.BR-TNP).

TNP-self is associated with a loss in ability of the cultures to generate TNP-specific plaque-forming cells (PFC) (31). This could be a result of suppression of the antibody response either by cytotoxic effectors themselves or by a separate population of suppressor cells generated concomitantly with the effectors. Because spleen cells from B10.BR or B10.A, but not from B10 mice, generate cytotoxic responses to limiting doses of TNP-self (including cultures stimulated with TNP-conjugated proteins), it might be predicted that $H-2^b$ mice would be higher genetic responders than $H-2^k$ or $H-2^{b/k}$ mice for TNP-specific antibody responses to TNP presented in association with antigens. Consistent with this prediction is the observation that B10.D2 and B10 mice were reported to be respective high and intermediate antibody responders to TNP-conjugated mouse serum albumin (MSA), whereas B10.A and (B10.A \times B10) F_1 mice were low responders (32). This raises the possibility that the H-2-linked lack of

antibody response to TNP-MSA in F₁ mice is associated with the ability of the *H-2^a* haplotype to respond well in CML to low doses of TNP-self. From the results of Fig. 9 and (30) we would predict that F₁ T-helper and B-precursor cells would be high or intermediate antibody responders to TNP-MSA when accessory cell function is provided by the *H-2^d* or *H-2^b* parental haplotype.

The TNP-dose-dependent differences observed in the present study could be TNP-specific, i.e., they could be specific for the modifying hapten or infectious agent, or these results could imply the existence of a general H-2-linked response pattern to limiting antigen doses which would be H-2 self specific and not determined by the particular antigen recognized in association with self. It should be noted in this context that H-2-restricted cytotoxic responses to limiting doses of virus appear in preliminary experiments to exhibit a similar strain-dependent pattern (W. E. Biddison and G. M. Shearer, unpublished observations). This also agrees in general with the H-2-restricted CTL (33) and skin graft (34) results to the HY antigen in which responses involving *H-2^k* appear to be stronger than those involving *H-2^b* or *H-2^d*. Our observation that the genetic differences between B10 and B10.BR mice could be detected by lowering the stimulating cell number (modified with an excess of 10 mM TNBS) suggests that a component other than TNP is limiting. That component could be *H-2^b* and *H-2^d* self-recognition determinants. It is possible that the self-recognition determinant coded for by *H-2K^k* genes provides an unusually efficient structure for CTL, and thereby represents the exception rather than the examples shown for *H-2^d* and *H-2^b*. Preferential recognition of *K^k* self determinants over *D^b* and *D^d* have been reported in viral (9-11) and TNP (1-3) CML studies, and the responses demonstrated to low doses of TNP-self in *H-2^k* and *H-2^a* mice may reflect immunodominance of *H-2K^k* (9).

The present study demonstrates that TNP-specific H-2-restricted cytotoxic responses are controlled by H-2-linked genes, the effects of which can be clearly detected only when the responding cells are exposed to relatively low concentrations of TNP. Although TNP is not an infectious or pathogenic agent, the ease with which different amounts of this hapten can be conjugated to cell surfaces makes the system potentially useful as a model for investigating H-2-restricted viral systems. The possibility exists that similar MHC-linked genetic effects which are not detected at relatively high doses of immunogen may be demonstrable at lower immunogen doses for other MHC-restricted CTL systems such as viruses. Antigen dose-dependent genetic regulation by the MHC could be critical for initiating T-cell immune reactivity after chronic infections.

Summary

Spleen cells from B10.BR and C57BL/10 (B10) mice were compared for their ability to generate primary in vitro cytotoxic responses to syngeneic cells modified with different concentrations (from 10 to 0.031 mM) of trinitrobenzene sulfonate (TNBS) (TNP-self). Although both strains generated effector cells to TNP-self in the range of 10-0.25 mM TNBS modification, effector activity of B10 cells was weaker than that of B10.BR cells. B10 spleen cells did not respond to syngeneic stimulating cells modified at 0.1 mM or lower, whereas B10.BR cells generated effector activity even when stimulated by TNP-self modified with as low as 0.031 mM TNBS. Fluorescence analysis of the modified cells using the FACS II indicated that equivalent

quantities of TNP were conjugated to the surfaces of B10.BR and B10 spleen cells for any given concentration of TNBS modification. Similar strain-dependent differences were observed when the TNP was diluted out in the cultures by reducing the number of stimulating cells modified with 10 mM TNBS. These response patterns were verified by stimulating cultures of B10.BR and B10 spleen cells either with TNP conjugated to bovine serum albumin or bovine gamma globulin (B10.BR but not B10 cells responded to TNP-conjugated proteins) or with TNBS-modified glass-adherent spleen cells. The strain-dependent differences could also be detected at the effector phase, because optimally stimulated B10.BR, but not B10 effector cells, could lyse 0.1 mM TNBS-modified syngeneic target cells. The genetic parameters associated with the response and nonresponse patterns of B10.BR and B10 mice were further investigated by comparing the cytotoxic responses to low doses of TNP-self of spleen cells from the following strains: (a) C3H/HeJ ($H-2^k$) and C3H.SW ($H-2^b$); (b) BALB.K ($H-2^k$) and BALB.B ($H-2^b$); and (c) B10.A ($H-2^a$) and B10.D2 ($H-2^d$). The $H-2^k$ and $H-2^a$, but not the $H-2^b$ and $H-2^d$, strains generated cytotoxic responses to TNP-self when the syngeneic stimulators were modified with 0.1 mM TNBS. Further studies using (B10 \times B10.BR) F_1 responding cells and parental or F_1 -modified stimulating cells, indicated that the F_1 cells generated cytotoxic activity to low doses of TNP in association with $H-2^k$ but not in association with $H-2^b$ self products. The results of this study indicate that H-2-linked genetic factors, expressed in the target as well as in the responding and/or stimulating cell populations, control the ability of inbred mouse strains to generate cytotoxic effector cells to low doses of TNP-self. Such dose-dependent genetic effects may be important in the regulation of immune responses activated in vivo by chronic exposure to infectious agents.

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