

H-2-RESTRICTED CYTOTOXIC EFFECTORS GENERATED IN VITRO BY THE ADDITION OF TRINITROPHENYL-CONJUGATED SOLUBLE PROTEINS

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Major histocompatibility complex (MHC)¹-restricted T-cell-mediated cytotoxicity has been demonstrated for virus-infected (1-5) and chemically modified (6-10) murine cells, as well as for cells expressing weak transplantation antigens (11, 12). In each of these examples, the restriction was shown to map to the *K* and/or *D* regions of the *H-2* complex. To investigate the immunogenic parameters involved in the sensitization of cytotoxic effector cells, it was of interest to study whether or not cytotoxic responses could be generated against soluble antigens or antigenic components which are not covalently linked to cell surface products, and if they could, whether or not products of the MHC would play a role in such sensitization. Related to this issue is the observation that inactivated Sendai virus, which cannot infect cells, was associated with the cell surface resulting in the generation of *H-2*-restricted, Sendai-specific cytotoxic effector cells (13). Recently, it was shown that cells whose surfaces are modified with an amphipathic 2,4,6-trinitrophenyl (TNP) compound, TNP-stearyl-dextran (TSD), which is not covalently associated with cell surface components, are neither effective stimulators for the generation of cytotoxic effector cells, nor efficient targets for lysis by *H-2*-restricted, trinitrobenzene sulfonate (TNBS)-specific cytotoxic lymphocytes (14). The present report demonstrates that the addition of TNBS-conjugated soluble proteins such as bovine gamma globulin (TNP-BGG) or bovine serum albumin (TNP-BSA) to cultures of mouse spleen cells leads to the generation of cytotoxic effector cells which are both *H-2*-restricted and TNP-specific. A number of variables associated with this response were investigated.

Materials and Methods

Mice. The C57BL/10 congenic resistant lines, as well as the C3H.OH strains of mice used were purchased from The Jackson Laboratory, Bar Harbor, Maine. The A.TL, A.TH, A.AL, and B10.A(4R) strains were provided by Dr. David Sachs, Immunology Branch, National Cancer Institute.

¹ *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; AGG, β -alanylglycylglycyl; BGG, bovine gamma globulin; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NRS, normal rabbit serum; PBS, phosphate-buffered saline; RAMB, rabbit anti-mouse brain serum; SDS, sodium dodecyl sulfate; TNBS, trinitrobenzene sulfonate; TNP, 2,4,6-trinitrophenyl; TSD, TNP-stearyl dextran.

Cell Cultures. Mouse spleen cells (7×10^6) were cultured with either 3×10^6 irradiated (2,000 R) stimulating cells or with soluble protein antigens at 100 $\mu\text{g/ml}$ final concentration unless otherwise indicated, in RPMI 1640 tissue culture media (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (FBS) (Microbiological Associates, Bethesda, Md.), 5×10^{-5} M 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, glutamine, penicillin, and streptomycin, in a 2-ml volume in 24-well culture plates (Linbro Chemical Co., New Haven, Conn.) at 37°C in a humidified incubator (3% CO_2 , 97% air). TNP modification of stimulating cells was performed as previously described (6) using 10 mM TNBS (Pierce Chemical Co., Rockford, Ill.) unless otherwise stated.

The mouse spleen cells were harvested after 5 days of culture unless otherwise indicated and tested in a 4-h ^{51}Cr release assay (7). Cells suspended in Eagle's minimal essential medium containing 10% FBS were assayed at various effector:target cell ratios in round-bottomed microtiter plates (Cooke Laboratory Products Div. Dynatech Laboratories Inc., Alexandria, Va.) in a final volume of 200 μl . The target cells used were either RDM-4 ($H-2^k$) or EL-4 ($H-2^b$) lymphoid tumors carried in ascites form, or 48-h cultures of mouse spleen cells incubated with a 20- $\mu\text{g/ml}$ final concentration of *Escherichia coli* 055:B5 lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.). The target cells were labeled by incubation at 37°C for 1 h with $\text{Na}^{51}\text{CrO}_4$ (New England Nuclear, Boston, Mass., 0.2 $\mu\text{Ci}/2 \times 10^7$ cells). The targets were washed and modified by incubation with 10 mM TNBS at 37°C for 10 min. Excess TNBS was removed by washing the modified, labeled targets with Hanks' balanced salt solutions (HBSS) containing 10% FBS.

After the effector and target cells had been incubated for 4 h at 37°C in a CO_2 incubator, the microtiter plates were centrifuged and 100 μl of the supernate was removed and counted in a gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The percent of specific lysis was calculated as described elsewhere (6).

Stimulating and target cells were also prepared by incubating spleen and RDM-4 tumor cells respectively with the soluble protein antigens at 37°C (100 $\mu\text{g/ml}$ final concentration) for various periods of time followed by two or three washes of the cells with HBSS-FBS. These washed spleen and tumor cells were then tested for their ability to serve as stimulating and target cells, respectively.

Preparation of TNP Proteins. Proteins (BGG, serum fraction II and BSA, fraction V; Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) were dissolved in 0.4 M borate buffer pH 9.0, at a final concentration of 10 mg/ml of the reactive mixture, and TNBS was added to a final concentration of 20 mM and the mixture was incubated at 37°C overnight. These preparations were dialyzed sequentially against phosphate-buffered saline (PBS) pH 7.4, against PBS containing 5 mM glycylglycine, and twice more against PBS containing AG1 \times 8 beads (Bio-Rad Laboratories, Richmond, Calif.) at 4°C . Calculations of TNP derivatization were based on the value of 15×10^3 as molar extinction coefficient of TNP at 350 nm (15). Batches of TNP_{38-50} -BGG and TNP_{27-35} -BSA were used in these studies.

Immunofluorescence. All surface staining with anti-TNP was done as previously described (14) with rhodamine-conjugated F(ab')_2 fragments of the IgG fraction of affinity chromatography-purified sheep anti-TNP-keyhole limpet hemocyanin (KLH) antibodies.

Treatment of Effector Cells with Rabbit Anti-Mouse Brain Serum. Effector cells from B10.BR donors (10^7 cells/0.1 ml media) were incubated for 30 min at room temperature with a 1:20 dilution of rabbit anti-mouse brain serum (RAMB, a gift from Dr. R. Hodes, Immunology Branch, National Cancer Institute) which had been absorbed with mouse erythrocytes. This RAMB has been shown to be specific for T cells (16). The effector cells were then washed and incubated with a 1:5 dilution of guinea pig complement (Grand Island Biological Co.) for 30 min at 37°C . The effector cells were washed three times and tested in the ^{51}Cr release assay.

Cell Separations. Ascites tumor cells were passed through columns of Sephadex G-10 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), prepared as described by Ly and Mishell (17). 2.1 ml of RDM-4 ascites tumor cells at a concentration of $70 \times 10^6/\text{ml}$ in HBSS containing 1% penicillin streptomycin and 5% FBS was passed through a 40-ml column of Sephadex G-10 and 40 ml of the eluted volume was collected. Approximately 47% of the cells was recovered in the effluent. Latex uptake was used for the detection of phagocytic cells as follows. 2-ml samples (3×10^6 cells) were incubated overnight in a roller drum at 37°C with 10 μl of latex particles (18) in culture medium supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic

acid buffer (Microbiological Associates, Bethesda, Md.), washed three times with cold HBSS-FBS, centrifuged at 700 rpm for 5 min, and counted.

Radioiodination of Spleen Cells. B10.A spleen cells were either reacted in the presence of 1 mM TNBS for 10 min at 37°C, washed twice with HBSS and once with PBS, pH 7.4, or they were incubated for 2 h at 37°C in culture medium supplemented with 100 µg/ml of TNP-BSA, washed twice with HBSS-FBS and once with PBS. Lactoperoxidase-catalyzed iodination of surface proteins was carried out by a modification of the procedure of Vitetta et al. (19). Briefly, 2 mCi of ¹²⁵I-Na, 1.6 mg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.), and 40 µl of 0.03% H₂O₂ were added to 4 × 10⁸ cells in 4 ml PBS, followed by an additional 20 µl of H₂O₂ after a 10-min incubation at room temperature. The reaction was stopped after 15 min by the addition of a quenching solution composed of PBS pH 7.4 containing 0.01 M KI and 0.01 M cysteine. The cells were washed three times in the quenching solution and lysed in 4 ml of 0.01 M Tris, pH 7.4, 0.15 M NaCl, containing 0.5% Nonidet P-40 (Particle Data, Inc., Elmhurst, Ill.) for 30 min on ice. The cell lysates were centrifuged at 100,000 *g* for 60 min at 4°C.

Preparation and Analysis of Immune Precipitates. 0.1-ml samples of the lysates were incubated overnight on ice in the presence of 25 µl of either normal rabbit serum, rabbit anti-TNP-KLH serum prepared as previously described (14), rabbit anti-BSA (Miles-Yeda Laboratories, Rehovot, Israel) or mouse anti-*H-2* serum (A.BY anti-A) provided by Dr. David Sachs. The immunoprecipitates were obtained by the addition of *Staphylococcus aureus* (Cowan I) strain bacteria (10% suspension of formalinized bacteria; 375 µl for rabbit serum, 250 µl for mouse serum) as described by Cullen and Schwartz (20). After 30 min on ice, the precipitates were washed three times and eluted from the Cowan by boiling for 1 min in 0.4 ml of 0.06 M Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), and 0.28 M 2-mercapthoethanol. After centrifugation, the supernates were subjected to electrophoresis for 3 h on 10-cm polyacrylamide gels at 6 mA/gel (21). Gels were sliced, counted in a gamma counter, and the fraction numbers were adjusted from gel to gel for the migration of the phenol red dye.

Results

Generation of *H-2*-Restricted Cytotoxic Effector Cells by TNP-BGG. Spleen cells from B10.BR (*H-2^k*) donors were sensitized in vitro for 5 days by the addition of unmodified or TNBS-modified syngeneic spleen cells, BGG, or TNP-BGG and BSA, or TNP-BSA. In experiment 1, the effector cells were assayed on unmodified RDM-4 (*H-2^k*) and EL-4 (*H-2^b*) tumor target cells and on TNBS-modified RDM-4 and EL-4 at the effector:target cell ratios shown (Table I). The results indicate that the addition of TNP-BGG to the cultures led to the generation of effector cells which were at least as efficient in lysing RDM-4-TNBS target cells as were effectors generated by sensitization with B10.BR-TNBS-stimulating cells. The effectors generated by addition of TNP-BGG were ≅ 64-fold more efficient in lysing the *H-2*-matched, TNBS-modified RDM-4 targets than were the non-*H-2*-matched, modified EL-4 targets in the experiment shown. The 64-fold greater efficiency of lysis was estimated by comparing the effector:target cell ratios required to give equivalent lysis on modified RDM-4 and EL-4 targets; i.e., 0.625:1 for 20.3% on RDM-4-TNBS compared with 40:1 for 17.2% on EL-4-TNBS (line 4 of Table I). A similar estimation for effectors generated by sensitization with B10.BR-TNBS also indicated a 64-fold preferential lysis of RDM-4-TNBS compared with EL-4-TNBS. In other experiments using the same targets these effectors as well as the effectors generated by sensitization with TNBS-modified syngeneic spleen cells or by the addition of TNP-BSA lysed TNBS-modified, *H-2*-matched tumor targets from 16- (experiment 2) to 64-fold better than modified non-*H-2*-matched tumor targets. These results indicate that the response generated by the addition of TNP-conjugated

TABLE I
Generation of H-2-Restricted Effector Cells by the Addition of TNBS-Modified Syngeneic Cells or Soluble TNP-BGG or TNP-BSA to Cultures of B10.BR Spleen Cells

B10.BR Spleen cells sensitized by the addition of:	Percent specific lysis \pm standard error assayed on targets								
	RDM4	RDM4-TNBS				EL-4	EL-4-TNBS		
	40:1	40:1	10:1	2.5:1	0.625:1	40:1	40:1	10:1	2.5:1
Experiment 1									
B10.BR	[13.3]	—	[17.8]	[5.1]	[1.4]	[5.9]	[5.5]	[3.3]	[−0.5]
B10.BR-TNBS	-6.6 ± 0.7	—	44.2 ± 3.1	34.1 ± 4.0	10.5 ± 2.3	1.0 ± 1.9	13.5 ± 3.3	1.6 ± 2.8	0.8 ± 2.5
BGG	[17.7]	—	[21.5]	[6.1]	[0.7]	[7.6]	[12.7]	[3.4]	[0.4]
TNP-BGG	3.2 ± 1.9	—	45.3 ± 6.1	40.6 ± 2.3	20.3 ± 2.1	9.7 ± 2.5	17.2 ± 5.8	11.6 ± 3.0	5.0 ± 1.0
Experiment 2									
BSA	—	[25.1]	[15.3]	[6.2]	—	—	[8.8]	[2.8]	[0.2]
TNP-BSA	—	28.7 ± 3.7	27.3 ± 1.9	9.8 ± 1.9	—	—	9.1 ± 0.5	3.8 ± 1.0	2.5 ± 1.3

Values in brackets indicate the lysis above media controls by spleen cells cultured for 5 days in the presence of B10.BR or BGG without TNP, and serve as the controls for the respective specific responses generated by addition of B10.BR-TNBS or TNP-BGG. The percent specific lysis was calculated by subtracting the values in brackets from the total lysis, e.g., $44.2 = 62.0 - 17.8$. All specific lysis data shown in subsequent tables were calculated in this way.

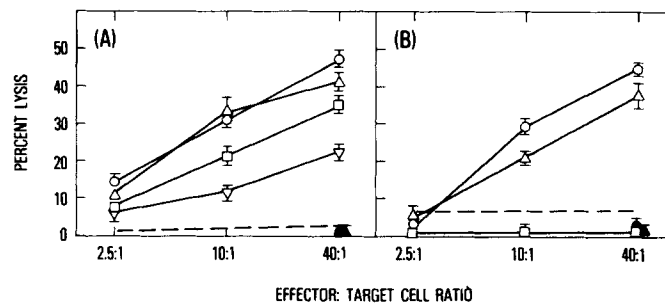


FIG. 1. Percent lysis by B10.BR effector cells after addition of different numbers of unmodified or TNP-modified syngeneic cells (A), or of different amounts of BGG or TNP-BGG (B), as a function of effector:target cell ratio. (A): (O), (Δ), (□), and (▽) indicate lysis of RDM-4-TNBS targets by B10.BR effectors generated by addition of 3×10^6 , 1×10^6 , 3×10^5 , and 1×10^5 B10.BR-TNBS stimulators, respectively. Dashed line indicates the level of lysis generated by addition of unmodified syngeneic cells. (●) and (▲) indicate lysis of EL-4-TNBS and RDM-4 targets, respectively, by effectors generated by addition of 3×10^6 B10.BR-TNBS. (B): (O), (Δ), and (□) indicate lysis of RDM-4-TNP targets by B10.BR effectors generated by addition of 100, 50, and 10 $\mu\text{g/ml}$ (final culture concentration) of TNP-BGG, respectively. Dashed line indicates the level of lysis generated by addition of unmodified syngeneic cells. (●) and (▲) indicate lysis of EL-4-TNBS and RDM-4 targets, respectively, by effectors generated by addition of 100 $\mu\text{g/ml}$ TNP-BGG.

BGG was H-2 restricted to the same extent as that resulting from sensitization with TNBS-modified syngeneic cells.

Effect of Antigen Concentration on the Generation of Cytotoxic Effector Cells. The phenomenon observed in the preceding section was examined further by comparing the number of TNBS-modified syngeneic spleen cells with the concentration of TNP-BGG required in the cultures for the generation of B10.BR effector cells. The results are summarized graphically at three effector:target cell ratios in Fig. 1. Addition of 3×10^6 and 1×10^6 B10.BR-TNBS cells were about equally effective in generating a cytotoxic response (Fig. 1 A). Sensitization with 3×10^5 and 1×10^5 B10.BR-TNBS cells generated

TABLE II

Mapping of the Restriction of B10.BR Effector Cells Generated by TNP-BGG to the K and D Regions of the H-2 Complex

B10.BR Spleen cells sensitized by the addition of:	Percent specific lysis \pm standard error detected on TNBS-modified targets indicated at effector:target = 10:1								
	RDM-4* (kkkkkkkk)†	RDM-4 (kkkkkkkk)	B10.BR-LPS (kkkkkkkk)	B10.A(4R)-LPS (kkbbbbb)	C3H.OH-LPS (ddddd)	C57BL/10-LPS (bbbbbbb)	A.TL-LPS (skkkkkkd)	A.TH-LPS (sssssssd)	A.AL-LPS (kkkkkkkd)
B10.BR	[6.8]	[19.5]	[14.7]	[14.1]	[1.5]	[1.1]	[0.4]	[2.6]	[4.4]
B10.BR-TNBS	-3.9 ± 1.6	35.4 ± 4.1	22.9 ± 4.9	33.0 ± 4.7	12.5 ± 2.5	3.7 ± 2.8	2.2 ± 2.3	3.8 ± 1.4	29.1 ± 2.6
BGG	[1.3]	[8.9]	[5.3]	[6.2]	[-1.5]	[2.2]	[-4.2]	[0.5]	[-2.8]
TNP-BGG	4.1 ± 1.1	37.4 ± 3.1	27.6 ± 2.9	35.8 ± 3.4	11.3 ± 1.3	6.5 ± 3.5	5.6 ± 1.9	5.1 ± 1.3	23.3 ± 5.7

* This column of data was assayed on unmodified RDM-4 at effector:target = 20:1.

† Indicates the *H*-2 haplotypes at region and subregion designations *K*, *I-A*, *I-B*, *I-J*, *I-E*, *I-C*, *S*, and *D*, respectively (22).

proportionally weaker responses. The cytotoxic response generated by the addition of TNP-BGG to the cultures was dependent upon the concentration of TNP-BGG at all concentrations tested: 100 μ g/ml was more effective than 50 μ g/ml, and 10 μ g/ml was ineffective (Fig. 1 B).

Mapping of the Restriction of Effector Cells Generated by TNP-BGG to the K and D Regions of the H-2 Complex. Earlier studies indicated that much of the *H*-2-restricted cytotoxic activity detected on TNBS-modified target cells was associated with products of the *K* and *D* regions of the murine MHC (7-9). To map the restriction of the effectors generated by TNP-BGG within the *H*-2 complex, cultures of B10.BR spleen cells were sensitized by the addition of B10.BR-TNBS spleen cells or TNP-BGG. The effectors generated 5 days later were assayed on RDM-4-TNBS tumor cells, as well as on a series of TNBS-modified, cultured, LPS-induced spleen blasts, at effector:target cell ratios of 20:1, 10:1, and 5:1. The results obtained using the 10:1 ratio are summarized in Table II. The lysis obtained by sensitization with either B10.BR-TNBS or TNP-BGG was detected on TNBS-modified LPS blast targets which expressed the *k* haplotype in *K* and *I-A* regions [B10.A(4R)], in the *D* region only (C3H.OH), or in any combination including *K*. It should be noted that lysis on the C3H.OH target, which expressed the *H*-2D^k haplotype, was weaker than on targets expressing the *k* haplotype in the *K* region. Appreciable lysis was not detected on modified targets which expressed the *k* haplotype throughout the *I* region but not at *K* or *D* (A.TL). No lysis was detected on unmodified RDM-4 (left column of Table II) nor on any of the unmodified LPS blast targets (data not shown).

Generation of H-2-Restricted Cytotoxic Effector Cells by Preincubation of Spleen Cells with TNP-Conjugated Proteins. To test whether or not TNP-conjugated proteins could interact with cell surfaces in a relatively short time period, spleen cells from B10.BR donors were incubated with BGG, TNP-BGG, BSA, or TNP-BSA for 1.5 or 3.0 h at 37°C in a CO₂ incubator. The cells, washed two or three times in BSS-FCS, and irradiated with 2,000 R, were used as stimulators of B10.BR spleen cells during 5 days of culture. The lytic responses of the effectors generated are shown in Fig. 2. Appreciable cytotoxic activity was detected by using cells incubated with TNP-BGG for 1.5 h (Fig. 2 A) and for 3 h (Fig. 2 B), although the level of cytotoxicity was slightly lower than when TNP-BGG was present during the first 5 days of culture (Fig. 2 C).

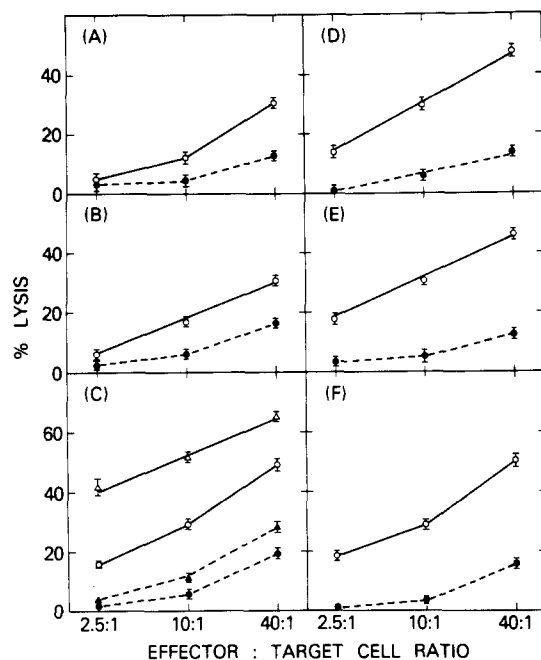


FIG. 2. Effect of TNP protein preincubation time with stimulator cells on generation of effector cells. (A, B) Lysis of RDM-4-TNBS targets by B10.BR effectors generated by addition of syngeneic spleen cells preincubated with 100 $\mu\text{g}/\text{ml}$ TNP-BGG (○) or 100 $\mu\text{g}/\text{ml}$ BGG (●) for 1.5 and 3.0 h, respectively. (C) Lysis of RDM-4-TNBS targets by B10.BR effectors generated by addition of 100 $\mu\text{g}/\text{ml}$ TNP-BGG (○), or 100 $\mu\text{g}/\text{ml}$ BGG (●) to the 5-day cultures, or by the addition of B10.BR-TNBS modified (Δ) or B10.BR (\blacktriangle) stimulating cells to the 5-day cultures. (D, E) Lysis of RDM-4-TNBS targets by B10.BR effectors generated by addition of syngeneic spleen cells preincubated with 100 $\mu\text{g}/\text{ml}$ BSA-TNP (○) or 100 $\mu\text{g}/\text{ml}$ BSA (●) for 1.5 and 3.0 h, respectively. (F) Lysis of RDM-4-TNBS targets by B10.BR effectors generated by addition of 100 $\mu\text{g}/\text{ml}$ TNP-BSA (○) or 100 $\mu\text{g}/\text{ml}$ BSA (●) to the 5-day cultures. Standard errors of the mean are shown.

Approximately equal levels of effector cell activity were detected when B10.BR spleen cells were incubated with TNP-BSA, whether the incubation period was for 1.5 or 3 h, or whether TNP-BSA was present during the 5 days of culture (Fig. 2 D, E, F). Addition of TNP-BSA to the spleen cell cultures for 5 days was not more effective in generating cytotoxic responses than were the 1.5- or 3-h preincubations. Furthermore, preincubation of spleen cells with TNP-BSA was more effective than with TNP-BGG, although equivalent lytic responses were observed for the two TNP-conjugated proteins when they were present in the cultures during the 5 days of sensitization.

Preincubation of Target Cells with TNP-Conjugated Proteins. As in the above experiments where TNP-conjugated proteins were preincubated with spleen cells for sensitization, RDM-4 tumor cells were incubated with 100 $\mu\text{g}/\text{ml}$ of BSA or TNP-BSA for 18 h at 37°C in a CO_2 incubator and then washed. Those RDM-4 cells to be used as targets were passed over a Sephadex G-10 column before incubation with BSA or TNP-BSA to remove phagocytic cells (6% latex-positive cells before column passage; 0.3% latex-positive after Sephadex G-10 column). The RDM-4 cells were then ^{51}Cr -labeled and used as targets

TABLE III
Lysis of Target Cells Prepared by Preincubation With TNP-BSA by Effectors Generated by Addition of TNP-Modified Syngeneic Cells or TNP-BSA

B10.BR Spleen cells sensitized by the addition of:	Percent specific lysis \pm standard error assayed at effector:target cell ratio of 40:1					
	Unfractionated cells				Cells fractionated over Sephadex G-10 columns*	
	RDM-4	RDM-4-TNBS	RDM-4(BSA)‡	RDM-4(TNP-BSA)‡	RDM-4(BSA)‡	RDM-4(TNP-BSA)‡
B10.BR	[7.7]	[31.0]	[11.7]	[9.7]	[6.2]	[11.3]
B10.BR-TNBS	4.0 \pm 2.1	38.0 \pm 4.6	0.5 \pm 1.8	18.8 \pm 2.5	1.7 \pm 1.9	29.4 \pm 1.8
BSA	[-1.5]	[17.6]	[4.4]	[3.2]	[0.7]	[5.9]
TNP-BSA	9.5 \pm 2.0	50.9 \pm 4.4	8.1 \pm 2.4	27.6 \pm 2.3	4.5 \pm 1.3	33.0 \pm 3.1

* RDM-4 tumor targets fractionated over Sephadex G-10 columns before incubation with BSA or TNP-BSA.

‡ RDM-4 tumor targets incubated with BSA or TNP-BSA for 18 h at 37°C in CO₂ incubator, and then washed before ⁵¹Cr-labeling.

for lysis by B10.BR effector cells which had been generated by culturing for 5 days with unmodified or TNBS-modified syngeneic cells, BSA, or BSA-TNP. The lysis obtained on this series of targets, as well as on unmodified RDM-4 and TNBS-modified RDM-4, is summarized in Table III. Effector cells generated by addition of B10.BR-TNBS, or TNP-BSA exhibited strong lysis on TNBS-modified RDM-4 cells or on RDM-4 cells incubated with TNP-BSA, whether or not the incubated cells had been passed over a Sephadex G-10 column. Appreciable lysis was not detected on targets incubated with BSA.

Those RDM-4 tumor cells to be used as targets were also incubated for shorter periods of time (i.e. 10 or 150 min) with 100 μ g/ml TNP-BSA before ⁵¹Cr-labeling, and they were used as targets for effectors which had been generated by addition of B10.BR-TNBS and TNP-BGG. Portions of these cell suspensions were stained with a rhodamine-conjugated anti-TNP antibody to determine whether or not TNP groups could be detected on the RDM-4 cell surface by fluorescence microscopy (14). The lysis of the target cells is presented in Table IV and the corresponding staining of the RDM-4 cells is shown in Fig. 3. Target cells modified with 10 and 1 mM TNBS were lysed equally at all three effector:target ratios by effectors sensitized with B10.BR-TNBS. Targets modified with 0.1 mM TNBS or preincubation with 100 μ g/ml TNP-BSA for either 150 or 10 min were not lysed as well as those modified at higher TNBS concentrations. Effectors generated by addition of TNP-BGG appeared to exhibit weaker lytic potential than those generated by sensitization with B10.BR-TNBS when assayed on modified targets treated with 10 and 1.0 mM TNBS, but not when assayed on targets treated with 0.1 mM TNBS or preincubated with TNP-BSA for 150 or 10 min.

In this experiment cytotoxicity was detected in cultures to which unmodified cells or proteins were added. This phenomenon of generation of cytotoxic effector cells in cultures not stimulated with modified cells is consistently observed on TNP-modified, *H*-2-matched tumor targets, and is proportional to

TABLE IV

Lysis of H-2-Matched Target Cells Prepared by Incubation With TNBS or TNP-BSA by Effector Cells Generated by Addition of TNBS-Modified Syngeneic Cells or TNP-BGG

Spleen cells indicated were sensitized by the addition of:	Percent specific lysis \pm standard error assayed on targets									
	RDM-4-TNBS 10 mM (10 min)*			RDM-4-TNBS 1 mM (10 min)			RDM-4-TNBS 0.1 mM (10 min)			RDM-4
	40:1	10:1	2.5:1	40:1	10:1	2.5:1	40:1	10:1	2.5:1	40:1
B10.BR:										
B10.BR	[59.6]	[32.9]	[11.9]	[38.5]	[14.7]	[2.2]	[23.5]	[9.8]	[-0.1]	[14.3]
B10.BR-TNBS	39.3 \pm 1.7	51.3 \pm 4.9	49.7 \pm 2.6	48.2 \pm 3.2	58.7 \pm 3.2	45.7 \pm 4.5	47.8 \pm 1.9	47.4 \pm 2.1	26.3 \pm 1.7	-15.1 \pm 1.5
BGG	[63.3]	[35.1]	[16.2]	[43.1]	[14.7]	[3.3]	[29.6]	[10.4]	[1.9]	[22.3]
TNP-BGG	22.9 \pm 3.9	37.8 \pm 5.4	20.6 \pm 2.0	33.7 \pm 6.1	47.4 \pm 3.2	29.7 \pm 2.7	42.3 \pm 2.8	53.2 \pm 2.7	23.3 \pm 0.8	-15.6 \pm 2.0
	RDM-4-(TNP-BSA) (150 min)			RDM-4-(TNP-BSA) (10 min)			RDM-4 (BSA) (150 min)		RDM-4 (BSA) (10 min)	
	40:1	10:1	2.5:1	40:1	10:1	2.5:1	40:1		40:1	
B10.BR:										
B10.BR	[33.3]	[16.4]		[19.1]	[5.3]	[-2.7]	[27.3]		[20.7]	
B10.BR-TNBS	40.2 \pm 1.7	40.5 \pm 3.4		40.6 \pm 2.9	37.1 \pm 3.9	18.6 \pm 1.9	-16.8 \pm 0.9		-11.8 \pm 0.6	
BGG	[37.4]	[20.7]	[8.3]	[23.5]	[1.0]	[1.3]	[29.2]		[27.2]	
TNP-BGG	34.1 \pm 1.8	45.1 \pm 4.1	25.8 \pm 3.0	39.2 \pm 2.8	46.3 \pm 4.7	20.3 \pm 2.2	-14.4 \pm 1.8		-15.5 \pm 2.0	

* Indicates period of time (in minutes) that target cells were incubated with TNBS, TNP-BSA, or BSA. Target cells were washed three times after incubation in an excess of FBS-containing media.

the degree of reaction of the cells with TNBS. Such autosensitization is not attributable to the addition of 100 μ g BGG or BSA to the cultures, since spleen cells cultured alone exhibit the phenomenon. This accounts for the apparent decrease in lytic efficiency on the 10 mM TNBS-modified target cells as the effector:target ratio increased from 10:1 to 40:1.

As shown in Fig. 3, the staining of these RDM-4 targets with rhodamine-conjugated anti-TNP antibodies parallels in general the lysis of the targets modified with TNBS or incubated with TNP-BSA as described in the data of Table IV. The staining of targets treated with 10 or 1.0 mM TNBS was bright (Fig. 3A) (14). Staining of cells treated with 0.1 mM TNBS was faint, but visible and uniform (Fig. 3B). Cells incubated with BSA for 150 min exhibited no detectable fluorescence (Fig. 3C). Targets incubated with TNP-BSA for 150 min (Fig. 3D) stained with an intensity similar to that seen for 0.1 mM TNBS-treated cells, although the cells incubated with TNP-BSA exhibited less uniform fluorescence than did TNBS-treated cells. Faint but detectable fluorescence was observed with the targets incubated for 10 min with TNP-BSA, showing a nonuniform patchy pattern of staining (Fig. 3E).

Role of T Lymphocytes in H-2-Restricted Cytotoxic Responses to TNP-Conjugated Proteins. To determine whether or not the H-2-restricted cytotoxic effectors generated by addition of TNP-conjugated proteins are T lymphocytes, effector cells were incubated with rabbit anti-mouse brain serum and/or complement. The cells were then tested for their ability to lyse RDM-4-TNBS target cells (Table V). Treatment with the antiserum and complement abolished the cytotoxic activity of effectors generated by sensitization with B10.BR-TNBS and greatly reduced the activity of effectors generated by addition of TNP-BGG. No effect was detected by treating the effectors with antiserum or complement alone. These results are consistent with the expression of the Thy-1 antigen on the surface of the effector cells generated by sensitization with

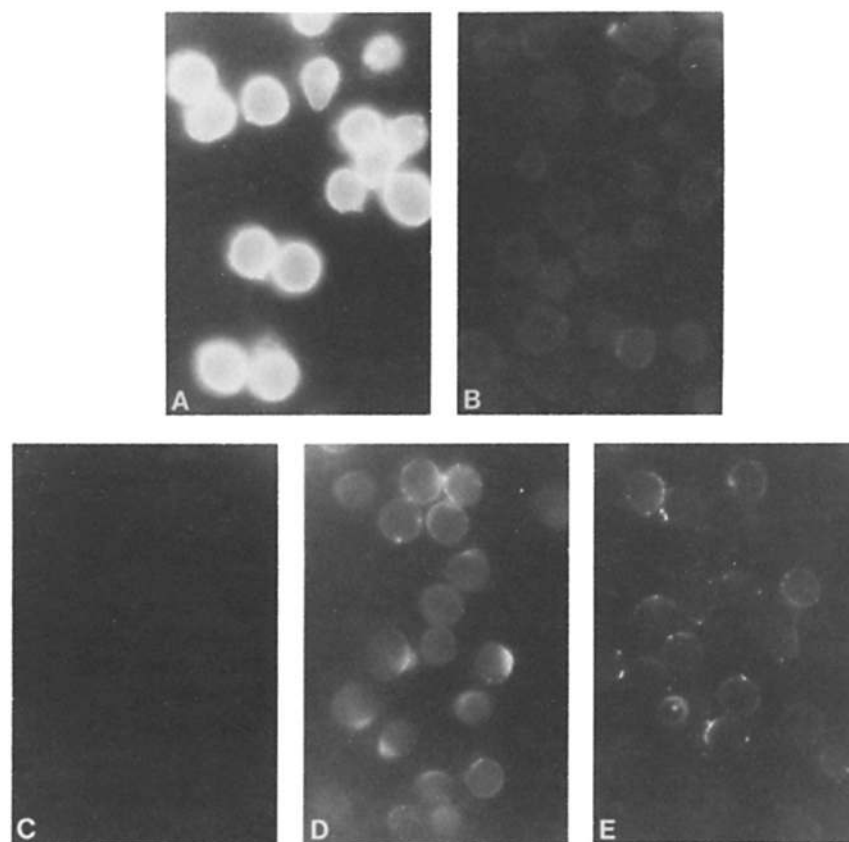


FIG. 3. Immunofluorescent staining of RDM-4 tumor target cells with anti-TNP antibody. Tumor target cells were treated with TNBS or TNP-BSA as described in the text, washed, then stained with rhodamine-anti-TNP antibody at 0°C, washed, and fixed with paraformaldehyde. Identical conditions of photographic exposure and processing were used for all samples. A. Cells modified with 1 mM TNBS, pH 7.4, 10 min, 37°C. B. Cells modified with 0.1 mM TNBS, pH 7.4, 10 min, 37°C. C. Cells exposed to 100 µg/ml BSA, 37°C, 150 min. D. Cells exposed to 100 µg/ml TNP-BSA, 37°C, 150 min. E. Cells exposed to 100 µg/ml TNP-BSA, 37°C, 10 min.

TABLE V
Sensitivity of Effector Cells Generated by Addition of B10.BR-TNBS or TNP-BGG to Cultures of B10.BR Spleen Cells to RAMB and Complement

B10.BR Spleen cells sensitized by the addition of:	Percent specific lysis \pm standard error obtained using effector cells treated with:*			
	Nothing	Complement	RAMB	RAMB + Complement
B10.BR	[16.6]	[20.7]	[12.5]	[6.6]
B10.BR-TNBS	23.8 \pm 2.1	22.4 \pm 3.1	21.0 \pm 1.4	-4.8 \pm 1.8
BGG	[10.7]	[9.1]	[6.8]	[-0.6]
TNP-BGG	30.9 \pm 1.1	30.5 \pm 2.6	32.3 \pm 1.6	7.7 \pm 1.8

* Effector:target cell ratio = 10:1 on RDM-4-TNP targets. 10^7 effector cells incubated with 1:20 dilution of RAMB at room temperature for 30 min. Cells were then incubated with 1:5 dilution of complement at 37°C for 30 min.

TREATMENT OF CELLS WITH:

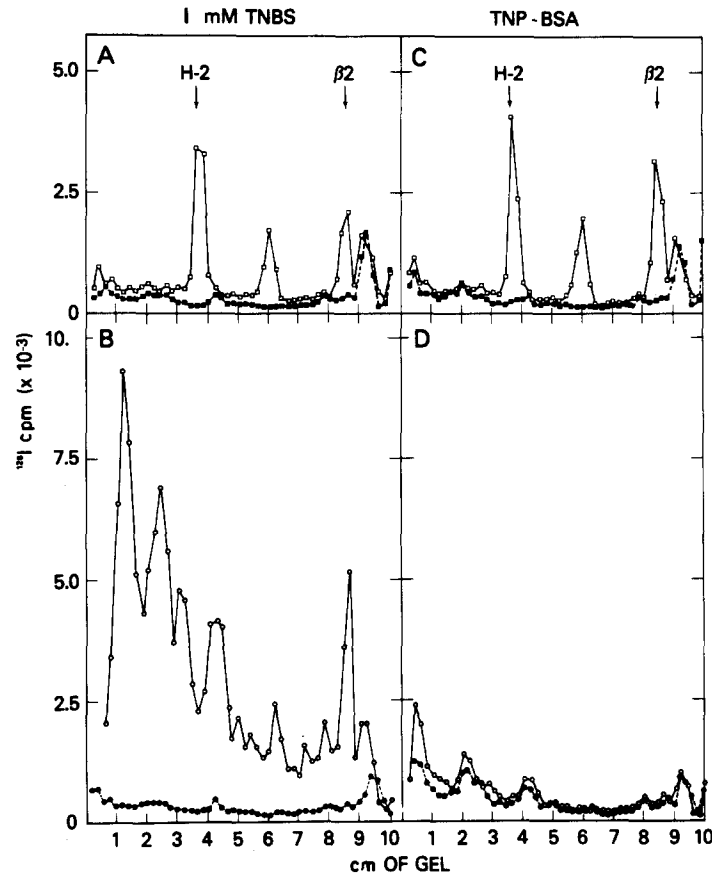


FIG. 4. SDS-gel patterns of radioiodinated proteins immunoprecipitated with NRS (■), anti-H-2 (□), anti-TNP (○), anti-BSA (●) from lysates of radioiodinated B10.A spleen cells modified with 1 mM TNBS (A, B), or preincubated with 100 μ g/ml of TNP-BSA for 2 h at 37°C (C, D).

B10.BR-TNBS or by addition of TNP-BGG. It is worth noting that the autosen- sitization detected on RDM-4-TNBS targets was also sensitive to RAMB and complement (see values in brackets).

Comparison of the SDS-Gel Patterns of Anti-TNP, Anti-BSA and Anti-H-2 Immunoprecipitates of TNBS-Modified Cells and Cells Preincubated with TNP-BSA. Experiments were performed to compare the mode of binding of TNP groups on cells treated with TNBS (23, 24) and on cells preincubated with TNP-BSA under conditions for which those cells stimulate the generation of H-2-restricted, TNP-specific cytotoxicity. B10.A spleen cells treated with 1 mM TNBS for 10 min at 37°C, or preincubated for 2 h at 37°C with 100 μ g/ml of TNP-BSA in culture medium were radioiodinated in the presence of lactoperoxidase, solubilized in Nonidet P-40, and the cell lysates were immunoprecipitated as described in Materials and Methods. The SDS-gel patterns of the immunoprecipitates obtained with normal rabbit serum (NRS) and anti-H-2 serum (Fig. 4 C) and with anti-TNP and anti-BSA serum (Fig. 4 D) for cells

incubated with TNP-BSA indicate that the TNP groups are bound to a protein that has the same SDS-gel pattern as that precipitated with anti-BSA, and is distinct from the proteins precipitated with anti-*H-2*. The SDS-gel patterns obtained for proteins precipitated with NRS and anti-*H-2* (Fig. 4 A) and anti-TNP and anti-BSA (Fig. 4 B) from cells reacted with 1 mM TNBS are shown for comparison. The general gel pattern for proteins immunoprecipitated with anti-TNP from the cells reacted with 1 mM TNBS is similar to that described by Forman et al. (24) in slightly different gel conditions. From their analysis of gel patterns after sequential immunoprecipitations with anti-TNP and anti-*H-2* or anti-Ig, these authors concluded that under their conditions, *H-2*-coded products as well as surface Ig were derivatized with TNP (23, 24). The results presented here suggest that the TNP groups which can be detected on the surface of spleen cells incubated for 2 h with 100 μ g/ml of TNP-BSA are still bound to the carrier BSA and did not covalently modify *H-2*-coded proteins.

Discussion

Covalent modifications of lymphoid cell surfaces can lead to MHC-restricted cell-mediated lympholysis (6-10). In the present report, cytotoxic effector cells from B10.BR mice were generated in the presence of soluble TNP-conjugated proteins. These effectors were capable of lysing TNBS-treated syngeneic target cells. As in the case of cells sensitized with TNBS-treated cells, preferential lysis was detected on TNBS-treated tumor targets, *H-2*-matched with the responding cells. Intra-*H-2* mapping of such restriction on LPS-stimulated, TNBS-modified blasts indicated that homology at: (a) *K* or *K* plus *I-A* was as effective as at the entire *H-2* complex; (b) the *I* region was ineffective; and (c) the *D* region was much less effective than *K* or *K* plus *I-A* in detecting effectors generated by either TNBS-modified spleen cells (6, 7, 9, 25-27) or TNP-BGG. The observation that the response of B10.BR spleen cells to TNP-BGG was much weaker when the effectors were assayed on TNBS-modified targets *H-2*-matched at *D*, than at *K* plus *I-A* is similar to the *Ir*-controlled weak responses of lymphocytes from donors expressing the *k* haplotype at the *K* plus *I* regions against TNBS-modified syngeneic cells (28). This similarity suggests that the same *Ir*-controlled regulatory functions are involved whether the TNP group is covalently linked to existing cell surface proteins or presented as adsorbed protein (Discussion). The generation of cytotoxic lymphocytes in the presence of TNP-conjugated soluble proteins was observed using spleen cells from a number of mouse strains of differing *H-2* haplotypes and genetic backgrounds (B10.BR, B10.A, C57BL/10, AKR/J, BALB/c), although the response was highest in the *H-2^k* strains tested (unpublished observations). Furthermore, the TNP-conjugated protein which elicited the cytotoxic response included TNP-ovalbumin and TNP-mouse serum albumin (unpublished observations).

Effectors generated in culture by addition of TNP-BGG were sensitive to rabbit anti-mouse brain serum and complement, and are therefore probably T lymphocytes. Two possible mechanisms could account for the lysis generated by TNP-conjugated proteins: T-cell-mediated cytotoxicity; or antibody-dependent cellular cytotoxicity (ADCC). ADCC has been observed using spleen cells from mice immunized against DNP-conjugated proteins (29, 30). The observation that the cytotoxicity generated in vitro by addition of TNP-conjugated

proteins was not inhibited by TNP-lysine at the effector phase (unpublished results) argues against this cytotoxicity being ADCC-mediated (30), and is similar to that reported for effectors generated by TNBS-modified syngeneic cells (6).

The observation that sensitization with TNP-conjugated proteins leads to the generation of *H-2*-restricted effectors suggests that the TNP group (either with or without the protein carrier) is presented on the cell surface, possibly presenting some groups in close proximity to *H-2* gene products. Both spleen and tumor cells incubated for 10–150 min with TNP-BSA were fluorescent when incubated with rhodamine-labeled anti-TNP antibodies (shown for tumor cells in Fig. 3; results not shown for spleen cells). Tumor cells incubated with TNP-BSA served as functional targets for *H-2*-restricted effectors generated by addition of either TNBS-modified cells or TNP-BSA. Spleen cells preincubated for 30 min or more with TNP-BGG or TNP-BSA, followed by washing and irradiation, served as stimulators of syngeneic responding cells for the generation of *H-2*-restricted effectors. In contrast to sensitization with TNP-BSA, it should be noted that addition of BSA to cultures of spleen cells did not lead to the generation of effectors detected on the targets preincubated with TNP-BSA. Since the TNP group appears not to be cleaved from the soluble protein carrier, and therefore BSA is also present on the target cells, the results presented here suggest that TNP-BSA exhibits greater cellular antigenicity than does unconjugated BSA.

The mechanism by which the TNP-conjugated proteins become associated with the cell surface remains to be elucidated. A few possibilities can be considered. The first is the possible covalent transfer of the TNP hapten from the soluble protein carrier to a cell surface protein. Although arylation reactions such as those of TNBS are generally irreversible, Shaltiel (31) has demonstrated that DNP conjugates of cysteine, tyrosine, and histidine (but not of amino groups) can be thiolized by SH compounds such as cysteine. Such a reaction, if it occurs with TNP, could potentially result in a protein-to-protein transfer. However, since TNBS reacts only with ϵ - and α -amino groups and cysteine SH groups in proteins (32, 33), and since BSA and BGG contain less than one reactive SH group per molecule, such a transfer appears to be very unlikely. Analysis of the SDS gel patterns (Fig. 4) of anti-TNP, anti-BSA, and anti-*H-2* immunoprecipitates of solubilized ^{125}I -labeled surface proteins of cells preincubated for 2 h with TNP-BSA indicates that the TNP moiety was still linked to BSA, and therefore was not covalently transferred to cell surface proteins.

Another possible mechanism by which soluble TNP-conjugated proteins could be associated with cell surface components, including *H-2*-coded products, involves the processing of the TNP-protein conjugates by macrophages. Incubation of guinea pig macrophages with dinitrophenyl-conjugated guinea pig albumin for brief time periods has been shown to result in antigen presentation which induces proliferation and lymphokine production by primed T-lymphocytes (34). The involvement of macrophages at least at the target cell level cannot account for this phenomenon, since a Sephadex G-10 column-passed macrophage-depleted population of tumor cells served as good targets for the *H-2*-restricted lysis after incubation with TNP-BSA (Table IV). In contrast to the incubation experiments with target cells, it is not known whether antigenic

processing occurs for these stimulators after the preincubation period during the 5 days of culture.

A third possibility is that the TNP-conjugated soluble proteins nonspecifically interact with cell surfaces, perhaps because of their increased hydrophobic character. However, such an interaction would not be expected to generate an antigenic cell-bound complex that would be recognized by effectors sensitized against TNBS-modified syngeneic cells, since cells modified with TSD did not function as efficient targets for effectors generated in the TNBS-modified cytotoxic system (14). In fact, it is difficult to reconcile the results of the present report with a recent study in which murine lymphocytes were modified with TSD, an amphipathic compound which modifies cell surfaces by noncovalent linkage (14). TSD-treated cells did not stimulate the generation of effector cells capable of lysing *H-2*-matched target cells modified with either TSD or TNBS (14). Furthermore, TSD-modified cells did not serve as targets nor as blocking cells for effectors generated by sensitization with TNBS-modified syngeneic cells (14). Such findings suggest that the random presentation of TNP groups on the cell surface, at a density equal to or greater than that obtained with TNBS-modification, is not sufficient to provide an antigenic unit (composed of TNP and the relevant self components) that will function either at the sensitization or lytic level. If particular MHC-coded structures are required in close association with TNP, it is possible that TSD has placed the TNP groups in an unfavorable position with respect to the relevant self components, whereas the random sticking of TNP-conjugated proteins to the cell surface provides some situations in which TNP is associated closely enough with the relevant *H-2*-coded self structures to provide an antigenic unit. It is possible that TNP-proteins could be preferentially bound to cell surface proteins (including *H-2* products), whereas TSD might be primarily bound the lipid bilayer. Another possibility is that the TNP group must be presented to the relevant lymphoid cells on an appropriate carrier, and that syngeneic cell surface determinants and proteins such as BSA and BGG are immunogenic carriers, whereas stearyl-dextran is not. This would imply that the soluble proteins are involved in the recognition—a possibility which remains to be tested.

The cytotoxic results obtained with the TNP-conjugated proteins in which cross-reactivity was observed with TNBS-modified cells are also difficult to reconcile with early published studies (10) in which the specificity of effectors generated by sensitization with syngeneic cells modified with TNBS did not lyse syngeneic targets modified with *N*-(2,4,6-trinitrophenyl)- β -alanylglycylglycyl (TNP-AGG) acylazide. At least two possibilities could account for this apparent discrepancy. First, it is possible that the specificity associated with recognition of the TNP group is dependent upon the number of methylene carbons in the adjacent amino acid. The predominant group reacting with TNBS on the cells and BSA would be the ϵ -amino group of lysine. In contrast, in the TNP-AGG structure, TNP is linked to the β -amino group of β -alanine. This might also account for the lack of cross-reactivity between TSD- and TNBS-modified cells. Alternatively, it is possible that the same clones of responding cells are activated by TNBS-modified syngeneic cells and the TNP-conjugated proteins, which are distinct from the clones triggered by TNP-AGG.

In such a model, it would have to be assumed that the interaction of the TNBS and the TNP-conjugated protein with the relevant *H-2*-coded structures are similar, but antigenically distinct from the interaction of TNP-AGG with such structures. The first of these possibilities could conform to a two-receptor model in which the TNP group plus the adjacent amino acid(s) would be recognized as a distinct entity from self *H-2* products. The second possibility could be consistent with a single receptor model involving the antigen presented in association with *H-2* products, but not necessarily involving covalent linkage to *H-2*-coded proteins. A similar interpretation has been given for results in which UV-inactivated Sendai virus-treated cells served as *H-2*-restricted targets for lysis by effectors generated by sensitization with UV-inactivated Sendai virus-coated stimulator cells (13).

It has been established in a series of earlier reports that cells covalently modified with TNBS can stimulate syngeneic mouse lymphocyte cultures to generate *H-2*-restricted, TNP-dependent cytotoxic effector cells (6-8). The results presented in this report broaden the limits of the experiments and interpretations imposed by covalent modification with TNBS to include TNP groups attached to soluble protein carriers. Thus, the chemically modified, *H-2*-restricted cytotoxicity model may not depend on actual covalent conjugation of the TNP group to *H-2*-coded cell surface products, and therefore could be similar to the *H-2*-restricted viral and weak transplantation antigen models (1-5, 11-13). These findings could also indicate the possible role of *H-2*-restricted cytotoxic reactions involving autoimmunity generated by soluble components associated with autologous cells.

Summary

Murine spleen cells from normal donors were cultured in vitro with trinitrobenzene sulfonate (TNBS)-conjugated soluble proteins, i.e., bovine gamma globulin (TNP-BGG) or bovine serum albumin (TNP-BSA). Addition of 100 μ g of any of these TNP-proteins to the spleen cell cultures led to the generation of cytotoxic T-cell effectors which were *H-2*-restricted and TNP-specific. The lytic potential of such effectors was comparable to that generated by sensitization with TNBS-modified syngeneic cells, and was restricted to haplotypes shared at the *K* or *K* plus *I-A*, or the *D* regions of the *H-2* complex. Greater effector cell activity was generated by addition of TNP-BGG against TNBS-modified targets which shared *K* plus *I-A* than against modified targets which shared the *D* region with the responding cells, which suggests that the same immune response genes are involved when the response is generated by the addition of TNP-conjugated soluble proteins or of TNBS-modified cells.

H-2-restricted, TNP-specific effector cells were generated by culturing mouse spleen cells with syngeneic cells which had been preincubated with TNP-BGG or TNP-BSA for 1.5 h. The addition of unconjugated soluble proteins to the cultures did not result in cytotoxic effectors detectable on *H-2*-matched targets, whether the targets were prepared by modification with TNBS, or by incubation with either the unconjugated or TNP-conjugated proteins. Depletion of phagocytic cells in the tumor preparation by Sephadex G-10 column fractionation before incubation with TNP-BSA had no effect on their lysis by the relevant effector cells. Immunofluorescent staining of tumor target cells with anti-TNP

antibodies indicated that TNP could be detected on the tumor cells within 10 min of incubation with TNP-BSA. The cytotoxic response generated by addition of the TNP-proteins to spleen cell cultures was found to be T-cell dependent at the effector phase, as shown by the sensitivity of the lytic phase to absorbed RAMB and complement. Furthermore, the response did not appear to be attributable to antibody-dependent cellular cytotoxicity.

Three mechanisms were considered which could account for the generation of *H-2*-restricted, TNP-specific, cytotoxic T-cell effectors by the addition of soluble TNP-proteins. These include covalent linkage of activated TNP groups from the soluble proteins to cell surface components, macrophage processing of the soluble conjugates and presentation to the responding lymphocytes in association with *H-2*-coded self structures, or hydrophobic interaction of the TNP-proteins to cell surfaces. Results obtained from sodium dodecyl sulfate gel patterns indicating that cell-bound TNP was still linked to BSA, and the observation that phagocytic-depleted cells could interact with the soluble TNP-proteins and function as *H-2*-restricted targets, appear not to favor the first two proposed mechanisms.

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