

SPECIFICITY OF CYTOTOXIC EFFECTOR CELLS
DIRECTED AGAINST TRINITROBENZENE
SULFONATE-MODIFIED SYNGENEIC CELLS
Failure to Recognize
Cell Surface-Bound Trinitrophenyl Dextran

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Cell-mediated lympholysis (CML)¹ has been generated *in vitro* against trinitrophenyl (TNP)-modified syngeneic murine spleen cells (1). The specificity of the effectors generated is such that the stimulator and target cells must both be modified by the same agent (2, 3), and must also express the same *H-2K* and/or *H-2D* haplotypes (4, 5). In previous studies of the specificity of the CML effectors directed against TNP self, the modifying agents were coupled to cell surface proteins via covalent linkage, primarily to the amino groups of lysines (6). The present report describes experiments in which TNP stearoyl dextran was inserted into the lipid bilayer of mouse spleen cells (used as sensitizing cells) and lymphoid tumor cells (used as target cells) for the generation of effectors directed against self-modified cells. The results indicate that when quantitatively equivalent amounts of TNP are present on the cell surface either in the form of TNP stearoyl dextran (TSD) or as a result of direct covalent modification of the cell surface with trinitrobenzene sulfonate (TNBS), only the latter is immunogenic for the generation of a TNP self CML. Furthermore, the TSD-modified cells do not serve either as lysable targets or as inhibiting cells of effectors generated by sensitization with TNBS-modified syngeneic cells.

Materials and Methods

Mice. The mice used in the experiments were males 6-9 wk of age. Both the B10.A and B10.BR, as well as the AKR/J, mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

In Vitro Cell Culture, TNP-Modification of Cells, and ⁵¹Cr Release Assay. The methods employed for the primary 5 day *in vitro* CML sensitization, the TNP modification of stimulating and target cells by covalent linkage of TNP, and the 4-h ⁵¹Cr release cytotoxic assay have been described in earlier publications (1, 4). For the covalent linkage of TNP to cells, both 10 and 1 mM TNBS were used. See separate section for description of TNP dextran fatty acid insertion into cell surfaces. The target cells used for the ⁵¹Cr-release assay and for the inhibition of lysis were RDM-4 (*H-2^k*) lymphoid tumor cells carried as an ascites tumor in AKR/J mice.

TSD. TSD was synthesized by a modification of the procedure described elsewhere (6a). To 10

¹ *Abbreviations used in this paper:* CML, cell-mediated lympholysis; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TNBS, trinitrobenzene sulfonate; TNP, trinitrophenyl; TSD, TNP stearoyl dextran.

vol of 5 mg/ml *N*-(2-aminoethyl) carbamylmethyl dextran (mol wt = 82,000) (7) in 0.04 M sodium borate, pH 9.0, were added 4 vol of dimethyl formamide and 1 vol of the *N*-hydroxy succinimide ester of stearic acid (8) at 2 mg/ml in dimethylformamide. After 2 h at 37°C, the remaining amino groups were TNP modified by addition of 2 vol of 0.10 M TNBS in phosphate-buffered saline (PBS), pH 7.0. After 2 h at 37°C, the TSD was dialyzed in PBS and AG1 × 8 (Bio-Rad Laboratories, Richmond, Calif.) beads at 5°C for 2 days. Each molecule of TSD contains 42 TNP groups and approximately five stearate groups.

Modification of Cells. TNBS modification was carried out as described previously (1, 4); in some cases the TNBS concentration during modification was reduced from 10 to 1.0 or 0.1 mM. TSD modification was carried out by suspending a pellet of 10^7 cells in 100 μ l of TSD at 1–2 mg/ml in PBS. After incubation at 37°C for 30 min, the cells were washed three times with culture medium.

Immunofluorescence. Anti-TNP was prepared by affinity chromatography on DNP lysine Sepharose (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) from the serum of a sheep hyperimmunized with TNP keyhole limpet hemocyanin. After gel filtration on G200, the IgG fraction was labeled with 50 μ g fluorescein isothiocyanate (FITC) per mg protein. F(ab')₂ fragments were prepared from the IgG fraction by pepsin digestion, and, after gel filtration on G200, labeled with 100 μ g FITC per mg protein. For staining of TNP-modified cells, 50 μ g of antibody was incubated with 5×10^6 cells in 500 μ l of PBS containing 1% bovine serum albumin at 0°C. After washing once with cold PBS, the cells were fixed with 2% paraformaldehyde in 0.125 M sodium cacodylate, pH 7.4, for 20 min at room temperature, washed twice with PBS, and examined in the fluorescence microscope or by flow microfluorometry.

Flow Microfluorometry. Stained and fixed cells were suspended at $\sim 4 \times 10^6$ cells/ml in PBS containing 10% fetal bovine serum. Flow microfluorometry was performed essentially as detailed by Loken and Herzenberg (9) by using a FACS I flow microfluorometer (Becton, Dickinson Electronics Laboratory, Mountain View, Calif.). A minimum of 40,000 cells were analyzed, with light scatter gating used to eliminate damaged cells (>5%). The laser (model 164, Spectra-Physics Inc., Laser Products Div., Mountain View, Calif.) was focused on the cell stream with a 30-mm focal length spherical lens, by using a 488-nm line at a 300-mW light output. The data obtained by the FACS I were stored and analyzed by using a GT44 Data Acquisition System (Digital Equipment Corp., Marlboro, Mass.).

Results

Qualitative Estimation of Cell Surface TNP Groups. Under the conditions described, TSD molecules from solution adsorbed to cell surfaces, allowing cells to acquire TNP groups by a procedure which should not result in the chemical modification of other cell surface molecules. The acquisition of TNP groups can be easily demonstrated by immunofluorescence, and we have used the binding of FITC-anti-TNP to estimate the relative amounts of available cell surface TNP hapten per cell. Fig. 1 shows the immunofluorescence of RDM-4 tumor cells modified by TNBS at 10, 1, 0.1 mM, and by TSD. It is clear that the TSD-treated cells were approximately as bright as those cells treated with 10 mM TNBS. In other experiments the TSD-treated RDM-4 cells were of intermediate brightness between those treated with 10 and 1 mM TNBS. Intact sheep IgG anti-TNP and F(ab')₂ fragments of this antibody gave identical results in these experiments. One qualitative difference between the anti-TNP staining of TSD- and TNBS-treated cells is the tendency of the former to cap while the latter show little or no tendency to patch or cap. This phenomenon has been studied intensively and will be reported elsewhere. All comparisons of anti-TNP immunofluorescence were made on cells stained at 0°C and fixed immediately. The tendency of the TSD molecules to patch even at 0°C can be seen in Fig. 1.

TSD-treated spleen cells also acquired TNP groups on their surface. Immunofluorescence with FITC-anti-TNP showed that such cells were similar to RDM-4

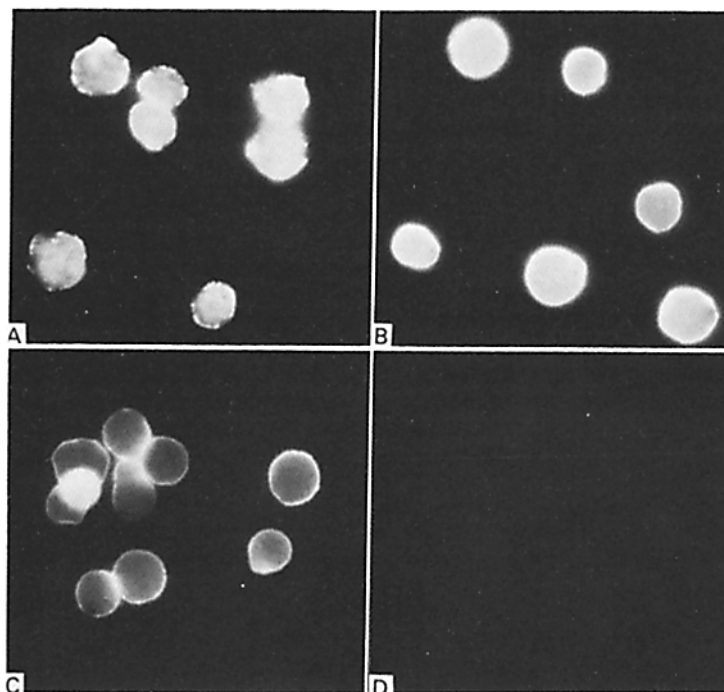


FIG. 1. FITC-anti-TNP staining of TNP-modified RDM-4 lymphoma cells. Tumor cells were TNP-modified by four different procedures, washed, stained with FITC-anti-TNP at 0°C, and fixed with paraformaldehyde. They were then photographed in the fluorescence microscope under FITC-specific illumination by using identical exposure times for all four types of cells. Subsequent photographic processing was likewise identical for all four cases. The TNP modification conditions, described in the text, are as follows: A, TSD; B, 10 mM TNBS; C, 1 mM TNBS; D, 0.1 mM TNBS.

in having as much accessible cell surface TNP as those treated with 1–10 mM TNBS. In comparisons of the stability of this cell surface TNP however, the TNP groups on TSD were less persistent. Although there was no detectable loss of TNP in either case after 1 h at 37°C, both TNBS- and TSD-treated cells had less TNP after 24 h than before culture. However, the TSD-treated cells appeared to have lost the majority of their accessible TNP groups (anti-TNP staining was still significantly above the control level). The TNBS-treated cells exhibited a more modest loss. After 48 h in culture the TSD-treated cells appeared the same as at 24 h, whereas the TNBS-treated cells stained more faintly than they did at 24 h, but were still somewhat brighter than the TSD cells after equal time in culture. RDM-4-TNP lymphoma cells modified by both techniques showed more rapid loss of accessible surface TNP groups than spleen cells and their stability was studied quantitatively as described below.

Quantitative Estimation of Cell Surface TNP Groups. A quantitative comparison of relative anti-TNP fluorescence intensity of these cells by using flow microfluorometry confirmed the qualitative impression from fluorescence microscopy. Fig. 2 shows the fluorescence intensity histograms for RDM-4-TNP cells made by modification with TSD, and 10, and 1 mM TNBS. Although this was a different experiment, the cells appeared microscopically similar to those shown

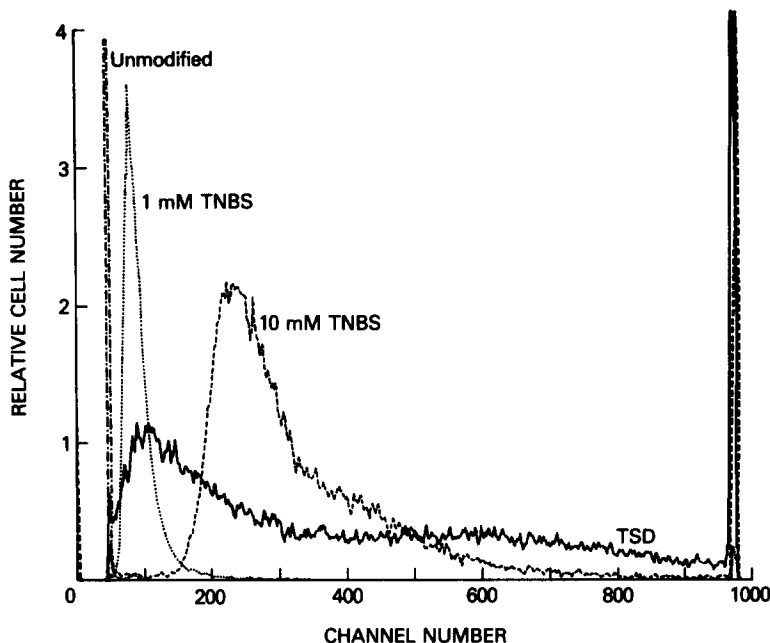


FIG. 2. Flow microfluorometry of TNP-RDM-4 cells stained with FITC-anti-TNP. Cells were modified and stained and fixed as in Fig. 1 except that in this experiment FITC F(ab')₂ anti-TNP antibody was used for staining. The histograms were run on the same gain. The channel number minus 40 is proportional to the fluorescence per cell. The cell populations are as follows. unmodified (· · · · ·); 1 mM TNBS (· · · · ·); 10 mM TNBS (- - -); TSD (—).

TABLE I
Quantitative FITC Anti-TNP Binding to TNP-Modified RDM-4 Cells as Measured by Flow Microfluorometry*

TNP modification	Mean fluorescence/cell (Channel no. -40)	Peak channel	Percent of cells above channel no. 900
TNBS, 10 mM	353.5	221	5.6
TNBS, 1 mM	107.7	76	1.3
TSD	384.1	88	11.3
None	6.3	45	<0.1

* Calculated from the FACS profiles shown in Fig. 2.

in Fig. 1, and were also fixed immediately after staining at 0°C. Fig. 2 shows that the distribution of TNP groups per cell was much more heterogeneous in cells treated with TSD than in cells treated with TNBS. However, as shown in Table I, the mean anti-TNP fluorescence per cell was greater for TSD-treated cells than for 10 mM TNBS-treated cells, although the peak value in the TSD cell distribution curve lies between the peak values of the distribution curves for 1 and 10 mM TNBS-treated cells. This was due to the presence of a substantial fraction (~30%) of the TSD-treated cells which were more intensely stained than the great majority (>90%) of the 10 mM TNBS-treated cells.

The stability of the cell surface TNP groups on RDM-4 cells modified by the

two different techniques was also examined over a 4-h culture period identical to that used in the CML assay (see below). The mean anti-TNP fluorescence per cell decreased to 85% of its original value for 1 mM TNBS-modified RDM-4 cells, and to 40% of its original value for TSD-modified RDM-4 cells. As can be seen from Table I this would still leave more average accessible TNP groups per cell on TSD-treated RDM-4 cells than on such cells treated with 1 mM TNBS. The possibility of enhanced TNP loss due to transfer of TSD from tumor target cells to cytotoxic effector cells during the CML assay incubations (see below) was assessed by mixing unlabeled spleen cells with TSD- and TNBS-treated RDM-4 cells at 10:1 and 40:1 ratios and incubating for 4 h. By using the scatter gating capacity of the FACS to examine the fluorescence of only the large cells in these mixtures, it was clear that the resulting histograms could be completely accounted for by assuming only the loss of TNP groups observed during culture of the tumor cells alone, plus the contribution of contaminating large unlabeled spleen cells.

Failure of TSD-Modified Cells to Serve as Sensitizing or Target Cells. To determine whether the TNP groups exposed on the cell surface by insertion of TSD into the lipid bilayer of cells would sensitize splenic lymphocytes to generate cytotoxic effectors or would serve as targets of cytotoxicity, B10.BR spleen cells were synthesized in vitro with either TNBS-modified or TSD-modified syngeneic spleen cells. The effector cells generated after 5 days of culture were assayed on ⁵¹Cr-labeled TNP-modified or TSD-modified RDM-4 target cells. The results, shown in Table II, indicate that sensitizing cells modified with TNBS at 10 or 1 mM stimulated the development of effector cells which lysed TNBS-modified target cells, but which did not lyse TSD-modified targets. Furthermore, when TSD-modified syngeneic sensitizing cells were used, no effector cells were generated which lysed either TNBS-modified or TSD-modified tumor target cells. The same result was verified in experiments by using the B10.A and C57BL/10 strains (data not shown). This result was also obtained when the cultures were restimulated with TSD-modified sensitizing cells 24 h after initiation of sensitization (line 5 of Table II). It was verified that TSD-modified target cells are lysable in an allogeneic sensitization, and did not detectably affect the interaction of effector cells with target cell alloantigens (line 7 of Table II).

To test cells bearing more stable surface TSD, TSD-modified spleen cells were also tested for their ability to be lysed by TSD- and TNBS-modified stimulating cells. As shown in the lower portion of Table II, such target cells were similar to TSD tumor cells in their lack of lysis by effector cells raised against TSD- or TNP-syngeneic spleen cells. Furthermore, as shown in the last line of Table II, modification of allogeneic stimulating cells with TSD had no detectable effect on the recognition of H-2^b alloantigens by B10.BR responding cells.

Comparison of Blocking of Cytotoxicity against TNP-Modified Targets with TNP-Modified and TSD-Modified Inhibiting Cells. Although TSD-modified cells were not immunogenic for the generation of a self-modified cytotoxic response, and were not lysable targets by effector cells generated in the TNBS-modified self CML (see Table II), the possibility existed that TSD-modified cells would specifically inhibit the lysis of TNBS-modified targets by effector cells generated against TNBS-modified syngeneic cells. To test the blocking ability of TNBS-modified and TSD-modified syngeneic cells, effectors generated by in

TABLE II
Comparison of TNP-Modified Cells as CML Stimulators and Spleen Targets

Sensitization		Percent specific lysis \pm SE on targets and at effector:target ratios shown:					
Stimulator	Responder						
Exp 1							
		RDM-4-TNBS, 10 mM (40:1)		RDM-4-TSD (40:1)		RDM-4 (40:1)	
-	B10.BR	[12.7]		[17.5]		[3.9]	
B10.BR-TNBS, 10 mM	B10.BR	42.6 \pm 2.0		-8.0 \pm 1.7		-5.5 \pm 1.6	
B10.BR-TNBS, 1 mM	B10.BR	39.5 \pm 3.1		-1.3 \pm 2.7		-4.5 \pm 2.0	
B10.BR-TSD	B10.BR	-8.0 \pm 1.9		-6.0 \pm 2.8		-3.7 \pm 1.7	
B10.BR-TSD (Restim) [‡]	B10.BR	-7.1 \pm 2.0		-9.2 \pm 1.9		-5.3 \pm 1.9	
		(4:1)		(4:1)		(4:1)	
-	B10	[1.9]		[0.6]		[-4.5]	
B10.BR	B10	26.9 \pm 0.7		44.3 \pm 3.8		48.1 \pm 3.4	
Exp 2							
		B10.BR Spleen-TNBS, 10 mM			B10.BR Spleen-TSD		
		(40:1)	(20:1)	(10:1)	(40:1)	(20:1)	(10:1)
-	B10.BR	[12.7]	[6.7]	[6.7]	[-4.0]	[-6.1]	[-6.1]
B10.BR-TNBS	B10.BR	23.6 \pm 4.7	25.2 \pm 2.1	18.9 \pm 4.3	-2.5 \pm 2.3	1.8 \pm 1.9	-1.9 \pm 1.5
B10.BR-TSD	B10.BR	-0.7 \pm 2.5	-6.0 \pm 1.4	-5.1 \pm 1.8	-1.2 \pm 1.6	0.5 \pm 1.6	-1.1 \pm 1.7
		EL-4					
		(4:1)	(2:1)	(1:1)			
-	B10.BR	[3.8]	[2.0]	[1.3]			
B10	B10.BR	80.6 \pm 4.1	68.7 \pm 3.5	43.3 \pm 2.6			
B10-TSD	B10.BR	81.4 \pm 4.3	65.9 \pm 4.3	46.6 \pm 4.0			

* Specific lysis was calculated by subtracting the lysis by unstimulated cultured cells (shown in brackets) from the total lysis. Spontaneous lysis of the target cells was 19.5, 24.4, and 20.9%, respectively.

[‡] Responder cells restimulated with 3×10^6 B10.BR-TSD spleen cells after 24 h of culture. Effector:target cell ratio = 20:1.

vitro sensitization against TNBS-modified syngeneic spleen cells were incubated for 30 min at 37°C with different numbers of unlabeled RDM-4 tumor cells, which were unmodified, modified with 10, 1, or 0.1 mM TNBS, or were modified with TSD. ⁵¹Cr-labeled RDM-4-TNP target cells were then added to the blocking effector cell mixture and centrifuged for 3 min at 400 rpm. The blocking effector target cell mixture was incubated for 3½ h for the ⁵¹Cr-release assay. The results of two independent experiments are summarized in Table III and Fig. 3. No inhibition of lysis was detected in the presence of 0.1 mM TNBS-modified RDM-4 or RDM-4-TSD blocking cells above that observed when the same number of unmodified RDM-4 cells were used. In the first experiment of Table III and in Fig. 3 some nonspecific inhibition of lysis was observed with the highest concentration of unmodified RDM-4 cells. Inhibition of lysis with 10 mM TNBS-modified blocking cells was consistently more effective than that observed with 1 mM TNBS-modified blocking cells. Thus, RDM-4 tumor cells modified with 10 or 1 mM TNBS inhibited the lysis of TNBS-modified target cells by the relevant effectors, whereas the TSD-modified RDM-4 cells which presented equivalent amounts of TNP on the cell surface did not inhibit lysis.

It is also noteworthy that when soluble TSD was allowed to interact with sensitized anti-self TNP effector cells for 30 min before the addition of TNBS-

TABLE III
Comparison of the Blocking of Cytotoxicity Against TNBS-Modified Targets with TNP-Modified and TSD-Modified Inhibiting Cells

Exp. no.	Responding and TNBS-modified stimulating cells	Blocking cells	Percent specific lysis at blocking:effector cell ratios of:		
			(4:1)	(1:1)	(1:4)
1	B10.A	RDM-4	10.5 ± 4.0	35.2 ± 4.0	47.3 ± 6.5
		RDM-4-TNBS, 10 mM	23.9 ± 3.5	8.1 ± 1.7	23.0 ± 2.6
		RDM-4-TNBS, 1 mM	32.3 ± 1.9	20.6 ± 3.3	30.1 ± 4.5
		RDM-4-TSD		46.4 ± 5.3	45.4 ± 5.7
2	B10.BR	RDM-4	45.9 ± 2.6	47.1 ± 4.1	51.6 ± 3.7
		RDM-4-TNBS, 10 mM	6.2 ± 0.7	12.5 ± 1.0	21.0 ± 1.6
		RDM-4-TNBS, 1 mM	24.7 ± 1.4	32.7 ± 1.5	34.2 ± 2.6
		RDM-4-TSD	39.0 ± 1.0	41.6 ± 1.6	45.9 ± 2.1

Number of effector cells per well was 2.5×10^5 . Effector:target cell ratio was 20:1.

Target cell was RDM-4-TNBS (10 mM).

Specific lysis in the absence of blocking cells was 38.6 ± 4.7 and 53.3 ± 1.2 for exps. 1 and 2, respectively.

treated tumor target cells, the presence of an excess of soluble TSD in the lytic assay did not affect the extent of lysis (data not shown). These results suggest that neither TNP presented on syngeneic cells via stearyl dextran nor TSD bound to the effector cells themselves are able to interact with high avidity with the receptor(s) involved in the *H-2*-associated recognition of TNBS-modified self by effectors generated against TNBS-modified syngeneic cells.

Discussion

Allogeneic cytotoxic effector cells, for which the response is directed against the natural *H-2* products, can be generated in a primary in vitro CML (10, 11). Similarly, haptens covalently linked to lymphocyte surface proteins appear to be capable of eliciting primary in vitro T-cell-mediated cytotoxic responses (1-5). In these examples, the specificity of the effector cells generated was directed against products of the *K* and *D* regions of the *H-2* complex (1, 2, 4, 5). The question can be raised whether covalent modification of cell surface proteins (possibly *H-2* products) with TNP is a requirement for the generation of a primary in vitro CML. The use of an amphipathic molecule such as TNP stearyl dextran, which can be inserted into the lipid bilayer of the cell membrane provides a system for investigating this question. Both the covalently-modified (TNBS-treated) cells and the TSD-modified cells (in which the hapten is not covalently linked to the cell surface proteins) provide equivalent amounts of the same TNP hapten exposed on the cell surface. Thus, in the present study it was demonstrated that the failure of TSD-modified cells to generate cytotoxic effectors, or to be recognized by the TNBS-modified self effectors is not due to a quantitative deficiency in the number of cell surface TNP groups accessible to

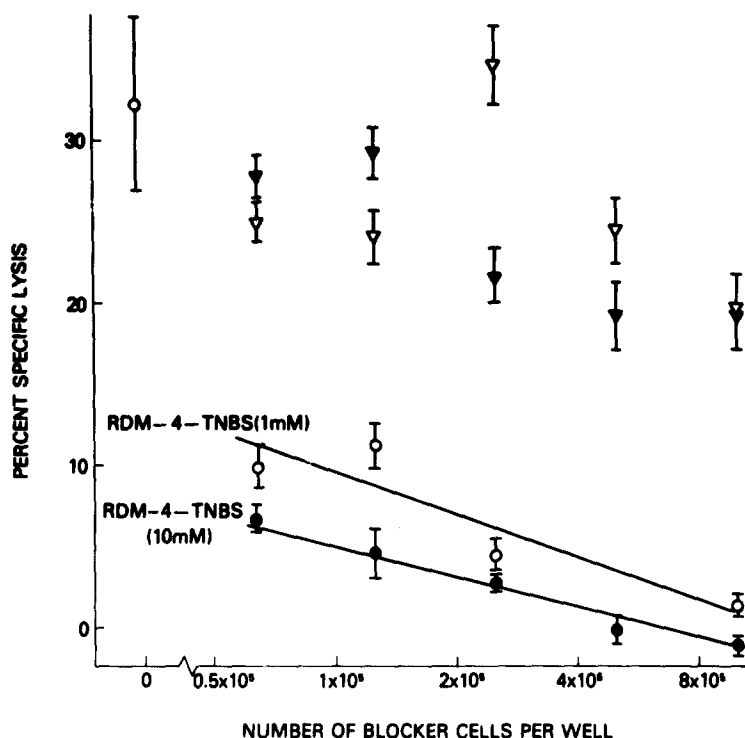


FIG. 3. Inhibition of the lysis of RDM-4-TNP-modified ^{51}Cr -labeled targets by B10.BR effectors by using TNBS- or TSD-modified, unlabeled RDM-4 blocking cells. The effector cells were generated by incubating B10.BR spleen cells with TNP-modified B10.BR cells for 5 days. Blocking cells were: RDM-4-TNBS (10 mM), (\bullet); RDM-4-TNBS (1 mM), (\circ); RDM-4-TNBS (0.1 mM), (\blacktriangledown); RDM-4-TSD (∇). The specific cytotoxic response without blocking was $32.7 \pm 6.1\%$. Nonspecific lysis by unsensitized cultured cells was 11.0%. Effector:target cell ratio = 20:1; target cells per well = 1.25×10^4 .

anti-TNP. These results imply that there may need to be a covalent linkage between the hapten and the proteins (possibly H-2 antigens) on the cell surface for efficient recognition to occur.

The loss of some of the TSD molecules from the spleen cells after 24 h of incubation could have prevented TSD-modified cells from sensitizing the cultures (perhaps even when the cultures were restimulated with TSD-modified cells, as in line 5 of Table II). We have shown, however, that the stability of cell surface TSD, although not as great as TNP self proteins, is still adequate to provide more than enough TNP groups for target cell recognition during the CML assay. We have furthermore sought to eliminate questions of the failure of this recognition being due to instability of TNP groups on TSD by using spleen cell targets having more stable TSD and by performing cold target blocking studies.

Two models have been proposed for the recognition of TNP self by effector T cells (12), differing in the number of independent receptors on the T-cell surface. In the first model, known as the altered self recognition model, the effector cells possess a single receptor recognizing self major histocompatibility complex

products which have been altered by the hapten (1, 12). The second, dual recognition model, proposes that the effector cells possess two separate receptors, one for hapten, and one for unaltered self MHC complex components (12). A number of experiments attempting to distinguish between these two alternatives have been performed (3-5, 12-14) and some of these suggest that the altered self model may be more likely, although definitive proof is still lacking. The present results are also more readily explained by the altered self model. If, as indicated above for the dual recognition model, two independent receptors exist on the effector cell surface, TSD-modified cells might be expected to be recognized by the TNBS self elicited effector cells, since we have shown that they possess equivalent TNP hapten and also MHC antigens recognizable by allogeneic effector cells (Table II, line 7). The failure of recognition in this case does not necessarily exclude the dual recognition model, since it could be argued that the TNP receptor has some steric requirements not met by cell surface TSD, or that the TNP receptor shows specificity for more than just the hapten and includes several neighboring amino acids (in addition to its other, MHC determinant receptor). The latter possibility appears plausible as suggested by the experiments of Janeway et al. (15). If neighboring amino acids for self antigens are included as part of the hapten and are recognized by the haptenic receptor, then it must be postulated that self components (even if they constitute only a few amino acids adjacent to the hapten) are involved in the specificity for both receptors of the dual recognition model. In such a model, TNP-altered self components would comprise one of the two receptors.

The experiments described in this report provide an extension of earlier investigations concerned with the fine specificity associated with the in vitro TNP-modified self CML (2-5, 16), and illustrate the versatility of this system for studying such issues. Similar to the interpretation from a previous report (3), the results are more compatible with a single than a dual receptor model, if it is assumed that the TNP group defines the hapten.

Summary

Mouse splenic lymphocytes and lymphoid tumor cells were modified with the trinitrophenyl (TNP) group either by treatment with trinitrobenzene sulfonate (TNBS) (which covalently modifies cell surface proteins) or with TNP stearoyl dextran (TSD) (which binds to the cell by noncovalent forces). These cell preparations were compared for their ability to: (a) sensitive syngeneic splenic lymphocytes leading to the generation of cytotoxic effector cells; (b) serve as lysable targets in a 4-h ^{51}Cr -release assay for effector cells generated in (a); and (c) act as blocking cells in the lysis of TNBS-modified targets lysed by TNP self effector cells generated in (a). In none of these three experimental systems did TSD-modified syngeneic spleen or *H-2*-matched tumor cells act either as a sensitizing immunogen or as a target antigen, despite the demonstration that quantitatively equivalent amounts of TNP were exposed on the cell surface in the TNBS- and TSD-modified cells. In contrast, TNBS-modified spleen cells sensitized syngeneic lymphocytes to generate effectors against TNBS-modified syngeneic targets. Furthermore, TNBS-modified, *H-2*-matched cells served as specific lysable targets and as inhibiting cells for such effectors. These results

indicate that the manner in which TNP is associated with the cell surface is important in the immunogenicity and antigenicity of hapten-modified syngeneic stimulating cells in generating *H-2*-associated cell-mediated lympholysis (CML) reactions. These findings raise the possibility that a covalent or at least a stable linkage with cell surface proteins (possibly *H-2*-controlled products) is important for immunological function. Furthermore, these observations do not favor the dual receptor model for *H-2*-restricted syngeneic CML if it is assumed in such a model that one receptor is specific for the TNP moiety and the second for unmodified self major histocompatibility products.

We thank Ms. Cindy Neels, Ms. Pam Gilheany, and Mr. Matthew Miller for expert technical assistance; Ms. Susan Sharrow for FACS experiments; and Mrs. Marilyn Schoenfelder for preparation of the manuscript.

Received for publication 28 February 1977.

References

1. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4:527.
2. Rehn, T. G., G. M. Shearer, H. S. Koren, and J. K. Inman. 1976. Cell-mediated lympholysis of *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)- β -alanylglycylglycyl-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. *J. Exp. Med.* 143:127.
3. Rehn, T. G., J. K. Inman, and G. M. Shearer. 1976. Cell-mediated lympholysis to *H-2*-matched target cells modified with a series of nitrophenyl compounds. *J. Exp. Med.* 144:1134.
4. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. 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. *J. Exp. Med.* 141:1348.
5. Forman, J. 1975. On the role of the *H-2* histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. *J. Exp. Med.* 142:403.
6. Okuyama, T., and K. Satake. 1960. On the preparation and properties of 2,4,6 trinitrophenyl-amino acids and -peptides. *J. Biochem. (Tokyo)*. 47:454.
- 6a. Wolf, D. E., J. E. Schlessinger, E. L. Elson, W. W. Webb, R. Blumenthal, and P. Henkart. 1977. Diffusion and patching of macromolecules on planar lipid bilayer membranes. *Biochemistry*. 16:3476.
7. Inman, J. K. 1975. Thymus-independent antigens: The preparation of covalent hapten-ficoll linkages. *J. Immunol.* 114:704.
8. Lapidot, Y., S. Rapport, and Y. Wolman. 1967. Use of esters of *N*-hydroxy-succinimide in the synthesis of *N*-acylamino acids. *J. Lipid Res.* 8:142.
9. Loken, M. R., and L. A. Herzenberg. 1975. Analysis of cell populations with a fluorescence activated cell sorter. *Ann. N.Y. Acad. Sci.* 254:163.
10. Häyry, P., and V. Defendi. 1970. Mixed lymphocyte cultures produce effector cells: An *in vitro* model for allograft rejection. *Science (Wash. D.C.)*. 168:133.
11. Wunderlich, J. R., and T. G. Canty. 1970. Cell-mediated immunity induced *in vitro*. *Nature (Lond.)*. 228:63.
12. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against

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altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature (Lond.)*. 251:547.

13. Pflizenmayer, K., A. Starzinski-Powitz, H. Rodt, M. Röllinghoff, and H. Wagner. 1976. Virus and trinitrophenol hapten-specific T-cell-mediated cytotoxicity against H-2 incompatible target cells. *J. Exp. Med.* 143:999.
14. von Boehmer, H., and W. Haas. 1976. Cytotoxic T lymphocytes recognize allogeneic tolerated TNP-conjugated cells. *Nature (Lond.)*. 261:141.
15. Janeway, C. A., Jr., P. H. Maurer, M. O. Dailey, and J. K. Inman. 1976. The specificity of cellular immune responses in guinea pigs. II. The structure of antigenic determinants leading to T-lymphocyte stimulation. *J. Exp. Med.* 144:1621.
16. Schmitt-Verhulst, A., D. Sachs, and G. M. Shearer. 1976. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Confirmation of genetic control of response to trinitrophenyl-modified H-2 antigens by the use of anti-H-2 and anti-Ia antibodies. *J. Exp. Med.* 143:211.