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

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Thymus-derived murine cytotoxic effector cells generated to viral or chemically modified autologous cells have been shown to selectively lyse target cells expressing the same *H-2* serological regions as the stimulator cells (1–11). In vivo, the cytotoxic effectors generated to lymphocytic choriomeningitis (LCM)[1] (2, 3, 5), ectromelia (4), or vaccinia (8) viral-infected autologous components were demonstrated to be specific only for target cells expressing in common with the stimulator cell at least one *H-2* serological region (2–5, 8) and infected with the same or a cross-reacting virus (8). In vitro, cell-mediated lympholysis (CML) reactions were generated by sensitization with trinitrophenyl (TNP)-modified autologous spleen cells (1, 6, 7). Splenic target cells were lysed by the effectors only if they were homologous with the stimulating cells at the *K* and/or *D* regions of *H-2*, and if they were modified with TNP (1, 6, 7, 9).

Since a number of viruses have now been shown to elicit cytotoxic reactions with this type of *H-2* and viral-associated specificity, it was of interest to determine whether other modifying agents known to possess haptenic functions in antibody production would stimulate cytotoxic effector cells with similar specificity requirements. The modifying agent *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycylglycyl-(N) was chosen for this study since Koren et al. have demonstrated that cytotoxic T-effector cells can be generated in vitro to N-modified, *H-2*-matched autologous spleen cells (11). The results presented in this report demonstrate that the cytotoxic effector cells generated in vitro by sensitization to N-modified autologous spleen cells are specific for N-modified target cells expressing K and/or D serological specificities identical to those of the

[1] *Abbreviations used in this paper:* ACK, ammonium chloride lysing solution; BSS, Hanks' balanced salt solution; CML, cell-mediated lympholysis; LCM, lymphocytic choriomeningitis; MHC, major histocompatibility complex; N, N-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanyl-glycyl-glycyl; PHA, phytohemagglutinin; TNP, trinitrophenyl.

modified stimulating cells. Similar to the observations made in the autologous TNP-modified model (6, 7, 9), the CML generated in certain strains were stronger to N-modified *K* than to N-modified *D*-region products, although the strain distribution patterns of preferential reactivity were different for the N and TNP models. Specificity for the modifying agent as well as for *H-2K*- and/or *H-2D*-region products was also demonstrated.

Materials and Methods

Mice. All mice used in these experiments were 7–9-wk-old males. The B10 congenic strains were obtained from The Jackson Laboratory, Bar Harbor, Maine. The B10.A(2R), B10.A(4R), and B10.A(5R) recombinant strains were obtained either from The Jackson Laboratory or were raised in our mouse colony from breeders obtained from The Jackson Laboratory or from Dr. Donald Shreffler, Department of Human Genetics, University of Michigan, Ann Arbor, Mich.

Tumor Cells. All tumors used in these experiments were serially passed at 7–9-day intervals in ascitic fluid. The tumors used and the strain in which each was passed were as follows: EL4 ($H\text{-}2^b$) in C57BL/6; P815 ($H\text{-}2^d$) in DBA/2; LSTRA ($H\text{-}2^d$) in BALB/c; RDM-4 ($H\text{-}2^k$) in AKR/J; LAF-17 ($H\text{-}2^a$) in B10.A. The cells were harvested 5–9 days after intraperitoneal injection and prepared as described below.

N Modification of Spleen and Tumor Cells for Sensitization and Targets. Tumor cells were obtained from the ascitic fluid of mice and filtered through nylon mesh to remove clumps. Fresh spleen cells were obtained from 7–9-wk-old male mice and suspended in Hanks' balanced salt solution (BSS) by gentle teasing with a blunt forceps. They were then aspirated through a syringe 3–4 times and filtered with nylon mesh. Erythrocytes were removed by suspension in ammonium chloride lysing solution (ACK) for 2 min. The lymphocytes were washed once in BSS and given a final wash in phosphate-buffered saline (PBS), pH 7.6. The stock reagent of N-azide was prepared as previously described (12, and footnote 2) and diluted to 0.04 M concentrations with dimethylformamide and stored at −50°C. For modification 15 μl of N azide, suspended in 0.25 ml PBS, pH 7.6, was added to the cell pellet which had previously been suspended in 1 ml PBS, pH 7.6. The cells were incubated for 20 min at 37°C in a shaking water bath. The cells were then washed two times in BSS with 10% fetal calf serum. Cells to be used as targets were labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) for 30 min before either N or TNP modification by incubation in a 37°C water bath.

TNP Modification of Spleen and Tumor Cells. Cell suspensions were prepared as described above except that the final wash after ACK lysing was in BSS. The cell pellet was then suspended in 2 ml of a phosphate-buffered solution of 10 mM 2,4,6-trinitrobenzene sulfonate (Pierce Chemical Co., Rockford, Ill.), pH 7.4, and incubated in a shaking water bath at 37°C for 10 min. The cell suspension was then washed twice in BSS supplemented with 10% fetal calf serum.

In Vitro Cell Culture and Assay for Cytotoxic Cells. Splenic lymphocytes were sensitized in vitro with N- or TNP-modified cells, as previously described for TNP (1). The culture medium was RPMI 1640 (Microbiological Associates, Bethesda, Md.) supplemented with 10% fetal calf serum (Microbiological Associates), 2 mM L-glutamine (National Institutes of Health Media Unit), 1 mM pyruvate (Microbiological Associates), 1 × nonessential amino acids (Microbiological Associates), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. After a 5-day incubation the cells were harvested and their cytotoxic activity measured as previously described (1). The mean and standard error of the percentage of specific cytotoxicity was calculated as described elsewhere (13). An effector target ratio of 40:1 was used except where noted. Nonspecific lysis of modified cells was determined from the amount of ^{51}Cr released from the appropriate ^{51}Cr-labeled target cells incubated for 4 h with effector cells which were incubated 5 days with no stimulator cells divided by the maximal ^{51}Cr release. Background lysis varied in these experiments from 15 to 50%.

Phytohemagglutinin (PHA) Stimulation of Spleen Cells. A fresh spleen cell suspension was obtained by teasing the spleens with a blunt forceps, aspirating the cells through a syringe, and

[2] Koren, H. S., J. R. Wunderlich, and J. K. Inman. 1975. T cell memory for the cytotoxic response to hapten-modified target cells. Submitted for publication.

then filtering the cells through nylon mesh. The cells were washed once in BSS before being suspended at a concentration of 5×10^6 per ml in RPMI 1640 culture media supplemented as described above plus 1 μg/ml of PHA (Burroughs Welcome & Co., Greenville, N. C.). After incubation for 2, 3, or 4 days, the cells were harvested immediately before use as targets.

Results

Target Cell Sensitivity to Effectors Generated by Sensitization with N-Modified Autologous Spleen Cells. Earlier studies of CML to TNP-modified autologous spleen cells indicated that appreciable lysis could be detected to TNP modified tumor or TNP-modified *H-2*-matched normal spleen cell targets at effector:target ratios of 20:1 (1, 6). However, at similar ratios, effector cells generated to N-modified syngeneic spleen cells were consistently detected using N-modified, *H-2*-matched tumor targets, but only occasionally when N-modified spleen cells were used as targets (11). To investigate the relative sensitivity of various N-modified lymphoid targets, C57BL/10 and B10.BR splenic lymphocytes were sensitized to N-modified autologous spleen cells. The effector cells generated were assayed on N-modified fresh spleen cells, 48–72-h PHA-stimulated N-modified spleen cells, or N-modified lymphoid tumor cells, syngeneic or allogeneic to the responding and stimulating lymphocytes. The results, shown in Table I, indicate that only modified targets homologous at *H-2K* and/or *H-2D* with the cells of the stimulating phase were lysed, and that N-modified tumor cells and PHA-stimulated N-modified spleen cells were more effective targets than unstimulated N-modified spleen cells. In all subsequent experiments, spleen cell targets for CML were PHA-stimulated.

Specificity of Effector Cells Requires both H-2 Homology and N Modification. To determine the specificity of effector cells generated by sensitization with N-modified autologous spleen cells, a series of unmodified and N-modified target cells syngeneic or congenic to the cells of the sensitizing phase were tested at different effector:target ratios for susceptibility to lysis by C57BL/10 effector cells. The results, shown in Table II, indicate that N modification is required for the lysis of both C57BL/10 autologous spleen cell targets and *H-2* matched tumor cell targets. The failure of C57BL/10 effector cells to lyse N-modified B10.D2 and P-815 target cells demonstrates a requirement for *H-2* homology between the targets and the cells of the sensitizing phase, as well as N modification.

Mapping of Effector Cell Specificity within the H-2 Complex. Previous reports have demonstrated that in vitro induction of CML to TNP-modified autologous spleen cells resulted in the generation of effector cells which were specific for TNP-modified spleen target cells sharing *K* and/or *D* end serological specificities with the TNP-modified stimulator cells (6, 7, 9). In the present study four C57BL/10 congenic mouse strains were used to determine whether similar *H-2* serological region homology is required to obtain lysis of N-modified, PHA-stimulated spleen targets. Splenic lymphocytes from B10.BR, B10.D2, B10.A, and C57BL/10 donors were sensitized to N-modified autologous spleen cells. The effector cells generated after 5 days of culture were tested for lysis of syngeneic, congenic, and allogeneic N-modified, PHA-stimulated spleen target cells. Results representative of these effector cell specificity experiments are summarized in Tables III–VI of this report. The first three columns of these tables indicate the strains of the responding, stimulating, and target cells, respec-

TABLE I

Induction of In Vitro Cytotoxicity by N-Modified Autologous Spleen Cells to N-Modified Spleen Cells, PHA-Stimulated Spleen Cells, and H-2-Matched Tumor Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
C57BL/10 (bbbbbb)	C57BL/10-N (bbbbbb)	B10-N (bbbbbb)	2.7 ± 1.6	All of *H-2*
		EL-4-N (bbbbbb)	27.2 ± 1.7	All of *H-2*
		C57BL/10-N′ (bbbbbb)	14.4 ± 1.5	All of *H-2*
		B10.A-N′ (kkkddd)	4.2 ± 2.3	None
		B10.BR-N′ (kkkkkk)	7.7 ± 2.3	None
		B10.D2-N′ (dddddd)	4.2 ± 1.1	None
B10.BR (kkkkkk)	B10.BR-N (kkkkkk)	B10.BR-N (kkkkkk)	14.5 ± 3.2	All of *H-2*
		RDM-4-N (kkkkkk)	26.0 ± 4.8	All of *H-2*
		B10.BR-N′ (kkkkkk)	22.2 ± 3.1	All of *H-2*
		B10.A-N′ (kkkddd)	12.9 ± 1.5	*K, I-A, I-B*
		C57BL/10-N′ (bbbbbb)	1.5 ± 2.0	None
		B10.D2-N′ (dddddd)	1.9 ± 3.0	None

Effector:target ratio, 20:1.
N′, PHA stimulated, N-modified spleen cells.

tively. The *H-2* alleles for the various regions of *H-2* are shown beneath each strain. The fourth column gives the mean percent of specific cytotoxicity with standard errors of the means. The last column indicates the *H-2* region common between the target cells and the responding and stimulating cells.

Representative data of CML experiments obtained using B10.BR effector cells generated by sensitization of splenic lymphocytes with B10.BR-N stimulating

TABLE II

Necessity of Both N Modification and H-2 Homology for Lysis of PHA-Stimulated Spleen Targets and Tumor Targets by C57BL/10 Effector Cells Sensitized by Autologous N-Modified Spleen Cells

Target cell	Specific lysis at effector:target ratio of:					Target cell *H-2* region common to responding and stimulating cell
	5:1	10:1	20:1	40:1	80:1	
			% ± *SE*			
C57BL/10 (bbbbbb)	(−)0.6 ± 2.0	2.2 ± 1.7	0.5 ± 1.4	(−)0.6 ± 2.1	1.5 ± 2.3	All of *H-2*
C57BL/10-N (bbbbbb)	0.1 ± 4.4	6.6 ± 2.7	15.4 ± 3.1	15.8 ± 2.7	24.0 ± 2.2	All of *H-2*
EL-4 (bbbbbb)	(−)1.1 ± 0.7	0.4 ± 0.4	(−)2.6 ± 0.8	(−)5.0 ± 0.9	(−)3.0 ± 1.0	All of *H-2*
EL-4-N (bbbbbb)	10.6 ± 0.8	16.7 ± 0.6	25.2 ± 1.5	31.4 ± 2.5	41.2 ± 1.9	All of *H-2*
B10.D2-N (dddddd)	3.5 ± 2.1	(−)2.4 ± 2.2	1.1 ± 2.2	2.6 ± 2.4	7.5 ± 1.2	None
P815-N (dddddd)	(−)0.5 ± 1.3	0.9 ± 0.6	0.1 ± 0.6	2.4 ± 0.2	4.0 ± 1.2	None

cells are summarized in Table III. Unmodified B10.BR target cells were not lysed by the effector cells, whereas N-modified syngeneic targets were lysed. N-modified B10.A and B10.A(2R) spleen cells, which share *K*, *I-A*, and *I-B H-2* regions with B10.BR were effective targets. The lysis of modified B10.A(4R) targets further localizes one area of homology to the *K* and *I-A* regions of *H-2*. The lower but significant lysis of the noncongenic C3H.OH-N targets by B10.BR effectors localizes a second region of *H-2* homology to the *D* region of the major histocompatibility complex (MHC). The above results contrast to the modified congenic B10.D2 and B10.A(5R) targets, which were not lysed and which share no known major regions of *H-2* homology with B10.BR responding and stimulating cells.

Effector cells generated from cultures of B10.D2 lymphocytes sensitized with B10.D2-N-modified spleen cells were also tested on a series of N-modified target cells. The results are shown in Table IV. N modification was required for lysis of *H-2*-matched L-1210 tumor cells. These effector cells lysed both B10.A-N and B10.A(5R)-N target cells, which share *I-C*, *S*, and *D* with B10.D2. This specificity was localized to the *D* half of *H-2* by the failure of these effectors to lyse B10.A(2R)-N targets. A region of *H-2* homology other than the *D* serological region is indicated by the lysis of the noncongenic C3H.OH-N targets, which share *K*, *I-A*, *I-B*, *I-C*, and *S* with B10.D2. Other congenic and noncongenic spleen cells which share no known *H-2* regions with B10.D2 were not effective targets.

Lymphocytes from the B10.A strain, which shares the *K*, *I-A*, and *I-B* regions with B10.BR and the *I-C*, *S*, and *D* regions of *H-2* with B10.D2, were also sensitized to N-modified autologous spleen cells and tested on a series of N-

TABLE III

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

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
B10.BR (kkkkkk)	B10.BR-N (kkkkkk)	B10.BR (kkkkkk)	2.1 ± 1.9	All of *H-2*
		B10.BR-N (kkkkkk)	23.1 ± 2.3	All of *H-2*
		B10.A-N (kkkddd)	16.2 ± 1.7	*K, I-A, I-B*
		B10.A(2R)-N (kkkddb)	24.2 ± 1.8	*K, I-A, I-B*
		B10.A(4R)-N (kkbbbb)	19.1 ± 3.0	*K, I-A*
		C3H.OH-N (dddddk)	11.6 ± 1.2	*D*
		B10.D2-N (dddddd)	(−)0.9 ± 1.3	None
		B10.A(5R) (bbbddd)	0.2 ± 3.9	None
		C57BL/10-N (bbbbbb)	1.1 ± 0.6	None

Effector:target ratio 40:1.

modified targets (see Table V). The B10.A effector cells lysed B10.A-N, B10.BR-N, B10.A(2R)-N, and B10.A(4R)-N to about the same extent. All of these targets share the *K* and *I-A* regions with B10.A. B10.D2-N, A.TL-N, and B10.A(5R)-N targets were also lysed by B10.A effectors, whereas C3H.OH-N targets were not. Finally, C57BL/10-N targets, which share no major *H-2* regions with B10.A, were not significantly lysed. Taken together, these results identify two distinct regions of homology between B10.A responder and/or modified stimulating cells and the modified targets, either region of which is necessary for appreciable lysis: one involving *K* or *K* plus *I-A* and a second including *D* or *D* plus *I-C* and/or *S*.

Sensitization of C57BL/10 splenic lymphocytes with N-modified autologous spleen cells generated effectors which lysed N-modified C57BL/10 and B10.A(5R)

TABLE IV
In Vitro Induction of Cytotoxicity of B10.D2 Spleen Cells to N-Modified Autologous Spleen Cells Assayed With N-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen PHA-Stimulated Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
B10.D2 (dddddd)	B10.D2-N (dddddd)	L1210 (dddddd)	−0.4 ± 1.4	All of *H-2*
		L1210-N (dddddd)	17.4 ± 1.0	All of *H-2*
		B10.D2-N (dddddd)	15.0 ± 4.6	All of *H-2*
		B10.A-N (kkkddd)	12.4 ± 1.2	*I-C, S, D*
		B10.A(5R)-N (bbbddd)	14.3 ± 2.3	*I-C, S, D*
		B10.A(2R)-N (kkkddb)	(−)2.2 ± 0.9	*I-C, S*
		C3H.OH-N (dddddk)	14.3 ± 2.3	*K, I-A, I-B, I-C, S*
		B10.A(4R)-N (kkbbbb)	3.4 ± 2.3	None
		C57BL/10-N (bbbbbb)	2.5 ± 1.5	None
		B10.BR-N (kkkkkk)	2.0 ± 1.5	None
		SJL-N (ssssss)	2.4 ± 2.2	None

Effector:target ratio 40:1.

targets (see Table VI). These targets respectively share all of *H-2* or *K*, *I-A*, and *I-B* with the cells of the sensitization phase. A lower but significant lysis was obtained using modified targets sharing either *I-B*, *S*, and *D* (B10.A[4R]) or *D* alone (B10.A[2R]) with C57BL/10. B10.D2-N and B10.BR-N spleen cell targets were not lysed by C57BL/10 effectors. These results indicate that either one of two major regions of MHC homology are also required between the modified target cells and the C57BL/10 responding and/or modified stimulating cells.

Differential Cytotoxic Reactivity of C57BL/10 Spleen Cells to K and D End Modified by TNP or N. In a previous study involving CML to TNP-modified

TABLE V

In Vitro Induction of Cytotoxicity of B10.A Spleen Cells to N-Modified Autologous Spleen Cells Assayed with N-Modified Syngenic, Congenic, Allogeneic, or Recombinant Spleen PHA-Stimulated Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
B10.A (kkkddd)	B10.A-N (kkkddd)	LAF-N (kkkddd)	19.7 ± 0.7	All of *H-2*
		B10.A (kkkddd)	0.5 ± 0.6	All of *H-2*
		B10.A-N (kkkddd)	19.1 ± 5.3	All of *H-2*
		B10.BR-N (kkkkkk)	14.9 ± 1.0	*K, I-A, I-B*
		B10.A(2R)-N (kkkddb)	12.4 ± 1.1	*K, I-A, I-B, I-C, S*
		B10.A(4R)-N (kkbbbb)	18.6 ± 1.5	*K, I-A*
		B10.D2-N (dddddd)	7.1 ± 1.2	*I-C, S, D*
		B10.A(5R)-N (bbbddd)	7.6 ± 1.6	*I-C, S, D*
		A.TL-N (skkkkd)	8.0 ± 1.0	*I-A, I-B, D*
		C3H.OH-N (dddddk)	4.5 ± 0.5	*I-C, S*
		C57BL/10-N (bbbbbb)	1.9 ± 1.1	None
		SJL-N (ssssss)	4.0 ± 1.7	None

Effector:target ratio 40:1.

autologous spleen cells, C57BL/10 responding lymphocytes generated effector cells with equal reactivity toward TNP-modified H-2K and TNP-modified H-2D serological region products (6). In contrast, it was consistently found that C57BL/10 responding lymphocytes generated effector cells which exhibited greater reactivity to N-modified *K*-end than to N-modified *D*-end products (see Table VI). Table VII compares the differential cytotoxic reactivity of C57BL/10 responding lymphocytes sensitized either with TNP- or N-modified autologous

TABLE VI

In Vitro Induction of Cytotoxicity of C57BL/10 Spleen Cells to N-Modified Autologous Spleen Cells Assayed with N-Modified Syngeneic, Congenic, Allogeneic, or Recombinant Spleen PHA-Stimulated Target Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
C57BL/10 (bbbbbb)	C57BL/10-N (bbbbbb)	C57BL/10 (bbbbbb)	0.1 ± 1.3	All of *H-2*
		C57BL/10-N (bbbbbb)	31.4 ± 3.2	All of *H-2*
		B10.A(5R)-N (bbbddd)	22.5 ± 2.5	*K, I-A, I-B*
		B10.A(4R)-N (kkbbbb)	13.7 ± 2.6	*I-B, I-C, S, D*
		B10.A(2R)-N (kkkddb)	10.1 ± 1.7	*D*
		B10.D2-N (dddddd)	6.5 ± 1.4	None
		B10.BR-N (kkkkkk)	4.4 ± 0.6	None

Effector:target ratio 40:1.

stimulating cells. These effector cells were assayed on target cells *H-2* matched with the stimulating cell at the *K* or *D* end and modified with the same reagent to which the effector cell precursor had been stimulated. These results verify that equivalent cytotoxic reactivity was obtained to TNP-modified *K*- or *D*-region products, whereas stronger lympholysis was detected to N-modified *K*-end products than to N-modified *D*-end products.

Specificity of Lympholysis as a Function of the Modifying Agent. To determine whether the modifying agent plays a significant role in the specificity of the cytotoxic reaction to modified autologous spleen cells, responding lymphocytes from three B10 congenic mouse strains were sensitized to autologous spleen cells modified either with TNP or N. The effector cells generated in each sensitization were assayed separately on *H-2*-matched tumor targets modified with either TNP or N. The results, summarized in Table VIII, demonstrate that effector cells generated by sensitization with autologous spleen cells modified with one of the reagents lyse only *H-2*-matched targets modified with the same reagent. Thus, no cross-reactivity was detected for CML generated against TNP-modified and N-modified autologous spleen cells.

Demonstration that K and/or D Homology is Required between N-Modified Target and N-Modified Stimulating Cells. In the preceding four tables H-2

TABLE VII

In Vitro Induction of Differential Cytotoxic Reactivity of C57BL/10 Spleen Cells to Chemically-Modified K and D Region Serological Products by Sensitization to either TNP- or N-Modified Autologous Spleen Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell *H-2* region common to responding and stimulating cells
			% ± *SE*	
C57BL/10 (bbbbbb)	C57BL/10-TNP (bbbbbb)	B10.A(5R)-TNP (bbbddd)	12.6 ± 2.3	*K, I-A, I-B*
		B10.A(4R)-TNP (kkbbbb)	12.2 ± 1.8	*I-B, I-C, S, D*
		B10.A(2R)-TNP (kkkddb)	15.7 ± 2.1	*D*
	C57BL/10-N (bbbbbb)	B10.A(5R)-N (bbbddd)	14.4 ± 2.2	*K, I-A, I-B*
		B10.A(4R)-N (kkbbbb)	2.8 ± 3.1	*I-B, I-C, S, D*
		B10.A(2R) (kkkddb)	6.6 ± 2.0	*D*

Effector:target ratio 40:1

homology at the *K* and/or *D* serological regions was always present among responding, modified stimulating, and modified target cells when significant lysis was obtained. None of the results described thus far discriminate among the possibilities that the intra-H-2 homology obtained is required: (*a*) between responding lymphocytes and modified stimulating cells; (*b*) between responding lymphocytes and modified target cells; (*c*) between modified stimulating cells and modified target cells; or (*d*) among all three of these cell types. To investigate this problem, (C57BL/10 × B10.BR)F_1 responding lymphocytes were sensitized with C57BL/10, B10.BR, or (C57BL/10 × B10.BR)F_1 N-modified stimulating cells. The effector cells generated were assayed on EL-4-N (*H-2*^{b}, matched with C57BL/10) and on RDM-4-N (*H-2*^{k}, matched with B10.BR) target cells. The results are shown in Table IX. Effector cells sensitized with modified stimulating cells from either parent lysed only modified target cells which were *H-2* matched with the modified stimulating cell of that parent. These data are compatible only with possibilities (*c*) or (*d*). In contrast to the data obtained with splenic lymphocytes from the parental B10 congenic strains, (C57BL/10 × B10.BR)F_1 responding lymphocytes sensitized with F_1-N stimulating cells generated effectors that resulted in the lysis of both N-modified *H-2*^{b} and *H-2*^{k} targets.

A similar experiment involving (B10.D2 × B10.BR)F_1 responding lymphocytes sensitized with B10.BR-N stimulating cells and assayed on RDM-4-N (*H-2* matched) and P-815-N (*H-2* unmatched with the stimulator) targets is shown in

TABLE VIII

Specificity of Cytotoxic Reactivity of B10 Congenic Spleen Cells as a Function of either TNP or N Modification of the Autologous Sensitizing Spleen Cells

Responding cells	Stimulating cells	Target cells	Specific Lysis
			% ± *SE*
C57BL/10	C57BL/10-TNP	EL-4-TNP	47.6 ± 1.5
		EL-4-N	(−)1.8 ± 1.1
	C57BL/10-N	EL-4-TNP	1.8 ± 2.1
		EL-4-N	26.1 ± 1.2
B10.BR	B10.BR-TNP	B10.BR-TNP	44.0 ± 2.8
		B10.BR-N	2.5 ± 1.7
	B10.BR-N	B10.BR-TNP	3.6 ± 1.5
		B10.BR-N	24.4 ± 1.7
B10.D2	B10.D2-TNP	LSTRA-TNP	13.5 ± 1.5
		LSTRA-N	(−)2.0 ± 1.8
	B10.D2-N	LSTRA-TNP	2.6 ± 1.0
		LSTRA-N	10.7 ± 2.0

Effector:target ratio 20:1

Table X. These data confirm the results of the previous experiment, since the effector cells generated lysed RDM-4-N but not P-815-N target cells. In addition, an attempt was made to block the lytic phases with non-^{51}Cr-labeled modified and unmodified RDM-4 and P-815 cells. An appreciable reduction in lympholysis was observed only when the effectors were preincubated for 30 min with the N-modified *H-2*-matched RDM-4 cells.

Discussion

The results presented in the first six tables of this report consistently demonstrated that the cytotoxic specificity of effector cells generated in a CML to N-modified autologous spleen cells involved both *H-2* serological region homology and N modification of both stimulating and target cells. By testing the effector cells on N-modified targets differing from the responding and stimulating cells within *H-2*, it was possible to identify two distinct *H-2* regions in each inbred strain toward which the specificity was directed.

The mapping studies of effector cell specificity to *N*-modified autologous cells presented here are similar to those reported earlier using TNP-modified autologous cells (6, 9). Effector cells from the B10.BR (Table III) and B10.A (Table V) strains sensitized to N-modified autologous spleen cells exhibited cytotoxic specificity to N-modified targets sharing *K* or *K* plus *I-A* with the cells of the stimulating phase. A significant but lower level of reactivity was generated to N-modified targets sharing *H-2D* in the case of B10.BR and to N-modified targets sharing alleles in the *D* half of *H-2* in the case of B10.A. It is interesting that reactivity to N-modified targets sharing *K* end was greater than that to N-modified targets sharing *D* end. This pattern of preferential reactivity to *K* end is similar to that observed for CML in these two strains to TNP-modified autologous stimulating cells (6, 9).

TABLE IX

In Vitro Induction of Cytotoxicity of (C57BL/10 × B10.BR)F_1 Spleen Cells by C57BL/10, B10.BR, or (C57BL/10 × B10.BR)F_1 N-Modified Spleen Cells Assayed with N-Modified Tumor Cells

Responding cells	Stimulating cells	Target cells	Specific lysis	Target cell homology with:	
				Responding cells	Stimulating cells
			% ± *SE*		
(C57BL/10 × B10.BR)F_1 $\left(\frac{bbbbbb}{kkkkkk}\right)$	C57BL/10-N (bbbbbb)	EL-4-N (bbbbbb)	39.1 ± 2.2	Yes	Yes
		RDM-4-N (kkkkkk)	4.0 ± 2.0	Yes	No
	B10.BR-N (kkkkkk)	RDM-4-N (kkkkkk)	21.9 ± 3.7	Yes	Yes
		EL-4-N (bbbbbb)	8.0 ± 1.0	Yes	No
	(C57BL/10 × B10.BR)F_1-N $\left(\frac{kkkkkk}{bbbbbb}\right)$	EL-4-N (bbbbbb)	18.7 ± 1.3	Yes	Yes
		RDM-4-N (kkkkkk)	14.2 ± 1.6	Yes	Yes

Effector:target ratio 20:1

B10.D2 effector cells were demonstrated to exhibit significant cytotoxicity toward N-modified targets sharing one or two *H-2* regions with the responding or stimulating cells (Table IV). As shown by the use of N-modified recombinant congenic strains, the regions probably involved *D* alone and some regions not including *D*, presumably the *K* serological region as shown by the C3H.OH targets. Again, these B10.D2 data quantitatively resemble those obtained with TNP-modified autologous spleen cells in that equal cytotoxic reactivity was detected toward *K* or *D*-matched N-modified targets. The level of cytotoxicity of B10.D2 effector cells was lower than that generated by B10.BR and B10.A to their respective N-modified targets, as was seen in the TNP system (6, 9).

Responder lymphocytes from C57BL/10 mice generated effector cells which lysed N-modified targets homologous with the cells of the stimulating phase at *K*, *I-A*, and *I-B* or at the *D* serological region (Table VI). However, in contrast to the TNP-autologous CML in which C57BL/10 effector cells lysed equally well TNP-modified target cells matched at the *K* or *D* end with the cells of the sensitization phase (upper part of Table VII), the N-autologous CML in this strain lysed N-modified targets matched at the *K* end greater than N-modified targets matched at the *D* end (lower part of Table VII). These differential patterns of reactivity could be due to differences in immunogenicity of the antigenic specificities associated with the *K* and *D* ends and/or to *H-2*-linked immune response genes controlling reactivity to these specificities. The data presented in this study do not distinguish between these possibilities. A recently

TABLE X

In Vitro Induction of Cytotoxicity of (B10.D2 × B10.BR)F_1 Spleen Cells by N-Modified B10.BR Spleen Cells Assayed with N-Modified Tumor Targets: Blocking of Effector Cells with H-2-Matched Nonradioactive Tumor Cells

Responding cells	Stimulating cells	Blocking cells	Target cells	Specific lysis	Reduction in cytotoxicity*
				% ± *SE*	%
(B10.D2 × B10.BR)F_1 $\left(\frac{ddddddd}{kkkkkk}\right)$	B10.BR-N (kkkkkk)	—	P815-N (ddddddd)	2.5 ± 0.9	—
		—	RDM-4-N (kkkkkk)	30.4 ± 2.2	—
		P815 (ddddddd)	RDM-4-N (kkkkkk)	27.9 ± 2.0	—
		P815-N (ddddddd)	RDM-4-N (kkkkkk)	29.3 ± 1.4	(−)5.0
		RDM-4 (kkkkkk)	RDM-4-N (kkkkkk)	23.6 ± 2.1	—
		RDM-4-N (kkkkkk)	RDM-4-N (kkkkkk)	13.6 ± 1.0	42.4

Effector:target ratio 40:1.
Blocking cell:target ratio 20:1.
* Percent reduction in cytotoxicity equals percent specific lysis in presence of unmodified blocking cells minus percent specific lysis in presence of modified blocking cells divided by percent specific lysis in presence of unmodified blocking cells.

published report indicates that differential reactivities to TNP-modified *K* and *D* region products involves both immune response genes acting at the level of the responder lymphocytes and differential immunogenicity at the level of the stimulating cell (7). Studies using F_1 and recombinant animals are currently in progress to investigate these possibilities in the N system.

Since it was necessary to modify the target cells to obtain lysis by effector cells generated by chemically-modified autologous spleen cells (1, 6, 7, 9, 11 and Tables II–VI), the specificity requirements of the N and TNP modifying agents were investigated. The results of Table VIII demonstrate that effector cells generated by sensitization with TNP-modified autologous cells do not lyse N-modified syngeneic targets and vice versa. These findings are compatible with one of three possibilities: (*a*) that "hapten specificity" is involved and that *H-2* homology is required between effector and target cells by cell-to-cell interaction requiring *H-2* identity possibly similar to that reported by Katz et al. for antibody production (14); (*b*) that these modifying agents induce distinct and specific conformational changes in the structure of *H-2* serological region products, but

do not themselves compose part of the antigenic structure; or (*c*) that TNP and N constitute integral components of a new antigen created by the modification that involves altered *H-2* serological region products.

The possibility of a major "hapten-specific" CML with intra H-2 identity required for effector-target cell interaction can be excluded by the F_1 data presented in Tables IX and X. If possibility (*a*) were correct, then F_1 effector cells generated by sensitization with N-modified cells from either of the parents should have lysed N-modified targets from both parents. These findings demonstrate that intra-*H-2* homology is required between N-modified stimulator and N-modified target cells, or among responding, stimulating, and target cells. This experimental design and the conclusions drawn are similar to other examples of CML to viral (3, 4, 8)- and chemically (6, 9)-modified autologous cell models.

While possibility (*b*) cannot be evaluated by data presented in this report, the observations that TNP- and N-modified chicken erythrocytes block effector cell lysis of the appropriate TNP- or N-modified target cells, respectively (15, and Koren, unpublished observations) suggest that these moieties may be integral components of new antigens created by modification of H-2 products. However, the demonstration that unmodified chicken red blood cells will not block while chemically-modified chicken red blood cells do block does not prove specificity. The blocking could be due to a nonspecific toxic effect of the modified chicken red blood cells. For instance, it must be shown that TNP chicken red blood cells do not block other cytolytic reactions such as an allogeneic CML or preferably a CML model more similar to the N system. Koren (manuscript in preparation) has found such controls difficult to obtain reproducibly. In contrast to modified chicken red blood cell blocking, inhibition with chemically-modified spleen cells occurs only when there is *H-2* homology among stimulating, target, and blocking cells (6, and Table X). It is not understood at the present time why *H-2* homology is required for blocking of lysis by modified spleen cells, whereas no such homology appears to be necessary for blocking by modified chicken erythrocytes.

The data available in the N modification system as well as other systems (3, 4, 6, 8, 9, 16) are compatible with the hypothesis that certain viral and chemical agents can modify *H-2*-related cell surface components (possibly serological region products) to create new antigenic determinants. These "neo-self antigens" appear to include as part of the viral (3, 4, 6, 8, 16) or chemical (6, 9) recognition structure the respective modifying agent.

The (C57BL/10 × B10.BR)F_1 responding lymphocytes sensitized to N-modified autologous F_1 cells generated a broader range of effector cell specificities than would either of the homologous parents (Tables IX, III, VI). If an expanded range of cytotoxic specificities were advantageous for survival, as might be expected in a viral model (17), then a selective pressure for polymorphism in the MHC would exist (6, 17). Such a pressure could be exerted by functionally distinct H-2 products at the level of formation of the new antigen (modified serological region products) and/or at the level of response potential to that antigen (immune response genes).

Summary

Splenic lymphocytes from four C57BL/10 congenic mouse strains were sensitized in vitro to *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycylglycyl-(N) modified autologous lymphocytes. The effector cells generated after 5 days of culture were assayed on a series of either N-modified phytohemagglutinin-stimulated spleen cells or N-modified tumor cells. The results indicated in all cases that both N modification of the targets and *H-2* homology between the modified stimulating and target cells are required for lysis to occur. In each case the effector cells were found to lyse N-modified target cells only when there was homology at either or both ends of the major histocompatibility complex (MHC) between the stimulator and target cells. B10.BR lysed targets sharing alleles at *K* (or *K* plus *I-A*) and/or at *D*. B10.A effector cell specificity was mapped to *K* (or *K* plus *I-A*) and/or the *D* half of the MHC (*D* or *D* plus *I-C* and/or *S*). The two regions of specificity determined for B10.D2 effector cells were *D* (or *D* plus *S* plus *I-C*) and a region not including *D* of the MHC. C57BL/10 effector cells lysed N-modified targets only if there was target cell *H-2* homology at *K*, *I-A*, and *I-B* or at the *D* serological region. As in the trinitrophenyl (TNP) system (6) B10.BR and B10.A effector cells lysed targets sharing *K* end *H-2* serological regions greater than target cells sharing *D*-end serological regions. The C57BL/10 effector cells were shown to react to the *K* end greater than the *D* end, which differed from the equal reactivity seen in the TNP system for this strain. The data are consistent with the hypothesis that the antigen recognized by the effector cell includes an altered H-2 serological cell surface product. That the reaction is not "hapten specific" and the *H-2* homology is required only for effector:target cell interaction was excluded by the use of two F_1 combinations in which lysis of only N-modified target cells sharing the *H-2* haplotype with the stimulating parental strain was obtained. Finally, it was demonstrated that N and TNP modification create distinct new antigenic determinants, since an effector cell sensitized to one modifying agent will lyse only *H-2*-matched target modified with that same modifying agent.

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Bibliography

1. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4:527.
2. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.)* 248:701.
3. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature (Lond.)* 251:547.
4. Gardner, I. D., N. A. Bowern, and R. V. Blanden. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the H-2 gene complex. *Eur. J. Immunol.* 5:122.

5. Doherty, P. C., and R. M. Zinkernagel. 1975. H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* **141**:502.
6. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. *J. Exp. Med.* **141**:1348.
7. Schmitt-Verhulst, A., and G. M. Shearer. 1975. Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes. *J. Exp. Med.* **142**:914.
8. Koszinowski, V., and H. Ertl. 1975. Lysis mediated by T cells and restricted by H-2 antigen of target cells infected with vaccinia virus. *Nature (Lond.)* **255**:552.
9. 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.
10. Gardner, I., N. A. Bowern, and R. V. Blanden. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. *Eur. J. Immunol.* **4**:63.
11. Koren, H. S., J. R. Wunderlich, and J. K. Inman. 1975. In vitro development of cytotoxic T cells specific for hapten-associated antigens on syngeneic cells. *Transpl. Proc.* **3**:169.
12. Inman, J. K., B. Merchant, L. Claflin, and S. E. Tracey. 1973. Coupling of large haptens to proteins and cell surfaces: preparation of stable, optimally sensitized erythrocytes for hapten-specific hemolytic plaque assays. *Immunochemistry.* **10**:165.
13. Martin, W. J., J. R. Wunderlich, F. Fletcher, and J. K. Inman. 1971. Enhanced immunogenicity of chemically coated syngeneic tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* **68**:469.
14. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral responses to hapten protein conjugates. *J. Exp. Med.* **137**:1405.
15. Dennert, G., and L. E. Hatlen. 1975. Induction and properties of cytotoxic T cells specific for hapten-coupled tumor cells. *J. Immunol.* **114**:1705.
16. Doherty, P. C., and R. M. Zinkernagel. 1974. T cell mediated immunopathology in viral infections. *Transplant. Rev.* **19**:89.
17. Doherty, P. C., and R. M. Zinkernagel. 1975. A biological role for the major histocompatibility antigens. *Lancet.* **1**:1406.