T CELLS SPECIFIC FOR HAPTEN-MODIFIED SELF ARE PRECOMMITTED FOR SELF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS BEFORE ENCOUNTER WITH THE HAPTEN*

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One of the most striking developments in immunology in recent years has been the emergence of the concept that thymus-derived lymphocytes (T cells) recognize foreign, non-major histocompatibility complex (MHC) antigens in association with self MHC antigens. Several models have been proposed to account for these findings (1–5). There are basically two types of theories. One states that T cells have a single receptor with which they recognize a modification of self MHC antigens induced by the foreign antigen ("altered self"). The other states that T cells have two receptors or receptor sites, which must act in concert in order that activation occur, one receptor being specific for self MHC antigens and the other specific for the foreign antigen. At present, it is not possible to discriminate between these two possibilities. Indeed, inasmuch as most studies of MHC restriction have involved primed cells performing some effector function, it is not yet possible to state whether MHC restriction exists in T cells before their encounter with antigen, or whether it follows upon antigenic stimulation.

One approach to the problem of MHC restriction that has proven to be very valuable is T-cell cytotoxicity to 2,4,6-trinitrophenyl (TNP)-modified self target cells (5). In this model, it has been shown that cells from F_1 mice, immunized with TNP-modified parental cells, preferentially kill TNP-modified targets derived from the immunizing parental strain, as compared with TNP-modified targets from the other parent. The present experiments were initially undertaken to answer the question of whether F_1 mice contain a single set of precursors for TNP-reactive killer cells, which become committed to killing TNP-modified cells of one parent only after immunization, or, alternatively, have two sets of precursors, each already committed to recognize TNP-modified cells of one or the other parental MHC type. To study the state of commitment

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of the cells before immunization, the technique of antigen-driven, 5-bromo-deoxyuridine (BUdR) and light suicide was used (6–8). By this means, F_1 cells responding to TNP-modified parental cells of one type could be eliminated, and the residual cells restimulated to see whether they retained the ability to generate cytotoxic effectors specific for TNP-modified cells from the other parental strain. These experiments gave results consistent with the recent studies of Zinkernagel et al. (9, 10) and of Bevan (11) in showing that F_1 T cells are precommitted to recognize TNP coupled to cells of one or the other parental strain.

In carrying out these experiments, two other questions could readily be asked. The first involved the nature of the precursor of killer T cells capable of killing TNP-modified self cells, but which are induced by stimulation with allogeneic cells (5, 12). Such cells have previously been shown to differ from TNP-self killers induced by TNP-self stimulating cells in their specificity, a result we have confirmed. We can also state that such cells arise from different precursors than cells responding to TNP-self stimulators, because they are not affected by BUdR and light suicide with TNP-parental stimulators, under conditions where the response to the TNP-parental stimulator is totally eliminated.

The second question involved testing whether cells acutely depleted of alloreactive cells by BUdR and light suicide could respond to TNP-modified allogeneic cells of the type used to induce suicide. Although several authors have found such responses with acute depletion techniques (13, 14), our results strongly suggest that precursors for TNP-modified allogeneic cells are rare in normal mice. This result is again consistent with the elegant studies on virus-specific cytotoxic cells recently reported by Zinkernagel et al. (9, 10).

Materials and Methods

Mice.   Mice of the strains CBA/H, A.CA, and (CBA/H × A.CA)F_1 were bred at the University of Uppsala (Uppsala, Sweden). Mice of the strains CBA/J, BALB/c, C57BL/6J (B6), DBA/2, A.SW, AKR/J, and (AKR × DBA/2)F_1 were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice of the strains A.TH, A.TL, and (A.TH × A.TL)F_1 were bred at Yale University (New Haven, Conn.). These mice were a generous gift of Dr. R. K. Gershon. The MHC haplotypes of these strains are given in Table I.

In Vitro Cultures.   Spleen cell suspensions were prepared in Dulbecco's modified phosphate-buffered saline (PBS-D) by flushing them from the spleen with PBS-D with a syringe and needle. The red cells were lysed by brief (3 s) hypotonic shock, and the cells were then washed and counted in a hemacytometer; greater than 95% were viable by trypan blue dye exclusion. They were cultured in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) to which was added 10 ml 200 mM L-glutamine, 10 ml 1 M Hepes, 10 ml 7.5% $NaHCO_3$, 20 ml penicillin-streptomycin, 0.5 ml 0.1 M 2-mercaptoethanol, and 50 ml heat-inactivated fetal calf serum per liter. 30×10^6 responder cells were mixed with 30×10^6 allogeneic or 45×10^6 TNP-modified stimulator cells, which had been inactivated with 2,000 rads X-irradiation, in a volume of 14 ml medium in upright 50-ml tissue culture flasks (Falcon 3013, Falcon Plastics, Div. BioQuest, Oxnard, Calif.). The cultures were carried out in an atmosphere of 5% CO_2 in air at 37°C. These conditions were found to be optimal for these responses, and reproducibly led to nearly complete suicide of precursors. TNP modification of stimulator cells was performed as described by Shearer (5).

BUdR and Light Suicide.   Preliminary experiments showed that the timing of addition of BUdR and time of lighting were critical for complete suicide (Table II). From this, we have evolved the following procedure, based on experience and subsequent experiments (8): 27 h after initiating the cultures, freshly prepared BUdR (ICN Pharmaceuticals, Cleveland, Ohio) is added to the cells and mixed to achieve a final concentration of 3×10^{-6} M. After 69 h of culture, the

TABLE I

MHC Haplotypes of the Mice Used in These Experiments

Strain	Haplotype of origin of MHC regions			
	K	I	S	D
A.CA	f	f	f	f
CBA/H, CBA/J, AKR/J	k	k	k	k
DBA/2, BALB/c	d	d	d	d
C57Bl/6J (B6)	b	b	b	b
A.SW	s	s	s	s
A.TH	s	s	s	d
A.TL	s	k	k	d

TABLE II

Timing of BUdR and Light Is Critical in Suiciding TNP-Self Killer Cells

Time of adding BUdR (3×10^{-6} M)	% Specific ^{51}Cr release from TNP-CBA/H targets of cultures lit		
	48 h	72 h	96 h
h			
24	19	−5	26
48	−	2	23
72	−	−	27

CBA/H spleen cells were stimulated with TNP-CBA/H cells for 5 days and then assayed for cytotoxic activity on CBA/H and TNP-CBA/H targets as an effector: target ratio of 20:1; no release was seen on CBA/H targets. Untreated cultures gave 46% specific release on TNP-CBA/H targets. BUdR was added to various cultures at the times stated, and the cultures were exposed to light for 90 min at the stated times. Underlined value gives the greatest suicide.

flasks are removed from the incubator, the caps closed to prevent pH change, and the flasks placed upright over a fluorescent tube, separated from it by the thin portion of a Pasteur pipette, taking care not to disturb the cells. Lighting is for 90 min. The caps are then loosened, and the flasks returned to the incubator. After another 24 h, the cells are placed over the light a second time. They are then recovered from the flasks, pooled, washed three times, counted, and recultured with fresh stimulator cells. As a positive control, fresh cells are also cultured at this time. Finally, to control for suppressive effects, suicided cells are mixed 1:1 with fresh, syngeneic cells and cultured with the suiciding stimulator (or related stimulators). After a further 4 days in culture, the cells are recovered from the flasks, washed once, and assayed for cytotoxic activity on a variety of targets.

Cytotoxicity Assay and Calculation of Data. The cytotoxicity assay is a modification of that described by Simpson et al. (15). Targets are either spleen cells or peritoneal exudate cells induced by injection of 3 ml of 3% thioglycolate broth 3 days before use. Attackers are adjusted to identical counts of viable cells before assay. Some titrations are carried out, but as these only confirm the findings with undiluted cells, they have not been reported. The medium we use is RPMI-1640 with 10 mM Hepes, 0.075% NaHCO$_3$, and 5% heat-inactivated fetal calf serum. The assays are carried out in "V bottom" microtiter trays. Half the supernate is recovered 3.5 h later after centrifuging the plates and counted in a gamma counter, (LKB ultrogamma II, LKB Instruments, Inc., Rockville, Md.). The percent-specific ^{51}Cr release is calculated using the formula:

% Specific ^{51}Cr release

$$= \frac{\text{experimental counts} - \text{control (medium or normal cells) count}}{\text{total counts} - \text{control counts}}$$
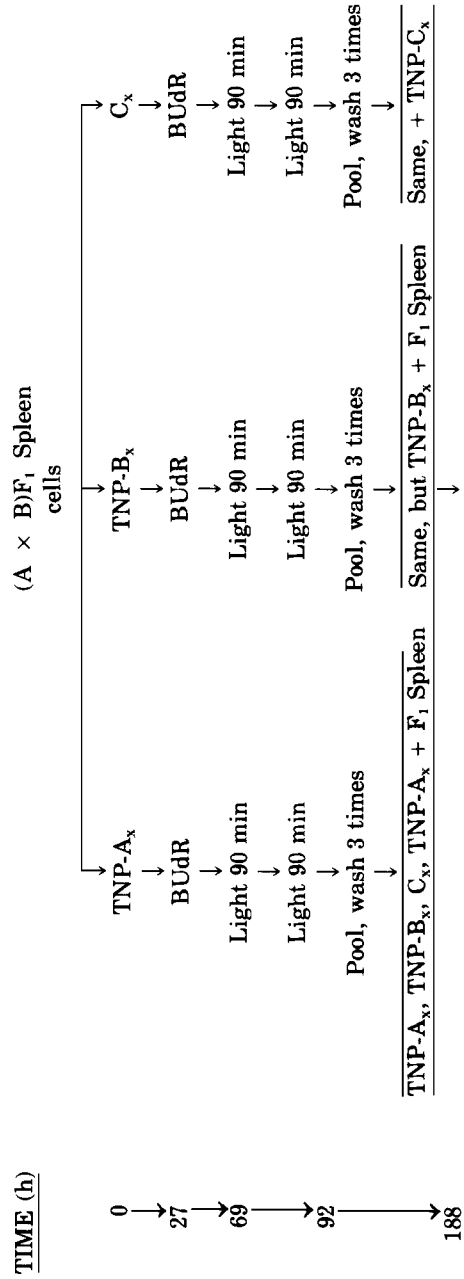
Total counts are obtained from samples lysed under identical conditions with 1% Triton X-100, and spontaneous release values of 20–30% are usual. Standard errors, which are not reported, are generally <1–2%.

Results

The general scheme of the experiments is outlined in Fig. 1. F_1 spleen cells are stimulated either with TNP-modified parental or allogeneic cells, and cells responding to the initial stimulus are suicided with BUdR and light. This treatment removes both cytotoxic effector cells (Table II) and their precursors (Tables III–VII). The remaining cells are then cultured with various stimulator cells, and each group is assayed for cytotoxicity on a variety of target cell types. Each table in this paper represents results of a single experiment. To simplify the presentation of the results, each line is numbered, and the experiments are discussed according to the questions being asked, rather than one experiment at a time. Responses to unmodified, syngeneic, or parental targets were generally less than background lysis, and have been omitted. On rare occasions, lysis of unmodified targets was significantly above background, in which case they were subtracted from the release obtained with TNP-modified targets of the same type. This response to unmodified self or parental cells is probably analogous to that reported by Peck et al. (16), and was seen only with freshly cultured cells when stimulated by allogeneic cells. Its absence in all the groups in which precultured (and suicided) cells were used probably means that cells killing unmodified targets are eliminated in the primary, suiciding cultures.

The Specificity of Cytotoxic Effector Cells for TNP-Modified Targets. As has been shown by Shearer et al. (5), spleen cells stimulated with TNP-autologous cells will kill preferentially TNP-modified targets identical at the K and D loci of the MHC to the original stimulator and responder cell combination. This is seen throughout these experiments (Tables II–IX). Furthermore, F_1 cells stimulated with TNP-modified parental cells lyse TNP-modified targets of the original immunizing parental strain preferentially, with two exceptions (Table VI, line 36; Table VII). The former exception was found only in this one experiment and is unexplained, whereas the latter demonstrates that when the parental strains are identical at the K and D loci, even if they differ throughout the I region of the MHC, then killers induced by TNP-modified cells of either parental strain will kill TNP-modified targets of either parental type. This finding is consistent with Shearer's previous results, and strongly suggests that killing directed at TNP-modified I region determinants does not play a significant role in our experiments.

The experiment reported in Table VIII demonstrates that although T cells stimulated with TNP-self cells will kill TNP-self cells, they will not kill allogeneic targets, and kill only poorly on TNP-modified allogeneic targets. By contrast, cells stimulated with allogeneic cells kill both allogeneic targets of the stimulating type and TNP-modified targets that are syngeneic to the responder cells. Table IX demonstrates that the specificity of these two types of T killer cell, as revealed by cold-target inhibition studies, is also different. Thus, although killing of TNP-self targets by TNP-self-activated cells is not inhibited by cold allogeneic targets, even if six independent haplotypes are mixed, killing

TIME (h)

$0 \rightarrow 27 \rightarrow 69 \longrightarrow 92 \longrightarrow 188$

$(A \times B)F_1$ Spleen cells

TNP-A_x TNP-B_x C_x

BUdR   BUdR   BUdR

Light 90 min   Light 90 min   Light 90 min

Light 90 min   Light 90 min   Light 90 min

Pool, wash 3 times   Pool, wash 3 times   Pool, wash 3 times

TNP-A_x, TNP-B_x, C_x, TNP-A_x + F_1 Spleen   Same, but TNP-B_x + F_1 Spleen   Same, + TNP-C_x

CML assay of each group on: A, TNP-A, B, TNP-B, C, TNP-C, 3.5-h assay

FIG. 1.   Flow diagram for BUdR and light suicide.

TABLE III

BUdR and Light Suicide Can Selectively Eliminate Cells Giving Rise to Cytotoxicity*

Line	Stimulating cells		% specific ^{51}Cr release on targets		
	First (suiciding)	Second	TNP-CBA	DBA/2	TNP-DBA/2
1	Fresh	TNP-CBA	33.4	0.4	5.6
2	TNP-CBA	TNP-CBA	0.3	1.7	−1.7
3	TNP-A.CA	TNP-CBA	17.2	2.8	2.1
4	DBA/2	TNP-CBA	15.6	0.2	2.6
5	Fresh	DBA/2	9.0	10.7	19.4
6	DBA/2	DBA/2	8.5	3.2	7.9
7	TNP-CBA	DBA/2	10.0	16.9	18.2
8	TNP-A.CA	DBA/2	21.1	26.1	27.2
9	DBA/2	TNP-DBA/2	5.7	1.6	9.1

* Responder cells are (CBA × A.CA)F_1.

Effector to target ratio is 15:1; no significant release seen with CBA targets. "Fresh" means F_1 spleen cells prepared at the time of the "second" culture. "First" stimulator was the stimulator used to induce suicide in F_1 spleen cells. "Second" stimulator was that used in the "postsuicide" reculture of the cells. Actually, this represents a second primary culture.

TABLE IV

F_1 T Cells Are Precommitted to Recognize TNP Associated with the H-2 of One Parent*

Line	Stimulating cells		% Specific ^{51}Cr release on targets			
	First	Second	TNP-AKR	TNP-BALB	B6	TNP-B6
10	Fresh	TNP-AKR	20.2	2.2	3.5	4.1
11	TNP-AKR	TNP-AKR	−1.6	1.2	0.9	3.0
12	B6	TNP-AKR	10.8	0.5	3.1	2.0
13	Fresh	TNP-DBA/2	1.6	8.2	0.1	2.6
14	TNP-AKR	TNP-DBA/2	1.4	7.1	2.6	7.0
15	B6	TNP-DBA/2	4.0	7.1	5.1	8.0
16	Fresh	B6	−0.8	1.7	2.2	2.4
17	TNP-AKR	B6	6.5	4.0	23.9	24.7
18	B6	B6	2.7	1.7	3.5	8.9
19	B6	TNP-B6	2.4	1.8	4.0	11.8

* Responder cells are (AKR × DBA/2)F_1.

Effector to target ratio is 40:1.

of TNP-self targets by alloreactive T cells is blocked by the appropriate, unmodified allogeneic cells.

Thus, by these criteria, at least three types of killers directed at TNP-self cells can be defined in F_1 mice: there are effector cells specific for TNP-parental cells of one or the other strain, and those stimulated by allogeneic cells. In the suicide experiments, we will present evidence that these three types of effectors come from three distinct sets of precursor cells.

Selective Suicide of Precursors Specific for Allogeneic Cells or for TNP-Parental Cells. In our experiments, we consistently find that cells responsive to TNP-parental stimulators can be suicided independently of cells responsive to allogeneic stimulators (Table III, line 2 vs. 4, 6 vs. 8; Table IV, line 10 vs. 11, 17 vs. 18; Table V, line 28 vs. 30; Table VI, line 32 vs. 35, 37 vs. 40, 42 vs. 44 and

TABLE V

F₁ T Cells Are Precommitted to Recognize TNP Associated with the H-2 of One Parent: Failure to Detect Specific Suppressors*

| Line | Stimulating cells | | % Specific ^{51}Cr release on targets | | | |
	First	Second	TNP-AKR	TNP-BALB	A.SW	TNP-A.SW
20	Fresh (A)	TNP-DBA/2	12.1	17.1	1.7	−1.2
21	TNP-DBA/2 (B)	TNP-DBA/2	1.6	0.1	−1.1	−2.3
22	(A) + (B)	TNP-DBA/2	10.2	12.6	1.2	−1.4
23	A.SW	TNP-DBA/2	1.7	−0.1	−2.3	−2.5
24	Fresh	TNP-AKR	22.0	3.2	−0.6	−1.8
25	TNP-DBA/2	TNP-AKR	13.7	−0.2	−1.5	−1.8
26	A.SW	TNP-AKR	0.3	−0.5	−2.1	−2.1
27	Fresh (C)	A.SW	25.1	14.3	36.8	31.2
28	A.SW (B)	A.SW	0.8	0.2	−1.0	−2.6
29	(C) + (B)	A.SW	7.5	4.8	21.4	8.0
30	TNP-DBA/2	A.SW	16.8	10.5	27.1	17.0

* Responder cells are (AKR × DBA/2)F₁.
Effector to target ratio is 50:1; essentially no killing on AKR or BALB targets.

TABLE VI

Evidence of Three Distinct Precursor Cells Specific for TNP-Self*

| Line | Stimulating cells | | % Specific ^{51}Cr release on targets | | | |
	First (suiciding)	Second	TNP-AKR	TNP-BALB/c	B6	TNP-B6
31	Fresh (A)	TNP-AKR	26.5	6.4	−2.2	4.1
32	TNP-AKR (B)	TNP-AKR	2.6	−0.4	−2.1	1.1
33	(A) + (B)	TNP-AKR	26.1	9.8	−0.8	2.3
34	TNP-DBA/2	TNP-AKR	16.0	0.8	−1.3	0.7
35	B6	TNP-AKR	13.1	0.3	−0.5	0.3
36	Fresh (A)	TNP-DBA/2	14.0	13.7	−0.1	2.7
37	TNP-DBA/2 (C)	TNP-DBA/2	2.6	3.7	−1.9	1.3
38	(A) + (C)	TNP-DBA/2	6.7	10.5	−1.8	2.5
39	TNP-AKR	TNP-DBA/2	5.6	1.1	−3.1	−0.2
40	B6	TNP-DBA/2	2.8	6.7	−1.8	1.4
41	Fresh (A)	B6	4.6	11.1	27.1	15.4
42	B6 (D)	B6	2.6	5.9	2.7	3.9
43	(A) + (D)	B6	3.9	4.9	18.3	12.8
44	TNP-AKR	B6	−0.5	12.0	30.8	16.1
45	TNP-DBA/2	B6	11.9	11.3	33.7	14.9
46	B6	TNP-B6	1.4	3.0	−0.5	0.8
47	Fresh B6 spleen	TNP-B6	8.2	4.3	−1.0	8.2

* Responder cells are (AKR × DBA/2)F₁.
Effector to target ratio is 37:1 except for mixture experiments where a ratio of 75:1 was used to compensate for dilution of responsive cells with unresponsive cells. Unmodified AKR and BALB/c targets showed only background lysis.

45; Table VII, lines 55 and 57 vs. 59, 61 vs. 63 and 64). Thus, we can say that our suiciding conditions are both selective and adequate for these experiments.

Loss of Response of Suicided Cells is Not Due to the Generation of Suppressor Cells. We have done several control experiments to test whether specific

TABLE VII

BUdR and Light Suicide of F_1 T Cells Specific for TNP-Modified Parental Cells Is Selective for K and D Determinants in the Parental H-2 *

| Line | Stimulator cells | | % Specific ^{51}Cr release on targets | | | |
	First	Second	TNP-A.TH	TNP-A.TL	B6	TNP-B6
48	Fresh (A)	TNP-A.TH	16.2	15.4	3.4	10.4
49	TNP-A.TH (B)	TNP-A.TH	−0.9	−1.7	−0.5	0.4
50	(A) + (B)	TNP-A.TH	18.1	14.6	0.9	4.7
51	TNP-A.TL (C)	TNP-A.TH	−2.0	−0.1	0.4	0.2
52	(A) + (C)	TNP-A.TH	9.3	9.1	−2.4	0.8
53	B6	TNP-A.TH	1.1	1.3	−0.4	−0.5
54	Fresh (A)	TNP-A.TL	7.5	6.4	−0.3	1.7
55	TNP-A.TL (C)	TNP-A.TL	−2.7	−1.9	−1.7	−2.0
56	(A) + (C)	TNP-A.TL	18.5	13.6	−0.4	1.5
57	TNP-A.TH (B)	TNP-A.TL	−1.5	−1.6	−1.3	−1.0
58	(A) + (B)	TNP-A.TL	22.2	17.2	0.4	5.5
59	B6	TNP-A.TL	6.4	3.5	−0.4	0.3
60	Fresh (A)	B6	10.4	11.9	40.0	39.7
61	B6 (D)	B6	8.8	6.3	1.6	6.0
62	(A) + (D)	B6	15.7	17.0	43.0	33.0
63	TNP-A.TH	B6	16.7	12.2	31.4	32.9
64	TNP-A.TL	B6	16.5	14.4	34.4	32.7
65	B6	TNP-B6	5.3	4.9	0.9	8.8
66	Fresh B6 spleen	TNP-B6	3.2	2.6	0.4	11.9

* Responder cells are (A.TH × A.TL)F_1.
Effector to target ratio is 50:1 except for mixture groups where 100:1 was used to compensate for dilution of responsive cells 1:1 with unresponsive cells. A.TH and A.TL targets showed lysis to a low extent in a few groups, and this has been subtracted from the kill on TNP-modified targets.

TABLE VIII

Killing of TNP-Self by Alloactivated Cells

| Responders | Stimulators* | % Specific ^{51}Cr release on targets | | | |
		CBA	TNP-CBA	A.CA	TNP-A.CA
CBA	−	−3.1‡	1.9	−3.0	−2.3
CBA	TNP-CBA$_m$	−3.7	53.4	−2.5	8.6
CBA	A.CA$_m$	−3.9	15.1	32.4	38.0
A.CA	TNP-A.CA$_m$	−0.8	17.9	−5.1	32.8

* Stimulators inactivated by 30 μg/ml mitomycin C, 30 min, 37°C, followed by four washes.
‡ Mean of triplicate wells; standard errors all <2.0%.

suppressor cells could be responsible for the loss of response seen after suicide. When suicided cells are mixed 1:1 with fresh F_1 spleen cells and stimulated with the same stimulator used for the suiciding culture, to which the suicided cells can no longer respond, the response obtained is not significantly different from that expected from the fresh F_1 cells cultured alone with that stimulator (Table V, line 21 vs. 22; Table VI, line 31 vs. 33, 36 vs. 38, 41 vs. 43; Table VII, line 48 vs. 50 and 52, 54 vs. 56 and 58, 60 vs. 62). This strongly suggests that our suiciding conditions deplete responsive cells by eliminating them rather than

TABLE IX

Cold-Target Inhibition Studies of the Alloreactive Cell Killing TNP-Self Targets

Reaction being inhibited*	% ^{51}Cr Release in the presence of the inhibitors‡					
	Medium	CBA	TNP-CBA	A.CA	TNP-A.CA	Mixture§
CBA anti-TNP-CBA on TNP-CBA	53.4	42.5	<u>14.1</u>	—¶	37.5	38.2
CBA anti-A.CA on TNP-CBA	15.1	14.2	<u>0</u>	<u>2.8</u>	<u>2.4</u>	<u>1.5</u>
CBA anti-A.CA on TNP-A.CA	38.0	19.5	21.4	<u>5.4</u>	—	<u>5.2</u>

* Effector to target ratio is 40:1; same cells as in Table VI.
‡ Inhibitor to target ratio is 80:1.
§ Mixture of equal numbers of spleen cells from mice of haplotypes: *b, d, f, q, r, s*.
 Underlined groups show significant inhibition compared with unmodified CBA blockers.
¶ Not tested.

by inducing specific suppressor cells. Nor do we have any evidence for nonspecific suppressor cells among these precultured and suicided cells (8, 17, 18), suggesting that such cells are also suicided in these cultures.

Selective Suicide of Precursors of Killer Cells Specific for TNP-Modified Cells of One or the Other Parental Strain. In most of these experiments, using F_1 responder cells derived from parental strains differing throughout the MHC, suicide with TNP-modified cells from one parental strain did not eliminate the response to TNP-modified cells of the other parental strain (Table III, line 2 vs. 3; Table IV, line 11 vs. 14; Table V, line 21 vs. 25; Table VI, line 34 vs. 37). Only one exception to this was seen (Table VI, line 32 vs. 39). However, this exception is perhaps misleading because in this experiment, cross-reactivity between TNP-AKR and TNP-DBA/2 targets was greater than expected (Table VI, line 36). The reasons for this cross-reactivity are not known, but it is interesting that this cross-reactivity at the effector level occurred only in the one experiment where cross-reactivity was also seen in the suiciding experiment.

When the K and D Loci of the Parental Strains Are Identical, Precursors for TNP-Modified Parental Cells Are Identical. We wanted to determine whether the selective suicide of F_1 responses to TNP-modified parental stimulators was due to recognition of *K/D* or of I region determinants. We, therefore, repeated these experiments using (A. TH × A.TL)F_1 responder cells. The two parental strains are identical at the *K* and *D* loci, but differ throughout the *I* region (Table I). In this instance, suicide with TNP-modified cells of either parental strain eliminated the response to TNP-modified cells of both parental strains (Table VII, lines 49, 51, 55, 57). Thus, selective suicide is specific for *K* and *D* locus antigens and most likely represents suicide of precursors of the cytotoxic effector cells themselves. Whether this technique is also capable of suiciding helper cells for these responses requires further experimentation.

Suicide of Cells Responsive to TNP-Parental Cells Does Not Eliminate Cells Stimulated by Allogeneic Cells to Kill TNP-Parental Targets. As noted above, allogeneic stimulators can induce cytotoxic effector cells that kill TNP-modified self targets. Such cells differ in several ways from cytotoxic effectors induced by TNP-self cells. The present experiments show that the precursors of such cells are distinct also. Thus, suicide with TNP-parental cells does not eliminate the killing of TNP-parental targets induced by allogeneic stimulators (Table III,

line 5 vs. 7; Table IV, line 16 vs. 17; Table V, line 27 vs. 30; Table VI, line 41 vs. 45; Table VII, line 60 vs. 63 and 64). An exception to this is also seen in one experiment (Table VI, line 44). This latter group of cells was exceptional in other ways, as noted above, and this is the only experiment in which we have failed to see killing of TNP-parental cells after suicide when allogeneic cells are used as the second stimulators. It should be pointed out that the experiment in Table VII represents a strong argument that we are suiciding precursor cells in these experiments, and that activation of TNP-parental suicided cells with allogeneic stimulators to kill TNP-parental targets does not represent a compensation for inadequate help; in this experiment suicide was seen with either parental strain, even though they differ in the *I* region, which latter is thought to be involved in helper effects important for cytotoxic effector cell generation. Thus, we would conclude that alloreactive cells killing TNP-self targets come from different precursors than those activated by TNP-self.

Suicide with Allogeneic Cells Does Not Unmask a Response to TNP-Modified Allogeneic Cells. After suicide with allogeneic stimulators, the cells were restimulated either with allogeneic cells or TNP-modified allogeneic cells, and then tested with either allogeneic or TNP-allogeneic targets. It is essential, in such experiments, that both modified and unmodified stimulators as well as targets be used, as can be clearly seen in our results. We have very good elimination of alloreactive cells in several experiments, with no evidence of suppression. Such cells will respond to TNP-modified parental stimulators after suicide with allogeneic stimulators (see above). Nonetheless, such cells do not respond to TNP-modified allogeneic stimulators by making killer cells specific for TNP-modified allogeneic targets, when compared with their response to allogeneic unmodified stimulators (Table III, line 6 vs. 9; Table IV, line 18 vs. 19; Table VI, line 42 vs. 46; Table VII, line 61 vs. 65). In each case, the importance of the unmodified stimulator control can be seen. In the experiments in Tables VI and VII, the activity of the TNP-modified B6 stimulators was confirmed using B6 spleen cells as responders. Thus, we have no evidence that normal mice harbor significant numbers of precursor cells specific for TNP-modified allogeneic cells but normally masked by the strong response to alloantigen.

Discussion

These experiments were undertaken to define the numbers and types of precursors of cytotoxic effector cells specific for TNP-modified parental strain targets in normal (A × B) F_1 mice. Of four possible sets of precursors, we have found evidence for three. These are (*a*) precursors specific for TNP-modified cells of parental strain A; (*b*) precursors specific for TNP-modified cells of parental strain B; (*c*) precursors specific for TNP-modified parental (A or B) cells, but activated only by allogeneic stimulators and not activated by TNP-modified parental stimulators. We did not find any evidence for a fourth possible class of precursors, namely, those capable of responding specifically to TNP-modified allogeneic cells.

These data are entirely consistent with the elegant ontogenetic experiments of Zinkernagel et al. (9, 10) and of Bevan (11). These authors showed that F_1 stem cells differentiating in parental thymus preferentially respond to modified

cells of the parental thymus type. They did not find evidence, even in tolerance situations, for an ability of T cells to respond to fully allogeneic-modified stimulators. This result and ours (confirmed by Schmitt-Verhulst and Shearer [19]) conflict with the results of other authors who suggested that acute depletion of alloreactive cells revealed strong reactivity toward TNP-modified allogeneic stimulators (13, 14). The reason for these differences is not clear. Wilson et al. (13) used an entirely different technique to deplete alloreactive cells, which may be responsible for the differences in the results. They also do not present data on response to unmodified allogeneic stimulators in the mouse system, but such controls were performed in parallel experiments carried out in rats and did not show lysis of TNP-modified allogeneic targets. Thomas and Shevach (14) likewise did not report controls using unmodified allogeneic cells as second stimulators; such controls were, however, performed in preliminary experiments and again did not elicit responses to TNP-modified allogeneic cells (Shevach, personal communication). Although Thomas and Shevach used essentially the same technique as we used here to eliminate alloreactive cells, the protocol and the species were different. It is clear that further experimentation is needed to clarify this crucial point.

We were surprised by the finding that alloreactive cells capable of killing TNP-modified parental cells in F_1 mice differ from F_1 cells stimulated by TNP-parental cells to kill TNP-parental targets, since we had previously speculated that such cells were identical. From this, and from the experiments of Wilson and Heber-Katz (20, 21), we had postulated that a given T cell can respond to two types of antigen: foreign MHC antigens, and modified self MHC antigens. Our results in the present experiments would suggest that this is not the case. However, it may also be that TNP-parental stimulators are relatively weak activators of cytotoxic effector cell precursors, whereas allogeneic cells are strong stimulators. If this were so, then suicide with TNP-parental cells would eliminate only a small fraction of all possible responding cells, the remainder being much more readily activatible by allogeneic cells. Experiments to test this proposition are now in progress. Recent experiments by Bevan (12) have suggested that allogeneic killers could be raised when T cells are immunized with minor alloantigens. However, these experiments only show that alloimmune cells will lyse targets bearing minor antigens which are H-2 identical with the responder, and show cold-target inhibition data similar to that presented here; they do not directly deal with the nature of the precursors of these cytotoxic cells.

If our conclusions from these results are correct, they in turn suggest that T cells differentiate in response to self MHC antigens. Zinkernagel (9, 10) has shown that at least part of this differentiation occurs in the thymus, but there are additional steps involved that may occur postthymically as well. Whether these cells differentiate in response only to self MHC antigens, or whether their differentiation requires foreign non-MHC antigens in an inapparent form, can not be determined as yet. However, it is clear that such cells do evolve an ability to recognize self MHC antigens, whether one uses chimeric T cells or normal F_1 T cells, as in the present experiments. This commitment of self MHC precedes intentional immunization, as shown by these experiments, because if it followed immunization, TNP-modified cells from either parental strain should

suicide all TNP-reactive precursors in an F_1 mouse. These results would seem to us to be more compatible with a T-cell receptor that has two sites, one specific for self MHC antigens, and one specific for the foreign, non-MHC antigen. Both types of recognition show exquisite specificity (3, 5, 22, 23). Thus, even though one cannot state conclusively from these or other experiments whether the T-cell receptor has one or two binding sites, we feel strongly that available evidence, including the results of the present experiments, favor the two-receptor site model.

Summary

The technique of antigen-driven, 5-bromo-deoxyuridine and light suicide has been adapted to eliminate the precursors of cytotoxic effector cells both for alloantigen and for 2,4,6-trinitrophenyl(TNP)-modified stimulator and target cells. Using this technique, the following observations have been made. Precursors of killer cells specific for alloantigen can be suicided independently of precursors of killer cells specific for TNP-modified self cells. The loss of activity during this procedure is not due to either specific or nonspecific suppressor cells, as judged by mixing experiments. With responder cells from F_1 animals, it has been possible to show that precursors specific for TNP-modified cells from one parent are suicided independently of precursors specific for TNP-modified cells of the other parent, but only if the parental strains differ in the K and D regions of the H-2 complex. Cells of F_1 mice derived from K and D identical, I region different, parental strains were specifically suicided by TNP-modified stimulator cells from either parent. However, the cross-reactive killing of TNP-self targets induced by stimulation with allogeneic cells is not eliminated by first suiciding with TNP-parental cells, suggesting that the precursors of these two types of TNP-self killer cells are different. This is compatible with reported differences in their specificity, as confirmed in this report. Finally, deletion of alloreactive cells by this technique reveals little or no reactivity specific for TNP-modified allogeneic stimulator cells. In summary, these results strongly suggest that recognition of self MHC antigens is preprogrammed in peripheral T cells of normal animals, and is not acquired during the immunization process. They also suggest that cells specific for modified alloantigen are relatively rare in the strains of mice studied.

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