ANTIGEN RECOGNITION BY CLONED CYTOTOXIC T LYMPHOCYTES FOLLOWS RULES PREDICTED BY THE ALTERED-SELF HYPOTHESIS*

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Antigen recognition by cytotoxic lymphocytes (CTL)[1] and by helper T cells is restricted by gene products of the major histocompatibility complex (MHC), called H-2 in the mouse (1). CTL recognize antigens in the context of K- and D-region-encoded structures, whereas helper T cells recognize antigen in the context of I-region-encoded structures. This H-2 restriction appears to be very tight: CTL specific for a conventional antigen X (e.g., a viral or minor histocompatibility [minor H] antigen) plus one H-2 haplotype will not lyse target cells expressing antigen X in the context of a different H-2 haplotype. Two types of models have been proposed to explain this phenomenon. According to the altered-self hypothesis (2), one T cell receptor recognizes neither the H-2-restricting element nor the conventional antigen X by itself but an interaction antigen formed by X and self-H-2 in physical proximity. On the other hand, dual-recognition models (3–5) postulate two types of receptors on every T cell, one, the anti-self-receptor, specific for the restricting element, the other specific for conventional antigen X. Recently, support for this latter view has been drawn from experiments with chimeric mice where it was demonstrated that the mature T cell repertoire is preferentially restricted by those H-2 antigens expressed on radioresistant cells present during T cell ontogeny (3, 6). Thus, bone marrow stem cells from H-2 heterozygous (A × B)F_1 mice give rise to a predominantly A- or B-restricted T cell receptor repertoire, depending on the H-2 type (A or B) of the thymus in which the F_1-derived stem cells mature (3, 7). The selection for self-H-2-restricted T cells is, indeed, more easily understood in terms of a selection for cells with anti-self receptors. However, the nonabsolute nature of self-H-2 learning (6–10), and the presence of T cells restricted to foreign H-2 antigens in normal (11, 12) and neonatally tolerant mice (13) did leave room for one-receptor theories: In terms of the altered-self hypothesis, T cells that recognize self-H-2 plus X might be cross-reactive with allo-H-2 plus Y, i.e., the same interaction antigen may be formed in different ways. Such cross-reactivity has been referred to as "aberrant recognition" by Doherty and

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[1] *Abbreviations used in this paper:* CASUP, concanavalin A-induced spleen cell supernatant; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; LPS, lipolysaccharide; MHC, major histocompatibility complex; MLC, mixed lymphocyte cultures; TNP, trinitrophenyl; TNP-BSA, trinitrophenylated bovine serum albumin.

Bennink (11). Therefore, a T cell receptor repertoire could be selected for during thymic maturation with a preference for interaction antigens in which self-H-2 participates, but without eliminating reactivity to conventional antigens in the context of allo-H-2.

We provide direct evidence that a CTL can react with two different interaction antigens. The specificity of these CTL is predicted by the altered-self model of T cell recognition, but is quite inconsistent with dual-recognition models that postulate independent recognition of self-H-2 and foreign antigen X by separate receptor sites. As will be discussed, the experimental conditions required to demonstrate this point suggest that the nonabsolute nature of thymic self-learning can be explained in terms of the altered-self hypothesis.

Materials and Methods

Mice. C57BL/10 (B10, H-2^b), B10.D2 nSn (H-2^d), AKR/J (H-2^k), C3HeB/FeJ (H-2^k), (C3H × DBA/2)F_1, and (AKR/J × DBA/2)F_1 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. All other mice were bred in the animal facilities at Massachusetts Institute of Technology, Cambridge, Mass. These were: BALB/cAn (H-2^d), BALB.B (H-2^b), BALB.K (H-2^k), BALB.HTG (H-2^g), B10.BR SgSn (H-2^k), C3H.H-2^o (H-2^o), DBA/2 (H-2^d), and the following F_1 mice: (B10 × B10.D2), (B10.BR × B10.D2), (B10.BR × DBA/2), (B10.D2 × DBA/2), (BALB/c × BALB.B), (BALB/c × BALB.K), (BALB.B × B10.BR), and (BALB.B × B10.D2).

Chimeras. Radiation chimeras were prepared by reconstituting lethally irradiated H-2-homozygous mice of the strains B10, B10.D2, and B10.BR with H-2 heterozygous bone marrow stem cells from (B10 × B10.D2)F_1 or (B10.BR × B10.D2)F_1 mice. Bone marrow cells from the tibias and femurs were treated with a mixture of the monoclonal anti-Thy-1 antibodies 13-4 (14) and T24 (15), followed by rabbit complement. 10×10^6 to 20×10^6 surviving cells were injected into mice irradiated with 900–950 rad from a ^{137}Cs source 2–4 h before. The chimeras were used for the experiments at least 8 wk after irradiation.

Priming to Minor H Antigens. Normal F_1 mice and $F_1 \rightarrow$ parent chimeras of the B10 background were primed to minor H antigens of the BALB background with a single intraperitoneal injection of 10×10^6 viable spleen cells from H-2-matched F_1 mice of the BALB series. After ≥ 2 wk, spleen cells from the primed mice were stimulated in vitro (16).

Mixed Lymphocyte Cultures (MLC). For primary MLC, 25×10^6 spleen and lymph node cells from primed animals were cocultured with 25×10^6 irradiated (1,000 rad from a ^{137}Cs source) stimulator cells in 20 ml of RPMI 1640 medium containing 5% fetal calf serum as described previously (7). Cultures were incubated for 5 d at 37°C in an atmosphere of 5% CO_2 in air. For secondary MLC, 5×10^6 viable cells recovered from a 10-d-old primary MLC were incubated for 5 d with 25×10^6 irradiated stimulator cells.

Assay for CTL Activity. MLC cells were harvested, washed, and assayed in threefold serial dilutions for lysis of target cells as described (7). Target cells were concanavalin A (Con A)- or lipopolysaccharide (LPS)-stimulated spleen cells after 2–3 d of culture. Con A-stimulated targets were washed with alpha-methyl-D-mannoside before labeling with $Na^{51}CrO_4$. The assay was performed in a final volume of 1 ml medium in 12- × 75-mm plastic tubes cultured upright for 4 h at 37°C. Percent specific lysis was calculated as 100 × (counts per minute released with CTL − (counts per minute released alone)/(counts per minute released by detergent − counts per minute released alone).

Modification of Target Cells with Trinitrophenylated Bovine Serum Albumin (TNP-BSA). Modification of target cells with TNP-BSA was essentially done as described by Schmitt-Verhulst et al. (17) and Ballas and Henney (18). After labeling with ^{51}Cr, target cells were washed in serum-free medium, then incubated in serum-free medium containing 1 mg/m TNP-BSA (39 mol trinitrophenyl (TNP)/70,000 mol wt) for 1 h at 37°C and washed three times in serum containing medium.

Cloning of CTL. Viable cells recovered from an MLC were seeded at 0.3 cells/well in Costar 3596 trays (Costar, Data Packaging, Cambridge, Mass.) in the presence of 3×10^5 irradiated

BALB/c stimulator cells. The medium was supplemented with 25% (vol/vol) of 24-h supernate from Con A-activated murine spleen cells (CASUP) as a source of interleukin 2 (19) and 50 mM alpha-methyl-D-mannoside. After 1 wk, the cloning plates were fed with 0.1 ml/well of CASUP-supplemented medium containing 3×10^5 irradiated BALB/c stimulator cells. After an additional week, cultures with microscopically visible growth were transferred to 2-ml culture wells of Costar trays and were further expanded under the same conditions as detailed above, but with 4×10^6 irradiated stimulator cells per well.

Results

The Experimental System. We have studied the secondary in vitro CTL response of H-2-congenic mice of the B10 background (B10, H-2^b; B10.D2, H-2^d; B10.BR, H-2^k) against cells of the H-2-congenic series derived from BALB/c (BALB/c, H-2^d; BALB.B, H-2^b; BALB.K, H-2^k). These two background strains differ by >30 minor H antigens (20), and in vitro boosting of in vivo primed animals leads to strong, H-2-restricted CTL responses (16).

Host H-2 Preference of Anti-Minor H CTL Responses from $F_1 \rightarrow$ Parent Chimeras to F_1 Stimulator Cells. If a normal (B10 × B10.D2)F_1 mouse is primed and boosted with (BALB/c × BALB.B)F_1 cells, equal amounts of CTL activity are generated against BALB/c and BALB.B target cells (7) as shown in Table I. In chimeric animals constructed by injecting F_1 bone marrow cells into lethally irradiated hosts of the parental strains ($F_1 \rightarrow$ Parent chimeras), however, this immunization protocol results in a CTL population that is much more active against target cells expressing the H-2 haplotype of the chimeric host than against targets of the non-host parent H-2 type (6, 7). As shown in Table I, (B10 × B10.D2) → B10 chimeras responded to stimulation with (BALB/c × BALB.B) cells with >50- (chimera 1) and ~30-fold (chimera 3) more CTL activity against BALB.B than against BALB/c targets, and (B10 × B10.D2) → B10.D2 chimeras responded with >50-fold more activity against BALB/c than against BALB.B target cells. This host preference of CTL responses, which is also apparent in the chimeras constructed from (B10.BR × B10.D2)F_1 bone marrow injected into the two parental strains (Table I, chimeras 5–8), is a well-established phenomenon.

Specificity of Anti-Minor H CTL Responses from $F_1 \rightarrow$ Parent Chimeras Boosted with Stimulator Cells Expressing Only One Parental H-2 Haplotype. In the anti-minor H response of normal F_1 mice, the CTL activities restricted to the two parental H-2 haplotypes are mediated by two separate subpopulations of CTL (16, 21): (B10 × B10.D2)F_1 anti-(BALB/c × BALB.B)F_1 CTL contain one subpopulation specific for BALB/c and another one specific for BALB.B targets. This point is easily demonstrated by selective restimulation in vitro, where presentation of minor H antigens on F_1 responder cells plays no role (21). Since the anti-minor H response of $F_1 \rightarrow$ Parent chimeras to F_1 stimulator cells is not always completely restricted to the host H-2 type (6–8; and Table I), it seemed feasible to select these two subpopulations from the chimeras described in Table I. Table II shows the activity of cultures that were stimulated for two cycles of MLC with cells expressing BALB minor H antigens plus either the chimeric-host-type or non-host-type H-2. Strong CTL responses were generated in all cases. It should be noted, however, that after the first cycle of MLC, cultures stimulated with host-type H-2 plus minor H antigens contained far more cells, and that all groups were adjusted to the same responder cell density for secondary MLC. All cultures that were stimulated with BALB cells of the chimeric

TABLE I

Anti-Minor H CTL Responses of F_1 → Parent Bone Marrow Radiation Chimeras to Stimulators Expressing Both Parental H-2 Haplotypes

Chimera	Type	Primed and boosted with	E:T* ratio	Percent specific lysis of			Host preference‡
				BALB/c	BALB.B	BALB.K	
1	(B10 × B10.D2)F_1 → B10	(BALB/c × BALB.B)F_1	27	2.1	70.1	ND§	>50
			9	−1.5	54.8		
2	(B10 × B10.D2)F_1 → B10.D2	(BALB/c × BALB.B)F_1	45	66.9	0.3	ND	>50
			15	51.9	−0.4		
3	(B10 × B10.D2)F_1 → B10	(BALB/c × BALB.B)F_1	81	13.2	88.4	ND	~30
			27	6.2	77.6		
4	(B10 × B10.D2)F_1 × B10.D2	(BALB/c × BALB.B)F_1	30	80.6	−4.0	ND	>50
			10	67.4	−4.7		
5	(B10.BR × B10.D2)F_1 → B10.BR	(BALB/c × BALB.K)F_1	45	39.1	ND	71.7	7
			15	17.2		59.2	
6	(B10.BR × B10.D2)F_1 → B10.D2	(BALB/c × BALB.K)F_1	30	77.0	ND	20.8	25
			10	61.0		5.1	
7	(B10.BR × B10.D2)F_1 → B10.BR	(BALB/c × BALB.K)F_1	36	9.7	ND	77.8	27
			12	10.6		68.2	
8	(B10.BR × B10.D2)F_1 → B10.D2)	(BALB/c × BALB.K)F_1	81	47.4	ND	−3.3	>50
			27	44.8		−2.1	
Normal	(B10 × B10.D2)F_1	(BALB/c × BALB.B)F_1	66	71.3	71.6	ND	
			22	54.7	32.4		
Normal	(B10.BR × B10.D2)F_1	(BALB/c × BALB.K)F_1	90	56.6	ND	60.0	
			30	46.2		56.1	

Spleen and lymph node cells from primed bone marrow radiation chimeras of the types indicated were boosted in MLC and assayed for CTL activity 5 d later. Three individual experiments are shown: chimeras 1, 2, and the normal (B10 × B10.D2)F_1; chimeras 3 and 4; chimeras 5, 6, 7, and 8 and the normal (B10.BR × B10.D2)F_1. Spontaneous ^{51}Cr release of Con A blasts varied from 18.3 to 49.5%.

* Effector:target cell ratio.

‡ Relative activity on BALB target cells expressing non-host-type H-2 over activity on targets expressing non-host-type H-2 as determined from complete titration curves.

§ Not done.

host H-2 type lysed only target cells of the type used for in vitro stimulation. For example, chimera 1 (B10 × B10.D2)F_1 → B10, when stimulated with BALB.B cells, had high cytotoxic activity against BALB.B and none against BALB/c target cells. This result was expected from the experience with normal F_1 mice (21). When the specificity of chimeric CTL populations generated against BALB cells of the non-host type H-2 was studied, however, we were surprised to find that this strict specificity for stimulator H-2 type was not always observed: two out of eight CTL populations generated in this fashion also lysed BALB target cells of host type H-2, i.e. the H-2 type absent from the stimulator cells in vitro (Table II, chimeras 4 and 6). The specificity of the CTL response generated from chimera 6 [(B10.BR × B10.D2F_1) → B10.D2] against BALB.K stimulator cells is also shown in Fig. 1. BALB.K and BALB/c, but not BALB.B, B10.BR, or B10.D2 target cells were lysed. This cell population was chosen for further analysis.

Cold Target Competition Analysis of the CTL Population Recognizing BALB Minor H Antigens on H-2^k and H-2^d Target Cells. The presumably cross-reactive CTL population (Fig. 1) was restimulated in MLC twice with BALB/c cells and assayed against

TABLE II

Anti-Minor H CTL Responses of $F_1 \rightarrow$ Parent Chimeras to Stimulators Expressing Only One Parental H-2 Haplotype

Chimera	Type	Boosted with	E:T* ratio	Percent specific lysis of			Preference for‡ stimulator H-2
				BALB/c	BALB.B	BALB.K	
1	(B10 × B10.D2)$F_1 \rightarrow$ B10	BALB/c	36	76.8	1.7	ND	>50
			12	67.0	−2.7		
		BALB.B	54	−4.8	87.2	ND	>50
			18	−1.4	94.8		
2	(B10 × B10.D2)$F_1 \rightarrow$ B10.D2	BALB/c	60	85.6	−7.7	ND	>50
			20	87.5	−4.2		
		BALB.B	21	−3.3	58.9	ND	>50
			7	−2.2	27.3		
3	(B10 × B10.D2)$F_1 \rightarrow$ B10	BALB/c	36	76.4	12.3	ND	81
			12	70.1	7.4		
		BALB.B	54	−3.0	83.5	ND	>50
			18	−1.5	81.9		
4	(B10 × B10.D2)$F_1 \rightarrow$ B10.D2	BALB/c	54	93.2	6.9	ND	>50
			18	88.9	1.6		
		BALB.B	27	55.6	78.3	ND	5
			9	24.6	66.7		
5	(B10.BR × B10.D2)$F_1 \rightarrow$ B10.BR	BALB/c	6	74.9	ND	−10.8	>50
			2	55.4		−6.1	
		BALB.K	60	−3.3	ND	74.6	>50
			20	−0.8		74.2	
6	(B10.BR × B10.D2)$F_1 \rightarrow$ B10.D2	BALB/c	66	82.6	ND	−3.7	>50
			22	84.1		−3.0	
		BALB.K	30	34.1	ND	74.0	10
			10	28.8		66.9	
7	(B10.BR × B10.D2)$F_1 \rightarrow$ B10.BR	BALB/c	20	34.2	ND	−6.4	>50
			7	22.1		−4.5	
		BALB.K	54	−13.1	ND	73.8	>50
			18	−6.8		68.8	
8	(B10.BR × B10.D2)$F_1 \rightarrow$ B10.D2	BALB/c	60	61.2	ND	−9.9	>50
			20	52.2		−5.0	
		BALB.K	40	−9.6	ND	69.3	>50
			13	−9.5		62.8	

Spleen and lymph node cells from the same chimeras listed in Table I which had been primed in vivo with BALB F_1 cells were boosted in MLC with irradiated BALB cells of either chimeric host or non-host H-2 type. After 10 d, cultures were restimulated with the same type of stimulator cells and assayed for CTL activity 5 d later. Spontaneous ^{51}Cr release from the Con A blast cells varied from 18.4 to 43.6%.

* Effector:target cell ratio.

‡ Relative activity on stimulator-type over nonstimulator-type target cells as determined from complete titration curves.

§ Not done.

^{51}Cr-labeled BALB/c target cells in the presence of a 30-fold excess of unlabeled BALB/c, BALB.B, or BALB.K targets. As shown in Table III, both BALB/c and BALB.K unlabeled competitors inhibited the ^{51}Cr-release from BALB/c target cells, whereas unlabeled BALB.B blasts had no inhibitory effect. We conclude that the lysis

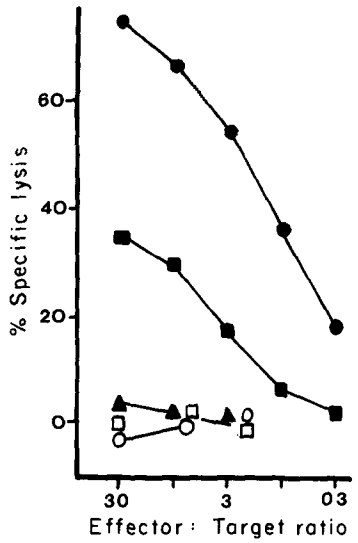

FIG. 1. Specificity of the CTL population derived from chimera 6 (Tables I and II) after two in vitro stimulations with BALB.K cells. Spleen and lymph node cells from a (B10.BR × B10.D2)F_1 → B10.D2 chimera that was primed to BALB minor H antigens were stimulated twice in MLC with BALB.K cells and assayed against Con A-induced blasts from various strains: BALB/c (■), BALB.K, (●), BALB.B (▲), B10.D2 (○), and B10.BR (□). Spontaneous release of ^{51}Cr varied from 26.9 to 49.5%.

TABLE III

Cold Target Competition Analysis of the Cross-Reactive CTL Activity

Unlabeled competitors	Percent specific lysis of ^{51}Cr-labeled BALB/c target cells at effector:target ratio	
	2.4:1	0.8:1
None	21.7	13.8
BALB.B	23.6	14.0
BALB/c	7.4	3.3
BALB.K	12.2	6.1

The CTL population described in Fig. 1 was boosted twice in MLC with irradiated BALB/c cells and assayed against ^{51}Cr-labeled BALB/c blast cells (spontaneous lysis: 13.8%) in the presence or absence of a 30-fold excess of unlabeled BALB.B, BALB/c, or BALB.K blast cells.

of BALB/c and BALB.K cells by this CTL population was mediated by the same cells.

Cloning of CTL That Lyse BALB/c and BALB.K Target Cells. In the next cycle of MLC, cells from the cross-reactive CTL population described above were dispensed into a total of 192 microwells at 0.3 cells per well in the presence of irradiated BALB/c cells and CASUP as a source of interleukin 2 (19). The microcultures were boosted in the same wells after 1 wk, and after 2 wk, 19 wells showed microscopically visible growth. 10 of these microcultures were transferred to 2-ml wells and restimulated twice with BALB/c cells in the presence of CASUP. This yielded sufficient cell numbers to screen the presumptive CTL clones on BALB/c and BALB.K target cells.

The results are given in Table IV. 7 out of 10 cultures had significant CTL activity and, more important, all active cultures lysed both BALB/c and BALB.K targets. This suggests that the CTL in the bulk culture were, at the time of cloning, already derived from one clone. Clone cr-3 was further expanded and used for specificity analysis. All the following experiments were performed both with the bulk culture and with several CTL clones, yielding identical results. Only data obtained with clone cr-3 will be presented.

Recognition of BALB Minor H Antigens on H-2^d and H-2^k Targets by Clone cr-3 is H-2 Restricted. Fig. 2 shows an experiment in which clone cr-3 was tested against BALB.B, B10, B10.D2, B10.BR, and (B10.D2 × BALB.B)F_1 and (B10.BR × BALB.B)F_1 target cells. As expected, target cells of the two parental strains genetically present in the responder, i.e., B10.BR and B10.D2, were not lysed. Also negative were BALB.B

TABLE IV

Cytotoxic Activity of Clones Derived from the Cross-Reactive CTL Population

Clone	Effector cell dilution	Percent specific lysis of	
		BALB/c	BALB.K
cr-2	1	18.8	36.4
	0.3	12.7	22.0
cr-3	1	17.8	39.4
	0.3	13.5	23.0
cr-4	1	−3.7	−2.0
	0.3	−2.9	−1.6
cr-5	1	−3.8	−0.3
	0.3	−4.6	−1.3
cr-9	1	18.7	38.4
	0.3	15.8	26.8
cr-10	1	13.4	32.1
	0.3	14.9	23.5
cr-15	1	19.3	40.1
	0.3	17.0	31.1
cr-16	1	16.3	28.9
	0.3	11.9	14.7
cr-17	1	4.9	5.2
	0.3	2.6	2.3
cr-19	1	19.7	34.1
	0.3	13.4	18.6

CTL clones were derived as described in Materials and Methods and further expanded in 2-ml culture wells in the presence of irradiated BALB/c stimulator cells and 25% CASUP. 0.7 ml of these cultures were harvested and the cloned cells were assayed in threefold dilutions as given in Materials and Methods against Con A blast cells from BALB/c (spontaneous lysis: 28.9%) and BALB.K (spontaneous lysis: 25.0%).

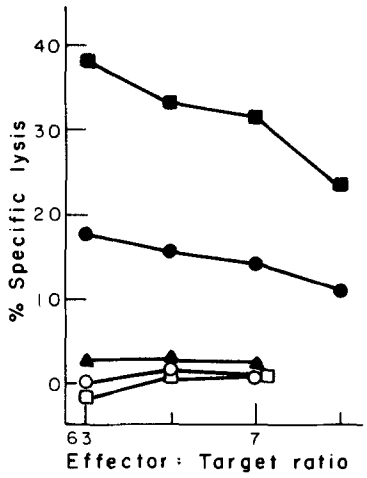

FIG. 2. Lysis of BALB-H-2^d and BALB-H-2^k targets by clone cr-3 is H-2 restricted. Clone cr-3 cells were assayed against LPS-induced blasts of BALB.B (▲), B10.D2 (○), B10.BR (□), (BALB.B × B10.D2)F$_1$ (■), and (BALB.B × B10.BR)F$_1$ (●). Spontaneous lysis varied from 18.3 to 37.9%.

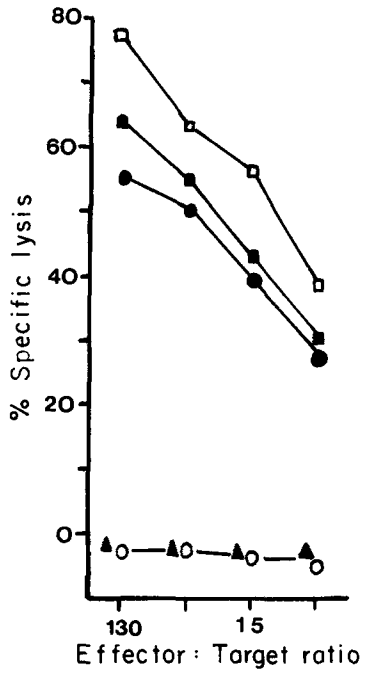

FIG. 3. Killing of BALB targets by clone cr-3 maps to K^k and D^d. Clone cr-3 CTL were assayed against LPS-induced blasts of C3HeB/FeJ (□), C3H.H-2^o (○), BALB/c (●), BALB.K (■), and BALB.HTG (▲). Spontaneous lysis varied from 31.9 to 41.9%.

target cells that express BALB minor H antigens in the context of H-2^b. Target cells derived from crosses between B10.BR or B10.D2 and BALB.B, however, were lysed by the CTL. In these targets, BALB minor H antigens are contributed by the BALB.B parent, and H-2^d or H-2^k antigens are contributed by the other parent. This formally demonstrated that recognition of BALB/c and BALB.K target cells by clone cr-3 is

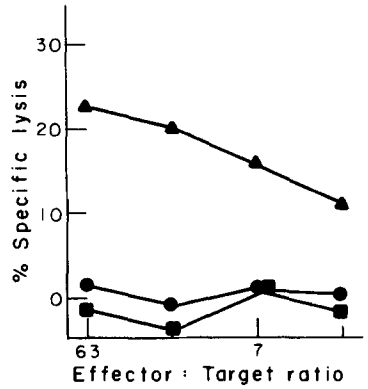

FIG. 4. Clone cr-3 CTL lyse (AKR/J × DBA/2)F_1 but not AKR/J or DBA/2 target cells. Clone cr-3 CTL were assayed against LPS-induced blasts of AKR/J (■), DBA/2 (●), and (AKR/J × DBA/2)F_1 (▲). Spontaneous lysis ranged from 33.8 to 38.1%.

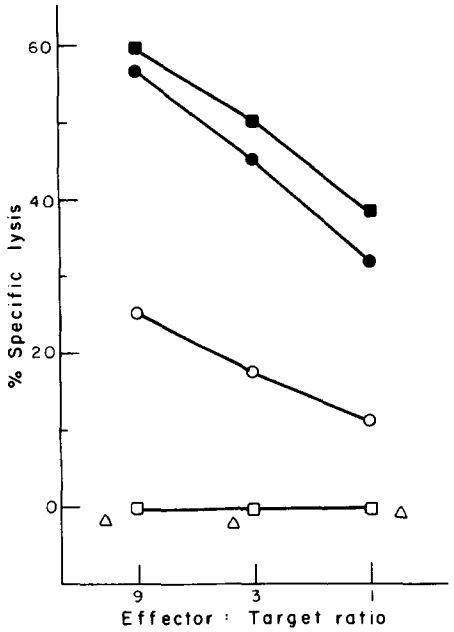

FIG. 5. A minor H antigen shared by BALB and DBA/2 is recognized in H-2^k-restricted but not H-2^d-restricted killing by clone cr-3. Clone cr-3 CTL were assayed against LPS-induced blasts of DBA/2 (△), (DBA/2 × B10.D2)F_1 (□), (DBA/2 × B10.BR)F_1 (○), BALB.K (●), and BALB/c (■). Spontaneous lysis varied from 21.1 to 25.4%.

H-2 restricted and not specific for an H-2-linked antigen mediating unrestricted killing (22–24).

Mapping of H-2 Restriction to K^k and D^d. Minor H antigens are partially shared by mice of various backgrounds. Thus, subsets of CTL populations generated in mice of the B10 background against cells of the BALB background can also lyse, for example, targets of the C3H background, provided the relevant H-2 antigens are also expressed. We have assayed the CTL activity of clone cr-3 against a panel of target cells

expressing all or part of the H-2^d or H-2^k haplotypes on various minor H backgrounds. Those results that led to conclusions about the specificity of clone cr-3 are presented in Fig. 3: C3H (K^kD^k), but not C3H.H-2^o (K^dD^k) blasts were lysed, indicating that the minor H antigen recognized in H-2^k-restricted killing is present on the C3H background and recognized in association with H-$2K^k$. The H-2^d-restricted killing was mapped to the D end of H-2^d by the lysis of BALB/c (K^dD^d) but not BALB.HTG (K^dD^b) target cells.

In K^k- and D^d-restricted Killing, Different Minor H Antigens Are Recognized. Fig. 4 shows a CTL assay in which clone cr-3 CTL were tested against ^{51}Cr-labeled blast cells of the strains DBA/2 (H-2^d), AKR/J (H-2^k), and of the F_1 cross between these two. Although target cells of neither parental strain were lysed, the CTL lysed (AKR/J × DBA/2) targets. This means that either in H-2^k-restricted killing, a minor H antigen is recognized that is present on DBA/2 but not on AKR/J cells, or conversely, that in H-2^d restricted killing an AKR/J minor H antigen absent from DBA/2 is recognized.

An additional experiment (Fig. 5), in which clone cr-3 CTL lysed (B10.BR × DBA/2)F_1, but not (B10.D2 × DBA/2)F_1 (or DBA/2) targets revealed that a minor H antigen shared by BALB and DBA/2 is recognized in H-2^k-restricted but not in H-2^d-restricted killing. We must, therefore, conclude that different minor H antigens associate with K^k and D^d on target cells that can be lysed by clone cr-3.

Further tests have shown that the AKR/J strain lacks both minor antigens, because target cells from AKR/J, (B10.BR × AKR)F_1, and (B10.D2 × AKR)F_1 mice are all not lysed. Also, it should be noted that similar results to those presented here in cold target competition analysis and in F_1-specific lysis have been obtained with clones cr-3 and cr-15 and with subclones of cr-15.

A CTL Line from Normal (H-2^k × H-2^d)F_1 Mice Specific for H-2^d plus Minor H Antigen and for H-2^k plus TNP-BSA. Spleen cells from (C3H × DBA/2)F_1 mice that were primed 3 mo previously by injecting B10.D2 cells were stimulated in MLC on day 0 and day 11 of culture with irradiated B10.D2 cells. At this time, the CTL population lysed B10.D2 targets very well (20% specific lysis at an effector:target ratio of 0.4:1),

TABLE V

Cold Target Competition Analysis of the Cytotoxic Specificity of the Long-term (C3H × DBA/2)F_1 CTL Line

Unlabeled competitor cells added to assay	Percent specific release of ^{51}Cr from	
	^{51}Cr-(B10 × DBA/2)F_1	^{51}Cr-TNP-BSA-C3H
None	41.0	29.8
(B10 × DBA/2)F_1	11.2	10.4
TNP-BSA-C3H	10.9	3.9
B10.BR	36.2	22.1
TNP-BSA-DBA/2	35.8	21.6
(C3H × DBA/2)F_1	34.5	21.5

Long-term MLC cells from (C3H × DBA/2) mice maintained in culture with CASUP 66 d were assayed for lysis of 4 × 10^4 ^{51}Cr-labeled LPS blast targets at a ratio of 7:1 in the presence or absence of 1.2 × 10^6 unlabeled LPS blasts. Spontaneous release of ^{51}Cr was 23.5% for (B10 × DBA/2)F_1 targets and 20.3% for TNP-BSA-C3H targets.

and did not lyse B10.BR or C3H targets, but did give weak lysis of TNP-BSA-C3H targets (20% specific lysis at an effector:target ratio of 25:1). These long-term MLC cells were subsequently stimulated at roughly 12-d intervals by coculture withTNP-BSA-coated C3H cells. CASUP at a final concentration of 25% was present in the medium after the second stimulation.

After 2–4 mo in culture, this long-term CTL line has the following specificity: it gives excellent lysis of B10.D2, TNP-BSA-C3H, and (B10 × DBA/2)F_1 targets but no lysis of B10.BR, B10, DBA/2, C3H, or TNP-BSA-DBA/2 targets. That (B10 × DBA/2)F_1 targets are sensitive to lysis, whereas B10 and DBA/2 targets are not is evidence that the activity is due to H-2^d-restricted recognition of foreign B10 minor H antigen and not to unrestricted killing (22–24).

The evidence that one and the same CTL can be specific for H-2^d plus minor H and for H-2^k plus TNP-BSA in this system comes from cold target competition experiments, an example of which is given in Table V. The lysis of ^{51}Cr-labeled (B10 × DBA/2)F_1 targets is inhibited by homologous targets and by TNP-BSA-C3H targets but not by TNP-BSA-DBA/2 or B10.BR targets. The same pattern of inhibition of ^{51}Cr-release is seen with labeled TNP-BSA-C3H targets as the indicator.

Discussion

The results presented here have implications for two important and related issues concerning T cell specificity: What is the basis of H-2-restricted antigen recognition? How does thymic self-H-2 select the T cell repertoire?

The results that speak to the first point can be summarized as follows. One CTL specific for one H-2 antigen plus conventional antigen X can also recognize a second H-2 antigen plus conventional antigen Y. This was demonstrated in two systems: in the first one, CTL clones were generated that were specific for H-2^d targets expressing one minor H antigen of the BALB background and for H-2^k targets expressing a different BALB minor H antigen. In the second system, we selected a CTL population that recognized B10 minor H antigens on H-2^d cells and TNP-BSA on H-2^k cells.

It is, of course, essential to our argument that indeed the same CTL recognized both H-2^d and H-2^k targets. In both experimental systems used, cold target competition experiments confirmed this (Tables III, V). In addition, the CTL recognizing BALB minor H antigens in association with H-2^k and H-2^d were cloned, without segregation of H-2^k- and H-2^d-restricted killing in any of the active clones isolated (Table IV). We have gone one step further and performed cold target competition experiments with the cloned CTL (data not shown). Again, BALB/c and BALB.K targets efficiently competed the lysis of the other, although the affinity of the cloned CTL appears higher for BALB/c targets than for BALB.K targets.

It is apparent from our analysis of the activity of the cloned CTL designated cr-3 that decreasing the density of the interaction antigen on the target membrane leads to a reduced rate of lysis. For example, compare the lysis of BALB/c targets in Fig. 3 with that of (BALB.B × B10.D2)F_1 targets in Fig 2, or the lysis of BALB.K and (DBA/2 × B10.BR)F_1 targets in Fig 5. In both these F_1 targets the density of the minor BALB antigen and of the restricting H-2 antigen (D^d or K^k) is approximately halved compared with the homozygous BALB target.

We are led to conclude that one CTL clone recognized both the combination of K^k with minor H antigen X and the combination of D^d with minor H antigen Y. In

terms of the altered-self hypothesis (2), which most conveniently explains this finding, this means that different conventional antigens can interact with different H-2 antigens in such a way that the complexes are recognized by the same T cell receptor. Two receptor models (3–5) do not explain our results because their straightforward application implies that CTL restricted to two different H-2 antigens cross-react via the anti-self receptor and should maintain the specificity for conventional antigen X. Since this is not the case, additional mechanisms have to be invoked; e.g., even in dual recognition by two receptors, close physical association between H-2 and X could be required. If K^k and D^d can only associate with two different minor H antigens, then a CTL clone with a cross-reactive anti-H-2 receptor and a cross-reactive anti-minor H receptor could explain the experimental data.

The most elegant explanation is given by the altered-self hypothesis, which predicts that self-H-2 and X are not recognized as separate entities but only the complex or interaction antigen can be bound by the T cell receptor. This hypothesis has also recently found support from the experiments of Kappler et al. (25). These workers fused antigen-specific, Ia-restricted T cells into T cell hybridomas that could then be induced to produce interleukin 2 when stimulated with antigen presented on cells of the appropriate Ia haplotype. Multiple fusions of T cells specific for different combinations of antigen and Ia into the same T cell hybridoma resulted in cell lines that were inducible only with the combinations of Ia and conventional antigens that the T cells were originally specific for. This lack of independent assortment of self H-2 recognition is most easily, though not exclusively, explained by T cell receptors specific for each combination of antigen and H-2.

Finally, the conditions under which we were able to demonstrate CTL specific for two combinations of major and minor H antigens should be discussed since it is relevant to the selection of the T cell repertoire. Briefly, we have primed (A × B)F_1 → A bone marrow radiation chimeras of one minor H background to minor H antigens of another background and selected for anti-minor H CTL restricted to B, i.e., the H-2 type absent from the chimeric host. It is well-established that when such chimeras are stimulated with antigen presented on (A × B)F_1 H-2 heterozygous cells, the vast majority of responding T cells is restricted to the H-2 type of the chimeric host A (Table I), or, in thymic chimeras, to the H-2 type of the thymus (3, 7). It was the nonabsoluteness of this self-preference (6–10), however, that led us to study the origin of B-restricted CTL in these chimeras. We wondered if in a CTL population restricted to non-self, the influence of self-H-2 antigens during T cell ontogeny might be detectable. We found that in two out of eight chimeras studied, anti-minor H CTL selected for restriction to B cross-reacted on target cells expressing the minor H antigen(s) plus A (Table II). We have never found such cross-reactive CTL in the anti-minor H response from normal (A × B)F_1 animals or in the A-restricted response of (A × B)F_1 → A chimeras (Table II). We explain this in the following way: Under the influence of thymic H-2 during T cell ontogeny, T cells are selected in such a way that they can recognize antigens that are close to self (26), i.e., interaction antigens formed by self-H-2 with conventional antigens. Such a selection can, by its very nature, not be absolute but will also generate T cell receptors capable of recognizing foreign H-2 antigens by themselves or in association with conventional antigens. As shown here, the same T cell that recognizes self-H-2 plus antigen X can, in fact, also be specific for foreign H-2 plus antigen Y. We suggest that in normal H-2 heterozygous

(A × B)F_1 mice, such cross-reactive T cells usually remain undetected because T cell responses restricted to A or B will largely make use of the portion of the T cell repertoire that has been selected for the recognition of A- or B-like interaction antigens, respectively. In $F_1 \rightarrow$ A chimeras, on the other hand, a B-restricted response, such as the one studied here, can only draw from the T cell repertoire selected for the recognition of A-like interaction antigens. Therefore, if the B-restricted response to a sufficient number of antigens is studied in such chimeras, CTL can be detected that are also specific for A plus foreign antigen.

As we have shown by the selection of CTL specific for minor H antigens plus H-2^d and for TNP-BSA plus H-2^k from a normal (H-2^d × H-2^k)F_1 mouse, T cells specific for more than one pair of H-2 and conventional antigens are, indeed, present in normal mice as well. We feel that in this particular experimental approach, the unusually strong immunogenicity of H-2K^k plus TNP (27) allowed us to detect these cross-reactive T cells without artificially biasing the T cell repertoire towards one of the parental H-2 haplotypes.

Taken together with earlier results on the self-H-2 preference of alloreactive T cells (26, 28), the present data suggests that all mature CTL precursors have been selected for the recognition of antigens that are close to self.

Summary

Radiation chimeras prepared by injecting H-2 heterozygous F_1 stem cells into lethally irradiated parental hosts show a marked, but not absolute, preference for host-type H-2 antigens in the H-2-restricted cytotoxic T lymphocyte (CTL) response to minor histocompatibility (minor H) antigens. We have selected for the anti-minor H CTL that are restricted to the parental H-2 type absent from the chimeric host and found that in two out of eight cases, such CTL lysed target cells of either parental H-2 type. From one of these CTL populations that lysed H-2^d and H-2^k target cells expressing BALB minor H antigens, clones were derived and further analyzed. The results showed that: (*a*) lysis of both H-2^d and H-2^k target cells was H-2 restricted; (*b*) H-2^d restriction mapped to D^d, and H-2^k restriction mapped to K^k; (*c*) testing against various H-2^d and H-2^k strains of different and partially overlapping minor H backgrounds as well as against the appropriate F_1 crosses revealed that in D^d- and K^k-restricted killing, different minor H antigens were recognized. In a second system, a CTL population was selected from normal (H-2^d × H-2^k)F_1 mice that was specific for H-2^d plus minor H antigens and for H-2^k plus trinitrophenylated bovine serum albumin. We interpret these findings in terms of the altered-self hypothesis: The association of one H-2 antigen with one conventional antigen X may be recognized by the same T cell receptor specific for the complex formed by a different H-2 antigen in association with a second conventional antigen Y. The implications of these observations for the influence of self H-2 on the generation of the T cell receptor repertoire are discussed.

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