SELF RECOGNITION BY T CELLS

I. Bystander Killing of Target Cells Bearing Syngeneic MHC Antigens

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T cell activation is restricted in the sense that T cells respond to antigens only in the context of self MHC antigens (1). It has been shown (2–4) that T cells learn about self in the thymus but the unanswered question is, how. Approximately 95% of the cells in the thymus die in situ (5, 6). To account for the low survival rate, and for MHC restriction, it has been proposed that the majority of immature thymocytes, whose receptors have little or no affinity for self MHC, do not receive the proper maturational signals and undergo programmed cell death (7, 8). Cells that are strongly self-reactive are deleted (9–12). The small proportion of thymocytes whose receptors have the "right" affinity (low but real) for self MHC antigens are positively selected to survive and are exported to the periphery. These cells would bind weakly to self alone, but they might bind strongly, that is, with activating affinity, to self plus antigenic peptides (13). This model predicts that all mature T cells should be able to recognize the selecting self-MHC molecules. However, the binding of T cells with cells bearing self MHC in the absence of specific antigen cannot, by definition, result in activation. With this limitation in mind can we determine if self recognition exists?

After binding to its specific target, an allospecific cytotoxic T cell becomes activated and kills the target. If this same CTL were also able to bind to a cell bearing syngeneic MHC antigens, lysis would not be observed because such an interaction would be of too low affinity to result in activation. However, Lanzavecchia has shown that once activated, cytotoxic T cells can kill any target to which they bind or by which they are bound (14). Based on the proposition that all mature T cells including CTL have been selected for low but positive affinity for self, an assay system has been developed to examine whether cloned allospecific CTL, after activation, can kill cells bearing syngeneic, but not third-party MHC antigens. The results of these investigations provide the first clear demonstration that CTL have detectable affinity for self MHC and support the positive selection model of T cell maturation.

Materials and Methods

Animals. CBA/J, C57Bl/6J, B10.D2, B10.BR, A/J, C3H.OH, and BALB/cBy mice purchased from The Jackson Laboratories (Bar Harbor, ME) were used as spleen donors.

Tissue Culture Medium. RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 50 μg/ml gentamycin, 2 mM L-glutamine, and 50 μM 2-ME, was used as tissue culture medium

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TABLE I

Target and Effector Cells Used in this Study

Cell	Name	MHC	Origin
Targets	EL-4	H-2^b	C57Bl/6 T cell lymphoma
	CTLL-2	H-2^b	C57Bl/6 T cell
	TIMI.4	H-2^b	C57Bl/6 thymoma
	P815	H-2^d	DBA/2 mastocytoma
	S49.1	H-2^d	BALB/c thymoma
	WEHI 7.1	H-2^d	BALB/c thymoma
	A20	H-2^d	BALB/c B cell lymphoma
	PSW	H-2^d	BALB/c lymphoma
	BW5147.3	H-2^k	AKR/J thymoma
	R1.1	H-2^k	C58/J thymoma
	C1.18.4	H-2^k	C3H myeloma
	L929	H-2^k	C3H fibroblast
	Raji	—	Human B cell lymphoma
CTL*	DAB-1,2,3,6,7,11	H-2^d	DBA/2 anti-C57Bl/6‡
	DAB-16,17	H-2^d	BALB/c anti-C57Bl/6
	KAD-1,2,3,4	H-2^k	CBA anti-BALB/c
	KAD-32	H-2^k	CBA anti-BALB/c
	BAD-1,2,3,4,5	H-2^b	C57Bl/6 anti-BALB/c
	BAD-61	H-2^b	C57Bl/6 anti-BALB/c

* CTL are grouped according to the cloning from which they were obtained.
‡ Clones were a generous gift of Dr. Joseph Portanova, Denver, CO.

(TCM).[1] Cell-free supernatants from 24-h Con A-stimulated rat spleen cell cultures (CAS), containing 20 mM α-methyl mannoside, were used as a source of IL-2.

Cells. Mice were killed by cervical dislocation. Spleens were obtained under aseptic conditions and single-cell suspensions prepared by gently pressing these organs through wire mesh screens. Murine and human tumor cell lines were used as targets in chromium release assays (Table I).

Con A-stimulated T Cell Blasts. Con A blasts were prepared by incubating 30 × 10^6 spleen cells in 10 ml TCM containing 2.5 μg/ml Con A (Type IV; Sigma Chemical Co., St. Louis) at 37°C for 72 h. Viable cells isolated by Ficoll-Hypaque density gradient centrifugation were propagated for an additional 10 d in TCM containing 20% CAS before their use as targets in ^{51}Cr-release assays.

LPS-stimulated B Cell Blasts. LPS blasts were prepared by incubating 30 × 10^6 spleen cells in 10 ml TCM containing 25 μg/ml LPS (*Salmonella typhimurium*; RIBI ImmunoChem Research, Inc., Hamilton, MT) and 20 μg/ml dextran sulfate (500,000 mol wt, 17% sulfate; Pharmacia Fine Chemicals, Uppsala, Sweden) at 37°C for 72 h. Viable cells isolated by Ficoll-Hypaque density-gradient centrifugation were resuspended in 5 ml TCM and layered over 5 ml of 50% isotonic Percoll (Pharmacia Fine Chemicals) in 17 × 100 mm Falcon tubes. After centrifugation (750 *g*, 20 min, 22°C), the B cell blasts were carefully removed from the TCM-Percoll interface using a Pasteur pipette. The cells were washed once and resuspended in TCM for ^{51}Cr labeling.

CTL Clones. Responder spleen cells (8 × 10^6) and mitomycin C-treated allogeneic stimulator spleen cells (2 × 10^6) were cultured together in 24-well tissue culture plates in 2 ml of TCM per well. After 7 d, CTL were harvested by Ficoll-Hypaque density gradient centrifugation. Lymphoblasts (10^6) were restimulated with mitomycin C-treated allogeneic spleen

[1] *Abbreviations used in this paper:* CAS, Con A-stimulated rat spleen cell cultures; TCM, tissue culture medium.

cells (10^7) in 10 ml of TCM per 25-cm^2 Falcon flask. After four rounds of weekly restimulation in the absence of added IL-2, viable CTL were cloned by limiting dilution in TCM containing 20% CAS and allogeneic spleen cells in 96-well flat-bottomed microtiter plates after the method of Glasebrook and Fitch (15). The clones thereby obtained were maintained by weekly stimulation with mitomycin C–treated allogeneic spleen cells in TCM containing 20% CAS; these clones do not replicate with IL-2 alone but must be propagated in the presence of the appropriate allostimulators. CTL clones were given the following designations based on their allospecificities: DAB (H-2^d anti-H-2^b); KAD (H-2^k anti-H-2^d); and BAD (H-2^b anti-H-2^d) (Table I). CTL were used in cytotoxic assays on days 7, 8, and 9 after stimulation.

^{51}Cr Labeling of Target Cells. Cells to be used as targets in ^{51}Cr-release assays were suspended at 1–5 × 10^6 in 100 μl of RPMI 1640 medium containing 5% FCS to which 100 μCi of ^{51}Cr (sodium chromate; ICN Radiochemicals, Irvine, CA) were added. The cells were then incubated for 90 min at 37°C, washed three times in RPMI 1640, and diluted in TCM for use.

^{51}Cr-release Assays. The experimental design and details of the three killing assays used in this investigation are shown in Fig. 1 and are discussed in the Results. In brief, ^{51}Cr-labeled target cells (5 × 10^3 cells in 50 μl TCM) were placed in individual wells of 96-well round-bottomed microtiter plates that contained either 50 μl TCM (direct killing assay), unlabeled stimulator cells (10^4) in 50 μl TCM (bystander killing assay), or Con A (20 μg/ml) in 50 μl TCM (lectin-mediated killing assay). 2 × 10^4 CTL in 100 μl TCM were immediately added, the plates were centrifuged (50 *g* for 5 min) to establish cell contact and incubated at 37°C. After 6 h of incubation, the plates were centrifuged at 200 *g* for 10 min and 100 μl of cell-free supernatant were collected from each well. Radioactivities in supernatants were

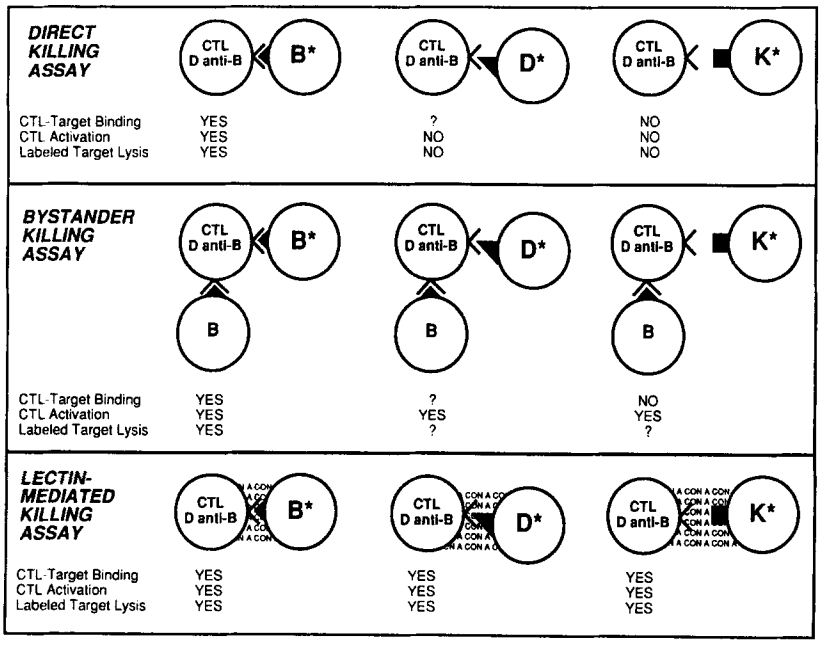

FIGURE 1. Experimental design. In the direct killing assay, 2 × 10^4 allospecific cloned CTL were incubated with 5 × 10^3 ^{51}Cr-labeled target cells of various MHC haplotypes. In the bystander killing assay, 10^4 unlabeled target cells bearing the activating alloantigens were included with the CTL and ^{51}Cr-labeled targets. The lectin-mediated killing assay was identical to the direct killing assay with the addition of Con A to a final concentration of 5 μg/ml. Specific lysis was measured after 6 h of incubation.

measured in a gamma counter. Percent specific lysis was calculated by the following formula: percent specific lysis = $100 \times [(cpm_{exp} - cpm_{spont})/(cpm_{max} - cpm_{spont})]$. Maximal release ($cpm_{max}$) was determined from supernatants of cells subjected to detergent lysis with 0.5% Triton X-100. Spontaneous release (cpm_{spont}) was determined from target cells incubated without added CTL.

Statistical Methods. Results are presented as the means ± SD for duplicate determinations. For significance determinations in the bystander killing assays, percent specific lysis values obtained in bystander and direct killing assays for a given ^{51}Cr-labeled target/CTL combination were compared using the Student's *t* test.

Results

Experimental Design. The experimental design for examining whether activated CTL can recognize and kill cells bearing self MHC antigens is shown in Fig. 1. Three types of cytotoxic assays were used. These were termed: (*a*) direct killing assay; (*b*) bystander killing assay; and (*c*) lectin-mediated killing assay. In the direct killing assay, the CTL are exposed to a single, chromium-labeled target; effector/target cell interactions that do not lead to activation of the CTL do not lead to cytolysis. In the bystander killing assay unlabeled "stimulator" cells bearing the appropriate allogeneic MHC antigens are used to activate the CTL, and ^{51}Cr-labeled target cells to detect cytotoxicity. If Lanzavecchia (14) is correct, activated CTL should kill any ^{51}Cr-labeled target cell to which they can bind. If activated CTL have sufficient binding affinity for self then chromium-labeled target cells bearing syngeneic but not "third-party" MHC antigens will be killed as bystanders. To show that failure of activated CTL to kill third-party bystander targets was due to a lack of recognition rather than resistance to lysis, the lectin-mediated killing assay was used. In this assay, Con A provides the activating signal and the "glue" allowing the CTL to bind and kill any target independent of MHC recognition (16).

Bystander Killing of Target Cells Bearing Syngeneic but not Third-Party MHC Antigens by Activated CTL. In a typical experiment, the CTL clone DAB-3 (H-2^d anti-H-2^b) was used in the three killing assays described above with ^{51}Cr-labeled cells of H-2^b, H-2^d, H-2^k and human origin (Table II). As predicted by their specificity, DAB-3 killed only H-2^b target cells in the direct killing assay; H-2^d (syngeneic) and H-2^k or human (third-party) targets were not lysed. In contrast, if unlabeled EL-4 (H-2^b) cells were included in the assay to act as "stimulator" cells then bystander killing of chromium-labeled target cells bearing H-2^d *but not* third-party MHC antigens was observed (Table II, *underscored data*). Although used as controls in the bystander killing assay, ^{51}Cr-labeled EL-4 and CTLL-2 are killed directly, and not as bystanders, because they bear the allelic MHC antigens (H-2^b) for which DAB-3 are specific. All of the target cells were killed in the presence of Con A, indicating that the inability of "activated" CTL to kill third-party targets in the bystander killing assay was not due to an inherent resistance to lysis.

To test whether bystander killing of syngeneic target cells was a general characteristic of activated CTL, the three killing assays were performed using 19 cytotoxic T cell clones from 6 independent clonings and a variety of chromium-labeled targets. Each of the assays was performed at least twice with all of the CTL-target cell combinations and in any given experiment at least six different CTL clones and eight targets were used in all three assays. The various CTL clones killed only their allospecific targets in the direct killing assay (data not shown). Representative results

TABLE II

Bystander Killing of Target Cells Bearing Syngeneic (H-2^d) but not Third-party MHC Antigens by DAB-3, an H-2^d anti-H-2^b-specific CTL Clone

^{51}Cr-labeled target	MHC	Percent specific lysis in: Direct killing assay	Bystander* killing assay	Lectin-mediated killing assay
EL-4	H-2^b	82 ± 4	82 ± 3‡	52 ± 6
CTLL-2	H-2^b	82 ± 1	80 ± 8‡	29 ± 1
P815	H-2^d	0 ± 0	36 ± 0§	58 ± 3
A20	H-2^d	0 ± 1	54 ± 0	35 ± 1
S49.1	H-2^d	0 ± 3	36 ± 2	34 ± 3
BW5147.3	H-2^k	0 ± 1	5 ± 0	65 ± 3
L929	H-2^k	0 ± 4	0 ± 1	29 ± 5
Raji	Human	0 ± 0	0 ± 0	42 ± 5

* Unlabeled EL-4 (H-2^b) were used to activate DAB-3 in the bystander killing assay.

‡ Although used as controls in the bystander killing assay, these targets, being H-2^b, are being killed directly by DAB-3.

§ Statistically significant values ($p < 0.01$) comparing specific lysis for each target in bystander vs. direct killing assays are underscored.

from bystander killing assays are shown in Table III. In the presence of unlabeled stimulator cells bearing allospecific MHC antigens (EL-4 [H-2^b] for DAB [d anti-b] clones; P815 [H-2^d] for KAD [k anti-d] and BAD [b anti-d] clones), all of the clones tested killed ^{51}Cr-labeled target cells bearing MHC antigens syngeneic to the CTL (*underscored data*). Third-party target cells, bearing neither syngeneic nor allospecific MHC antigens, were not killed as bystanders by the activated CTL. In lectin-mediated killing assays, all target cell lines were killed by all T cell clones (data not shown).

To show that syngeneic bystander killing was restricted to recognition of MHC-encoded molecules, LPS-stimulated spleen cell blasts derived from MHC-congenic mice were used as chromium-labeled target cells in specific and bystander killing assays. A representative experiment is shown in Table IV. Both direct and bystander killing of ^{51}Cr-labeled LPS blasts were MHC restricted. For example, in the direct killing assay mediated by the CTL clone DAB-16 (d anti-b), only blasts bearing H-2^b, the allospecific MHC antigens, were killed. In the bystander assay, this same clone killed chromium-labeled LPS blasts derived from C57Bl/6 (allospecific, H-2^b, 54% lysis) and B10.D2 (syngeneic, H-2^d, 57% lysis) mice; nonsignificant bystander killing (7% lysis) of LPS blasts derived from B10.BR (H-2^k) mice was observed. Thus, as was found with the tumor cell targets (Tables II and III), bystander killing of MHC-congenic LPS blasts was restricted to those bearing MHC antigens syngeneic to the individual CTL clones tested.

Fine Specificity of Syngeneic Bystander Killing. Antigen-specific as well as alloreactive CTL clones are highly specific and have been shown to be directed against a single MHC determinant, eg., H-2D, H-2K, or H-2L (17–19). Our results using target cells derived from MHC-congenic mice showed that syngeneic bystander killing is restricted to recognition of MHC-encoded molecules but failed to establish the fine

TABLE III

Bystander Killing of Target Cells Bearing Syngeneic but not Third-party MHC Antigens by a Variety of CTL Clones

		Percent specific lysis in a bystander* killing assay mediated by:																		
		DAB-clones (d anti-b)								KAD-clones (k anti-d)					BAD-clones (b anti-d)					
^{51}Cr-labeled target	MHC	1	2	3	6	7	11	16	17	1	2	3	4	32	1	2	3	4	5	61
EL-4	b	75	80	82	88	84	86	88	80	2	0	0	0	0	27	12	17	32	31	37
CTLL-2	b	63	77	80	75	54	75	41	—‡	0	0	3	6	—	—	—	—	—	—	—
TIMI.4	b	64	80	72	80	—	81	—	—	3	14	7	0	—	—	—	—	—	—	—
P815	d	51§	48	36	54	73	57	35	72	64	77	72	72	54	63	35	54	89	88	92
A20	d	43	59	54	31	56	26	—	42	96	90	91	94	—	—	—	—	—	—	—
S49.1	d	59	50	36	52	44	37	28	59	70	45	69	45	—	—	—	—	—	—	—
WEHI 7.1	d	63	50	53	79	—	58	26	—	84	92	77	83	—	—	—	—	—	—	—
PSW	d	75	74	73	79	63	57	—	—	59	79	75	71	—	—	—	—	—	—	—
BW5147.3	k	19	16	5	8	12	14	12	0	21	26	21	26	—	—	—	—	—	—	—
L929	k	0	5	0	1	0	0	3	1	16	18	19	21	—	—	—	—	—	—	—
C1.18.4	k	0	5	2	0	—	0	—	—	48	46	38	30	—	—	—	—	—	—	—
R1.1	k	—	—	—	5	—	—	—	—	—	—	—	—	36	4	0	1	10	0	2
Raji	human	0	0	0	0	0	2	0	0	2	2	3	1	—	—	—	—	—	—	—

* For the bystander killing assays, unlabeled EL-4 were used to activate the DAB clones; unlabeled P815 were used to activate the KAD clones.

‡ Not determined.

§ Statistically significant values ($p < 0.02$) comparing specific lysis for each target-CTL combination in bystander vs. direct killing assays are underscored.

specificity of this self recognition. Therefore, cells derived from MHC-recombinant animals were used as targets to determine the fine specificity of syngeneic bystander killing (Table V). Four of five DBA/2 anti-C57Bl/6 (d anti-b) CTL clones killed P815 ($K^d/D^d/L^d$) and C3H.OH-derived targets (K^d/D^k) *but not* A/J-derived targets ($K^k/D^d/L^d$) or R1.1 (K^k/D^k). Thus, the fine specificity of syngeneic bystander killing

TABLE IV

Both Direct and Bystander Killing are MHC Restricted

^{51}Cr-labeled target	Killing assay	Percent specific lysis induced by: DAB-11	DAB-16	BAD-61	KAD-32
B6 blasts ($H\text{-}2^b$)	Direct	46 ± 1	54 ± 4	10 ± 0	8 ± 4
	Bystander*	58 ± 13	52 ± 2	39 ± 9‡	11 ± 1
	Lectin-mediated	51 ± 4	67 ± 2	57 ± 4	82 ± 22
B10.D2 blasts ($H\text{-}2^d$)	Direct	0 ± 1	8 ± 2	74 ± 24	100 ± 27
	Bystander	57 ± 3	57 ± 12	91 ± 8	100 ± 4
	Lectin-mediated	42 ± 1	51 ± 9	100 ± 27	71 ± 9
B10.BR blasts ($H\text{-}2^k$)	Direct	15 ± 3	1 ± 0	0 ± 1	11 ± 0
	Bystander	13 ± 2	7 ± 2	13 ± 6	33 ± 5
	Lectin-mediated	47 ± 10	56 ± 5	56 ± 2	47 ± 6

* For bystander killing, unlabeled EL-4 were used to activate DAB-11 and DAB-16 and unlabeled P815 were used to activate BAD-61 and KAD-32.

‡ Statistically significant values ($p < 0.05$) comparing specific lysis for each target-CTL combination in bystander vs. direct killing assays are underscored.

TABLE V

Fine Specificity of Syngeneic Bystander Killing

Exp.	^{51}Cr-labeled target	H-2 phenotype K	I	D/L	Percent specific lysis in bystander* killing mediated by DAB-clones (d anti-b)‡ 1	2	3	6	11	17
1	P815	d	–	d	34§	32	44	38	37	29
	C3H.OH LPS blasts	d	d	k	32	32	37	30	31	10
	A/J LPS blasts	k	k	d	0	5	22	8	0	39
	R1.1	k	–	k	7	12	9	11	9	9
2	WEHI 7.1	d	–	d	43	47	38	36	45	50
	P815	d	–	d	60	64	53	60	58	32
	C3H.OH Con A blasts	d	–	k	50	51	46	39	48	4
	A/J Con A blasts	k	–	d	2	5	38	7	2	45
	YAC-1	k	–	d	0	10	41	10	1	53
	R1.1	k	–	k	6	3	2	1	7	8
Fine specificity of bystander killing					K^d	K^d	d	K^d	K^d	D^d/L^d

* Unlabeled EL-4 cells were used as stimulators in bystander killing assays.

‡ DAB-1, 2, 3, 6, and 11 are of DBA/2 origin; DAB-17 is of BALB/c origin.

§ Statistically significant values (Exp. 1, $p < 0.05$; Exp. 2, $p < 0.01$) comparing specific lysis for each target-CTL combination in bystander vs. direct killing assays are underscored.

mediated by these four clones, DAB-1, 2, 6, and 11 is H-2K^d. Using the same analysis the fine specificity of self recognition by the single BALB/c anti-C57Bl/6 (d anti-b) clone tested (DAB-17) was determined to be H-2D^d/L^d. Of interest, the remaining CTL clone DAB-3 (DBA anti-C57Bl/6) killed cells bearing either H-2K^d or H-2D^d/L^d as syngeneic bystanders.

Cloned CTL Are Not Susceptible to Syngeneic Bystander Killing. Activated CTL can recognize and kill target cells bearing syngeneic MHC antigens; however, CTL themselves bear self MHC antigens and should be killed as bystanders. If this were true, how could CTL be propagated in vitro if each time they were stimulated they killed one another? To address this paradox, CTL clones were used as target cells in bystander killing assays (Table VI). Syngeneic tumor cell targets were killed as bystanders;

TABLE VI

Cloned CTL Are Not Susceptible to Syngeneic Bystander Killing

Exp.	Group	^{51}Cr-labeled target	Percent specific lysis in bystander* killing mediated by: DAB-2	DAB-6
1	A	EL-4 (H-2^b)	80 ± 12	88 ± 4
	B	P815 (H-2^d)	48 ± 0	54 ± 3
	C	DAB-6 (H-2^d)	0 ± 1	0 ± 0
	D	BW5147.3 (H-2^k)	16 ± 1	8 ± 4
2	E	TIMI.4 (H-2^b)	80 ± 1	80 ± 3
	F	A20 (H-2^d)	45 ± 6	52 ± 1
	G	DAB-2 (H-2^d)	0 ± 1	0 ± 1
	H	C1.18.4 (H-2^k)	5 ± 1	0 ± 2

* Unlabeled EL-4 were used as stimulator cells.

however, DAB-2 and DAB-6 CTL clones were not (compare groups B and C and groups F and G). Similar results have been found for other CTL clones (data not shown).

Discussion

When a developing T lymphocyte rearranges its α and β antigen receptor genes, the process, it is assumed, is random and the two loci are rearranged independently. If this is true, then thymocytes must develop that bear all the receptors available to the individual. The environment places two constraints on the ultimate repertoire of the mature T lymphocytes: no cells should be strongly self-reactive while most or all cells should be self-restricted. These requirements can be met in the context of a model that deals with the affinity of binding of a T cell's receptor to "self" within the thymus. If, according to this model, an immature thymocyte binds a structure (usually MHC) on the surface of a bone marrow–derived cell (10, 20) with high affinity, the thymocyte is aborted. "High affinity" here is a purely operational term, meaning in the same range as would activate a mature T cell. Abortion of high affinity, self-reactive T cells assures that T cell autoreactivity is a rare event. Another way of putting this is that "negative" selection is very efficient, and there is experimental evidence to support that idea (9–12).

If receptors are randomly generated, and because somatic mutation of expressed receptors does not seem to occur (21, 22), the majority of developing thymocytes will have virtually no affinity for the MHC of the thymus they find themselves in. According to the model, these cells are neither positively nor negatively selected, and die in situ, their death being programmed (7, 8, 10). But a small proportion of thymocytes will have affinity for self-MHC that is too low to activate a T cell, but that could, for example, allow transient binding to a thymic epithelial cell and reception of a trophic signal from it. This signal would trigger maturational events such as acquisition of homing markers (23), glucocorticoid resistance (24), loss of CD4 or CD8 (25, 26), and export to the periphery. "Positive" selection for T cells with low affinity for self-MHC leads to self restriction in that these cells may have high affinity for self plus foreign antigens (2, 3, 13).

A prediction of the self-recognition model of T cell maturation, or positive-selection model as it has also been called, is that all mature T cells will have affinity for the self-MHC molecules used for selection; however, this affinity will not be high enough to activate the T cell and the interaction will be difficult to observe. The present studies were based on the notion that low affinity interactions between T cells and other cells bearing syngeneic MHC molecules could be observed if the T cells were first activated. The rationale for this idea was as follows. Antigen-driven T cell activation can be readily detected in vitro by assays that measure proliferation or acquisition of an effector function, e.g., lymphokine production or cytotoxicity. Self recognition by T cells cannot result in activation, and this binding, if it exists, cannot be detected by these conventional assays. Lanzavecchia has shown that once activated, CTL can kill any cell to which they are bound (14). Therefore, an assay system was developed to test the idea that activated allospecific CTL clones, by virtue of their ability to recognize self, could kill bystander cells bearing syngeneic but not third-party MHC antigens. This was found to be the case, and the results lend support to the self-recognition model of T cell repertoire selection.

Alloreactive CTL clones were used in killing assays on days 7–9 after restimulation with allogeneic spleen cells, a time at which they were likely to be in an inactivated or resting condition (27). When mixed with ^{51}Cr-labeled targets alone in the "direct" killing assay, the CTL clones killed only their allospecific targets; any interactions with target cells bearing syngeneic or third-party MHC antigens must have been below the threshold of activation required to elicit CTL effector function (13). However, after activation of the CTL by unlabeled allospecific target cells, the underlying ability of these effector T cells to recognize and bind to cells bearing self-MHC antigens was revealed in the "bystander" killing assay (Tables II–IV). The capacity of activated CTL clones to recognize self and not third-party MHC antigens was absolute in this relatively small sample; when activated, none of the 19 clones failed to kill syngeneic bystander targets and likewise no clones were found that killed bystanders bearing third-party MHC antigens (Table III). The failure of activated clones to kill third-party bystander targets was likely due to lack of recognition of third-party MHC antigens; all of the clones were able to kill all of the various targets in the presence of Con A (Tables II, IV, and data not shown).

As has been shown for antigen-specific killing (17–19), syngeneic bystander killing is restricted to a single MHC determinant; self-recognition by 5 of 6 H-2^d anti-H-2^b CTL clones tested was found to be restricted to either H-2K^d or H-2D^d/L^d *but not* both (Table V). This result provides the strongest support for the positive selection model of T cell maturation in that most T cells with "low but real" affinity for self-H-2K would not be expected to also have this "right" affinity for self-H-2D/L. It is reasonable to assume, therefore, that DAB-3, the only clone tested that killed bystander targets bearing H-2K^d or H-2D^d/L^d, is coincidentally crossreactive or is not a true clone. Subcloning of DAB-3 is currently being done in order to distinguish between these possibilities.

Further experiments are in progress to determine whether self recognition in the absence of specific antigen is characteristic of all T cells. Our results clearly show that activated CTL can recognize, bind, and kill bystander target cells bearing syngeneic MHC antigens, confirming the prediction of the self-recognition model that mature T cells should have low but detectable affinity for the self-MHC molecule used for their selection. In conjunction with the recent reports from several groups studying T cell development in vivo (12, 28–30), the present findings provide strong support for the model itself; that T cell repertoire selection is directed by self-MHC, or more concisely, by the ability of a small number of T cell precursors to recognize self-MHC antigens in the thymus allowing them to be positively selected to survive and mature (2–4).

Several additional points concerning syngeneic bystander killing should be considered. If syngeneic bystander killing also occurs in vivo, two potential problems need to be addressed. First, since CTL bear MHC antigens shouldn't they kill one another once activated? Second, in a virally infected tissue, for example, shouldn't uninfected cells be killed by MHC-restricted, virus-specific CTL? The data presented in Table VI address the first question and show that CTL are themselves resistant to syngeneic bystander killing. Consistent with this observation, several investigators have found that CTL clones are resistant to CTL-mediated cytolysis (31–33) and so the lack of killing of cloned CTL as bystander targets is probably not due to a lack of recognition by the activated CTL, but to relative resistance to killing. The

second question was not directly investigated. However, "innocent bystander" damage often occurs in tissues during immune responses in vivo, and while this damage is usually attributed to nonspecific phagocytes recruited to the site of inflammation, there is no evidence to refute the idea that some immunopathology may be due to syngeneic bystander killing by CTL. In addition, preliminary results suggest that CTL are able to kill bystanders only for a short time after activation (data not shown), and perhaps, as suggested by Lanzavecchia (14), only when a three-cell conjugate of CTL, specific target, and syngeneic target exists. Thus the in vitro conditions used to demonstrate bystander killing favor rapid and preferential formation of the appropriate conjugates, whereas in vivo such conditions may not exist.

Bystander killing by activated CTL clones has been observed by a number of groups (34–37); however, a preference for bystander targets bearing MHC antigens syngeneic to the effector cell has not been noted. In many of these studies the CTL were activated by much more extreme protocols than those used in the present study. Thus, bystander killing has been observed when the effector cells are activated by extensive crosslinking of the CTL receptor with specific antibodies (34, 35), or by calcium ionophores and phorbol esters (36, 37). Because these protocols may lead to increased conjugate formation or unusually prolonged activation, it is difficult to establish their bearing on the results presented here. With regards to bystander killing it is important to note that self recognition was not observed by Lanzavecchia in his "backwards" killing experiments (14), which formed the basis for the present syngeneic bystander killing assay. In his study, a single syngeneic bystander target was tested, this being a Th clone syngeneic to the CTL and which, like the CTL, bound to and was activated by the unlabeled stimulator cell. Whereas a third-party Th clone that bound directly to the CTL was killed as a bystander, the syngeneic Th clone was not. The complexity of this system makes interpretation difficult; however, Lanzavecchia remarks that Th clones are poor "stimulators" and therefore it is also possible that they are poor syngeneic bystander targets. This question is currently under investigation.

A final reference should be made concerning the observations that T cells do not respond to intact antigens but to antigenic peptide fragments bound within a cleft of the self-MHC molecules on APCs (38–40). In terms of the self-recognition model presented above, this finding would suggest that T cells are positively selected not by self-MHC alone but by self-MHC plus some self-peptide. If T cell repertoire selection is based on low affinity for self-MHC plus a self-peptide chosen at random, then the strict self recognition by activated CTL might be explained by one of the following. First, the target cells chosen for this experiment must present several self-peptides in the context of self-MHC including those used for positive selection of the CTL clones themselves during their maturation in the thymus. The possibility then exists that a target cell could be found that is not susceptible to syngeneic bystander killing because it does not bear the particular self-peptide. Second, as suggested by a model of positive selection in a recent review by Fink (41), the number of self-peptides used for selection may be quite limited and these are perhaps presented by all cells. This would explain why syngeneic bystander killing was observed in every CTL-bystander combination tested. Third, it is possible that selection occurs based on recognition of polymorphic determinants common to self-MHC regardless of the peptide bound, an idea consistent with the recent findings of MacDonald

et al. (28). This last explanation seems to be the most satisfactory as it is difficult to understand how strict self-MHC-restriction could result from selection based in part on randomly chosen self-peptides. Experiments are in progress to elucidate these possibilities.

Summary

Activated CTL can kill any cell to which they bind or by which they are bound. This observation has been used to determine whether alloreactive CTL can recognize cells bearing self-MHC. When activated by their specific targets, 19 CTL clones of 4 different specificities and origins killed bystander targets bearing syngeneic but not third-party MHC antigens. Using target cells derived from MHC-recombinant animals, syngeneic bystander killing was shown to be restricted to a single self MHC-encoded molecule. These results provide the first clear demonstration that T cells, or more precisely CTL, are capable of self recognition in the absence of their specific antigen. Our findings support the model that T cell repertoire selection occurs as a result of positive selection during maturation in the thymus of precursor cells whose antigen receptors have low but real affinity for self-MHC.

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