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Murine CD8+ T Cells that Specifically Delete Autologous CD4+ T Cells Expressing Vβ8 TCR: A Role of the Qa-1 Molecule

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Summary

Interactions mediated by TCRs expressed on different T cell subsets may play a role in immunoregulation. To investigate this idea, we studied the regulation of superantigen-induced TCR Vβ-restricted responses. We asked whether the in vivo regulation of CD4+ Vβ8+ T cells following SEB injection is controlled by CD8+ T cells. We found that in mice deficient in CD8+ T cells, the down-regulation of CD4+ Vβ8+ T cells below baseline is not observed. Moreover, following SEB administration, CD8+ T cells emerge that preferentially kill subpopulations of activated CD4+ Vβ8+ but not CD4+ Vβ8- T cells in vitro. This TCR Vβ-specific cytotoxicity is dependent on β2-microglobulin and is inhibited by antisera specific for Qa-1 but not by antibody to MHC class Ia. These data suggest the idea that the specificity of immune regulation may involve CD8+ T cell recognition of TCR Vβ determinants and Qa-1 molecules expressed on CD4+ T cells.

Introduction

The immune response is controlled in part by a complex interaction of T cells. Recently, superantigens have been used as a model to study these complex interactions both in vitro and in vivo, because they trigger immune responses by stimulating particular TCR Vβ chains. The administration of different superantigens to mice is followed by rapid changes in the populations of circulating T lymphocytes. For example, after intravenous injection of the superantigen staphylococcus enterotoxin B (SEB), there is an initial deletion (12–24 hr) followed by an expansion and a second phase of deletion (after day 4) of CD4+ and CD8+ T cells that express T cell receptor (TCR) Vβ8 chains (Gonzalo et al., 1994; Kawabe and Ochi, 1991; Rellahan et al., 1990). The increase in CD4+ Vβ8+ and CD8+ Vβ8+ T cells reaches a maximum on day 4 and by day 8 returns to background. However, the CD4+ Vβ8+ but not the CD8+ Vβ8+ T cell population is further deleted, becomes reduced to about 30%–40% below baseline, and remains at this reduced level for at least 21 days. The mechanism of this delayed deletion of CD4+ T cells following SEB administration is unknown. Although it is known that triggering of the TCR alone by either superantigen or anti-TCR antibodies can induce apoptosis (Takahashi et al., 1989; Lenardo, 1991; Boehme and Lenardo, 1993), other mechanisms requiring interactions with other immunoregulatory cells may also contribute to the deletion of CD4+ T cells. The mechanism of CD4+ T cell deletion in this context is of interest, because it might shed light on the mechanisms of T lymphocyte regulation in general.

In this regard, we previously demonstrated that CD8+ T cells mediate the resistance to murine experimental allergic encephalomyelitis (EAE), which is induced by the 1-9Nac peptide of myelin basic protein (Jiang et al., 1992). We confirmed that mice that had recovered from an initial episode of EAE were resistant to a second challenge of antigen. However, animals depleted of CD8+ T cells by anti-CD8 antibody in vivo were no longer resistant. A similar protective role of CD8+ cells was demonstrated by others studying the induction of EAE in CD8 gene knockout mice (Koh et al., 1992). Although the mechanism by which CD8+ T cell depletion rendered the animals susceptible to disease following a second challenge of antigen was unclear, we considered the possibility that CD8+ T cells functioned to down-regulate the CD4+ myelin basic protein-reactive effector T cells that mediate EAE.

To study more readily the role of CD8+ T cells in regulating CD4+ T cells, we asked whether, in the current studies, the deletion of CD4+ T cells expressing the Vβ8 TCR in SEB-treated animals involved CD8+ T cells. We used two kinds of CD8+ T cell–deficient animals to study the role of CD8+ T cells in regulating CD4+ T cells in vivo. First, in experiments analogous to those described in EAE, we investigated mice depleted of CD8+ T cells by injection of anti-CD8 antibody in vivo (Jiang et al., 1992). Second, we studied $\beta_2m^{-/-}$ mice, which are known to be deficient in CD8+ T cells (Zijlstra et al., 1990). In both systems, we provided evidence that CD8+ T cells participated in SEB-induced deletion of CD4+ Vβ8+ T cells in vivo. In an attempt to understand the possible mechanisms involved in such T–T cell interactions, we established an in vitro cytolytic system, and asked whether CD8+ T cells stimulated by SEB-activated autologous CD4+ Vβ8+ T cells will specifically kill CD4+ T cell targets expressing Vβ8 TCR. We demonstrated that CD8+ T cells derived from SEB-primed mice could be restimulated in vitro and were cytotoxic to the specific CD4+ T cell targets based on their TCR Vβ expression. Furthermore, this autologous TCR Vβ-specific cytolysis was β_2m dependent. Quite surprisingly, this cytolytic interaction between CD8+ effector cells and CD4+ targets was blocked by antisera to a MHC class Ib molecule, Qa-1, but not by antibody to classical MHC class Ia molecules. Thus, these data suggest that a subset of CD8+ T cells can be induced by activated CD4+ T cells to kill the inducer CD4+ T cells, through the recognition of their TCR Vβ chains or the peptides derived from their TCR Vβ chains in conjunction with Qa-1. We speculate that such a process may be involved in the regulation of other immune responses in vivo.

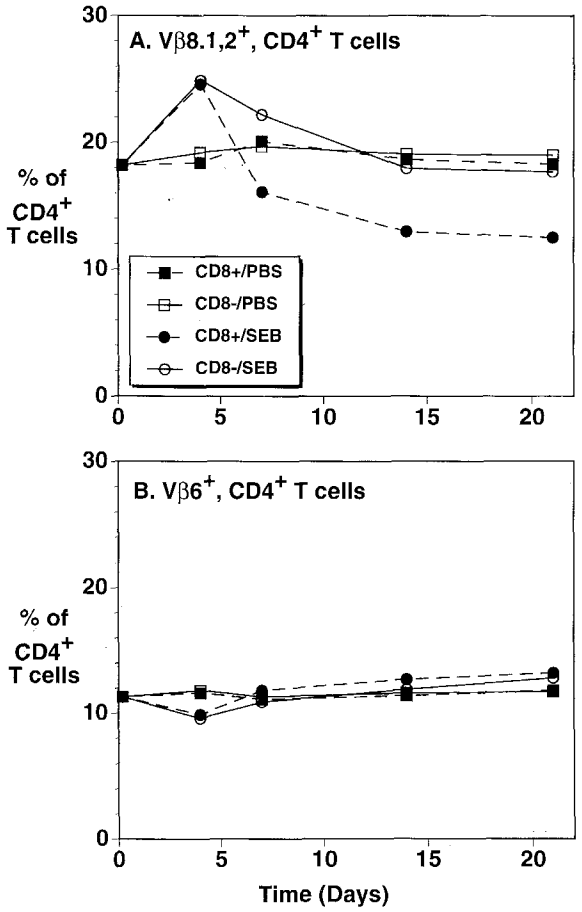

Figure 1. $CD8^+$ T Cell Depletion Protects $CD4^+$ $V\beta8.1,2^+$ T Cells from SEB-Induced T Cell Death

The experiments were done in BALB/c mice as described in Experimental Procedures. The group designations are the following: $CD8^+$/PBS, PBS-primed $CD8^+$ T cell nondepleted; $CD8^-$/PBS, PBS-primed $CD8^+$ T cell depleted; $CD8^+$/SEB, SEB-primed $CD8^+$ T cell nondepleted; $CD8^-$/SEB, SEB-primed $CD8^+$ T cell depleted. The value for day 1 is the average of >50 normal mice. For days 4, 7, 14, and 21, each point represents the average of the data of 12–18 mice from three separate experiments.

Results

The In Vivo Delayed Deletion of $CD4^+$ $V\beta8^+$ T Cells Following SEB Administration Is Markedly Reduced by In Vivo Treatment of Mice with Anti-CD8 Antibody

Following treatment of animals with SEB, there is an initial rapid deletion of $CD4^+$ T cells followed by an expansion of those T cells that express the characteristic Vβ segment reactive with SEB. Subsequently, there is a delayed deletion below background level of the T cells expressing the Vβs reactive with SEB (Gonzalo et al., 1994; Kawabe and Ochi, 1991; Rellahan et al., 1990). To address whether $CD8^+$ T cells are involved in this delayed TCR Vβ-restricted deletion following SEB administration to mice, we compared the effect of SEB on $CD4^+$ $V\beta8^+$ populations of T cells in normal mice and in mice depleted of $CD8^+$ cells by in vivo administration of anti-CD8 antibody. We injected 50 μg SEB intravenously into both untreated and $CD8^+$ T cell–depleted mice, and the portion of $CD4^+$ $V\beta8.1,2^+$ T cells (which are specifically interactive with SEB) in peripheral $CD4^+$ T cells of those mice was determined. As shown by Figure 1A and Table 1, in SEB-primed $CD8^+$ mice, the number of $CD4^+$ $V\beta8.1,2^+$ T cells increased until day 4 and then decreased, reaching a low point between day 7 and day 14, and remained low for at least another week. In the $CD8^+$ T cell–depleted mice treated with SEB, the number of $CD4^+$ $V\beta8.1,2^+$ T cells also increased after SEB injection, reached a peak on day 4, and then slowly returned to baseline levels by day 14. The decrease (25%–30%) below normal levels in the percentage of $CD4^+$ $V\beta8.1,2^+$ T cells that is observed in normal mice treated with SEB was completely eliminated by the depletion of $CD8^+$ T cells. Both SEB-induced T cell death and the protection due to $CD8^+$ T cell depletion were TCR Vβ specific, because there was no difference in the numbers of $CD4^+$ $V\beta6^+$ T cells between $CD8^+$ T cell–nondepleted and $CD8^+$ T cell–depleted SEB-primed mice (Figure 1B; Table 1). These data confirm that following SEB injection in normal mice, there is an initial proliferation of $CD4^+$ Vβ8 T cells followed by a decrease in the number of $CD4^+$ $V\beta8^+$ cells below baseline. Further, the data demonstrate that the decrease of $CD4^+$ $V\beta8^+$ T cells below baseline is $CD8^+$ T cell dependent, because this decrease is observed only in untreated mice containing $CD8^+$ T cells, but not in $CD8^+$ T cell–depleted mice.

The In Vivo Deletion of $CD4^+$ $V\beta8^+$ T Cells Following SEB Administration Is Abrogated in $\beta_2m^{-/-}$ Mice

To verify further the importance of $CD8^+$ cells in the in vivo deletion below the baseline of $CD4^+$ $V\beta8^+$ cells following SEB administration, we next studied $\beta_2m^{-/-}$ mice known to lack mature $CD8^+$ T cells. The $\beta_2m^{-/-}$ mice used were derived from a C57BL (B6) × 129 cross and are H-2^b (Zijlstra et al., 1989; Muller et al., 1992). We used both B6 and (B6 × 129)F2 mice as control animals. In two separate experiments, peripheral $CD4^+$ $V\beta8.1,2^+$ cells in SEB-primed $\beta_2m^{-/-}$ mice increased on day 4, returned to and remained at normal level after day 7 (Table 2). In contrast, the $CD4^+$ $V\beta8.1,2^+$ cells are reduced to greater than 30% of baseline in both SEB-primed control B6 and (B6 × 129)F2 mice. It is curious that in these experiments, unlike our experience in BALB/c mice, there was no $CD4^+$ $V\beta8^+$ T cell expansion observed by day 4 in both B6 and (B6 × 129)F2 mice. It is possible that Vβ8-specific $CD8^+$ T cells involved in the regulation of $CD4^+$ T cells arise earlier in these mice.

The In Vitro Generation of $CD8^+$ Killer Cells Specific for $CD4^+$ $V\beta8^+$ Target Cells

The requirement for $CD8^+$ T cells made it unlikely that the deletion of $CD4^+$ $V\beta8^+$ T cells following SEB administration was only owing to an endogenous self-destruction program activated by SEB. Moreover, the specificity of the deletion raised the possibility that the initial SEB-induced $CD4^+$ $V\beta8^+$ T cells further stimulated a population of $CD8^+$ T effector cells with anti-Vβ8 specificity (distinct from the $CD8^+$ $V\beta8^+$ T cells directly stimulated by SEB). We hypothesized that these $CD8^+$ T cells could then mediate the

Table 1. Analysis of Vβ8.1,2 and Vβ6 Expression on Peripheral T Cells in CD8+T Cell–Nondepleted and CD8+ T Cell–Depleted BALB/c Mice[a]

		Percentage of Total			
		CD4+ T Cells		CD8+ T Cells	
Anti-CD8 Treatment	Injection	Vβ8.1,2	Vβ6	Vβ8.1,2	Vβ6
No	PBS	18.7 ± 0.84	11.4 ± 0.20	25.5 ± 0.49	12.2 ± 1.4
Yes	PBS	19.1 ± 0.21	11.6 ± 0.32	–	–
No	SEB	13.0 ± 2.40	12.7 ± 0.44	29.1 ± 4.8	11.6 ± 3.0
Yes	SEB	18.0 ± 0.31	11.9 ± 0.90	–	–

[a] Experiments were done as in Figure 1. This table shows the data of expression of Vβ8.1,2 and Vβ6 in CD8+ T cell–nondepleted and CD8+ T cell–depleted mice 14 days after SEB injection. Each value represents data of 12–18 mice from three separate experiments.

deletion of CD4+ Vβ8+ T cells by a cytotoxic mechanism. To test this hypothesis in vitro, we first established a CD4+ Vβ8+ T cell line by stimulating purified CD4+ Vβ8+ T cells with SEB. This line was then irradiated and used to induce CD8+ Vβ8− T cell lines in vitro. Since the superantigen SEE does not react with T cells bearing Vβ8 TCR (Marrack and Kappler, 1990), we have used SEB and SEE to obtain CD4+ T cell lines bearing different TCR Vβ specificities, including the generation of Vβ8+ and Vβ8− cell lines.

To address the function and specificity of these CD8+ T cell lines, we asked whether these cells could specifically kill the CD4+ stimulating T cells that were used to induce them in the initial instance. In preliminary studies, we found that the CD8+ T cells initially induced by CD4+ Vβ8+ cells could specifically kill CD4+ Vβ8+ targets to a greater extent than CD4+ Vβ8− targets in a conventional ^{51}Cr release assay, but only after prolonged periods of incubation (>12 hr). However, incubation times greater than 12 hr were often associated with a high spontaneous release of ^{51}Cr from targets that significantly reduced the signal-to-noise level, increased the experimental variability, and made the interpretation of experiments difficult. During the course of these studies, we observed that the reduction (due to lysis) of CD4+ Vβ8+ T cells following coculture with effector CD8+ T cells and CD4+ Vβ8− T cells leads to a reduced ratio of CD4+ Vβ8+ to CD4+ Vβ8− that could be precisely enumerated by two-color FACS. This method of measuring specific cytotoxicity permitted analysis of cocultured effector and target cells for greater than 24 hr. To demonstrate that the FACS cytolytic T lymphocyte (CTL) method gives quantitative results comparable to conventional ^{51}Cr release assays, a murine alloantigen-specific CTL line of L3 cells (H-2^b) was tested simultaneously for its specific CTL activity to the specific targets, P815 cells (H-2^d), and nonspecific targets, EL4 cells (H-2^b), in both FACS and ^{51}Cr release assays. Similar CTL activities were obtained using both assays at effector:target ratio from 5 to 1.25 (Figure 2; Table 3). By FACS assay, the ratio of H-2^{d+} to H-2^{d-} targets clearly decreased in cultures containing anti-H-2^d killers (Figure 2b) compared with cultures without effector cells (Figure 2a).

Using the FACS CTL assay, we next tested the specificity of the CD8+ Vβ8− cell lines. In particular, we asked whether the CD8+ T cell line cells initially induced by CD4+ Vβ8+ cells specifically kill CD4+ Vβ8+ targets. The results, using four independent CD8+ Vβ8− T cell lines (CTL-1–4) cocultured for 24 hr with target populations containing varying numbers of CD4+ Vβ8+ and CD4+ Vβ8− T cells in five separate experiments, are shown in Table 4. The target cell populations were derived from cultures of CD4+ cells triggered by varying ratios of the superantigens SEB to

Table 2. Analysis of Vβ8.1,2 TCR Expression on Peripheral CD4+ T Cells in $\beta_2m^{-/-}$ mice after SEB Stimulation[a]

Experiment number	Mice	Injections	CD4+ Vβ8+/ Total CD4+ (Percentage) Time (Days) 4	7	14	21	60	120
1								
	B6	PBS	18.6	18.8	18.6	18.5	–	–
	B6	SEB	18.1	11.2	12.2	13.0	–	–
	$\beta_2m^{-/-}$	PBS	18.5	18.6	18.6	18.5	–	19.1
	$\beta_2m^{-/-}$	SEB	24.2	18.7	18.5	18.6	–	19.3
2								
	(B6 × 129)F2	PBS	18.6	18.7	19.0	18.9	18.8	–
	(B6 × 129)F2	SEB	17.0	12.5	13.3	13.5	14.3	–
	$\beta_2m^{-/-}$	PBS	18.4	18.9	18.6	18.8	19.1	–
	$\beta_2m^{-/-}$	SEB	20.2	20.0	19.1	18.9	18.4	–

[a] Experiments were done as described in Figure 1. B6 mice and (B6 × 129)F2 mice served as controls. There were 4 mice per group in each experiment.

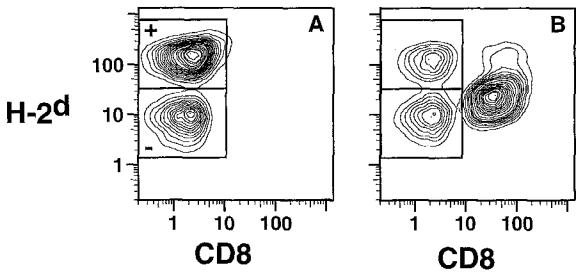

Figure 2. FACS Profiles of Data from Table 3, in FACS Assay, at E:T Ratio of 2.5:1

(A) The profile of control group (without L3). The ratio of specific targets/nonspecific targets was 2.0.

(B) The profile of experimental group (with L3). The ratio of specific targets/nonspecific targets was 0.70. Therefore, the specific CTL activity of L3 cells was calculated as (2.0 − 0.7) / 2.0 = 65%.

Table 3. A Comparison of CTL Activity of L3 Cells in Both FACS Assay and ^{51}Cr Release Assay

	Specific cytotoxicity (percentage) E:T ratio		
Target Cells	5:1	2.5:1	1.25:1
FACS Assay[a]			
P815 + EL4	75	65	43
^{51}CR Release Assay[b]			
P815	72	57	44
EL4	1.5	0.6	1.0

[a] In the FACS assay, specific targets P815 and nonspecific targets EL4 were mixed in a 2:1 ratio, then L3 cells were added to the mixed targets at different E:T ratio. Targets without L3 cells served as control. After 4 hr of incubation, the cells were stained with F-34.1.2 and APC-53-6.72, and the data was analyzed as described in Experimental Procedures.

[b] In the ^{51}CR release assay, P815 and EL4 cells were labeled with ^{51}Cr: then L3 cells were added to both targets at different E:T ratio separately. After 4 hr of incubation, the supernatant was harvested, the radioactivity was counted and specific cytolysis was calculated as described in Experimental Procedures.

SEE to generate cell lines with varying numbers of Vβ8$^+$ and Vβ8$^-$ (containing Vβ11$^+$) cells. The differences of the ratio of Vβ8$^+$ versus Vβ8$^-$ T cells in cultures with and without effectors reflects the specific deletion of Vβ8$^+$ cells. We simultaneously measured the ratio of Vβ11$^+$/Vβ11$^-$ cells as a specificity control. As shown, CD8$^+$ Vβ8$^-$ T cells deleted the CD4$^+$ Vβ8$^+$ T cells but not CD4$^+$ Vβ8$^-$ T cells. This was shown by the decreased ratio of CD4$^+$ Vβ8$^+$/CD4$^+$ Vβ8$^-$ (Vβ8$^+$/Vβ8$^-$) and, as expected, the increased ratio of CD4$^+$ Vβ11$^+$/CD4$^+$ Vβ11$^-$ (Vβ11$^+$/Vβ11$^-$) in target cells. One explanation of the preceding experiments was that specificity was not only a function of the TCR Vβ used by the target T cells, but was influenced by the different superantigens used to generate the targets. To exclude this possibility, we took advantage of the observation that SEB activates Vβ7$^+$ and Vβ17$^+$ as well as Vβ8$^+$ T cells in BALB/c mice (Marrack and Kappler, 1990), and used SEB to generate both CD4$^+$ Vβ8$^+$ and CD4$^+$ Vβ8$^-$ targets. These targets were derived either from purified total CD4$^+$ T cells stimulated by SEB (Table 5, experiments 1 and 2), or from purified CD4$^+$ Vβ8$^+$ and purified CD4$^+$ Vβ8$^-$ T cells stimulated by SEB separately, and mixed at a Vβ8$^+$/Vβ8$^-$ ratio of 1:1 before the test (Table 5, experiment 3). Again, only TCR Vβ8-specific cytotoxicity was observed. These data also rule out SEB carryover as a mechanism of TCR Vβ8-specific cytotoxicity, because if SEB carryover was involved in the CD8$^+$ T cell–mediated killing, there should have been comparable killing in both Vβ8$^+$ and Vβ8$^-$ populations. This was not observed, even though the Vβ8$^-$ T cell population contains SEB-reactive cells including those expressing Vβ7 and Vβ17. Taken together, these data strongly suggest that the CD8$^+$ T cell line initially induced by CD4$^+$ Vβ8$^+$ cells differentiate into killer cells that specifically lyse CD4$^+$ Vβ8$^+$ targets.

However, because the FACS assay quantitatively measures the fraction of Vβ8$^+$ cells present after 24 hr of cul-

Table 4. TCR Vβ8-Specific CD8$^+$T Cells Selectively Deleted CD4$^+$ Vβ8$^+$ T Cells but Not CD4$^+$ Vβ8$^-$ T Cells in Mixed T Cell Cultures

	Targets				Ratio of CD4$^+$ Vβ8$^+$/CD4$^+$ Vβ8$^-$			Ratio of CD4$^+$ Vβ11$^+$ CD4$^+$ Vβ11$^-$	
Experiment number	Cells	Stimulant[a]	CD8$^+$ Effector Cells	E:T Ratio	Control	Plus CD8$^+$ effectors	Percentage of specific deletion	Control	Plus CD8$^+$ effectors
1	CD4$^+$	SEB (1) plus SEE (0.1)	CTL-1	4:1	0.47	0.34	27.6	0.14	0.16 (0.16)[b]
2	CD4$^+$	SEB (1) plus SEE (0.1)	CTL-2	2:1	0.31	0.24	22.5	0.17	0.18 (0.18)
				1:1		0.23	25.8		0.18 (0.18)
3	CD4$^+$	SEB (1) plus SEE (0.1)	CTL-2	2:1	0.32	0.24	25.0	0.18	0.19 (0.19)
				1:1		0.23	28.1		0.19 (0.19)
4	CD4$^+$	SEB (1) plus SEE (0.025)	CTL-2	2:1	3.20	2.00	37.5	0.04	0.05 (0.05)
				1:1		1.94	39.3		0.06 (0.05)
5	CD4$^+$	SEB (1) plus SEE (0.025)	CTL-3	2:1	4.60	3.37	26.7	–	–
				1:1		3.54	23.0	–	–
			CTL-4	2:1		3.57	25.8	–	–
				1:1		3.59	22.0	–	–

Target T cells were purified CD4$^+$ T cells from BALB/c mice stimulated with SEB and SEE, and TCR Vβ8-specific CD8$^+$Vβ8$^-$ CTL lines were prepared as described in Experimental Procedures. Target cells were cultured alone (control) or mixed with the CD8$^+$ CTL lines (plus CD8$^+$ effectors) at the indicated ratios and cultured for 24 hr in the presence of IL-2 (10 U/ml). The cells were then divided into two tubes, washed, and stained with F-53.6.72 and biotin-F23.1 or biotin-RR-3.15 and analyzed as described in Figure 1. For data analysis, the CD8$^+$T cells were gated out and the data were expressed as the ratio of CD4$^+$ Vβ8$^+$ T cells versus CD4$^+$ Vβ8$^-$ T cells (Vβ8$^+$/Vβ^{8-}) or CD4$^+$ Vβ11$^+$ T cells versus CD4$^+$ Vβ11$^-$ cells (Vβ11$^+$/Vβ11$^-$).

The percentage of the specific deletion of CD4$^+$ Vβ8$^+$ T cells was calculated as:

$$\frac{\text{Vβ8}^+/\text{Vβ8}^- \text{ of control} - \text{Vβ8}^+/\text{Vβ8}^- \text{ of experimental}}{\text{Vβ8}^+/\text{Vβ8}^- \text{ of control}} \times 100\%$$

[a] To obtain different ratios of CD4$^+$ Vβ8$^+$/CD4$^+$ Vβ8$^-$ of target populations in SEB/SEE cultures, same amount of SEB (1 μm/ml) and different amount of SEE (μg/ml) were added in CD4$^+$ T cell cultures as indicated.

[b] The values in parentheses are the theoretical calculated values of the CD4$^+$ Vβ11$^+$/CD4$^+$Vβ11$^-$ ratio if CD4$^+$ Vβ11$^+$ T cells in the targets were not deleted by the CD8$^+$ T cells.

Table 5. TCR Vβ8-Specific CD8+ T Cells Selectively Deleted CD4+ Vβ8+ T Cells but Not CD4+ Vβ8− T Cells in Mixed T Cell Cultures

Experiment number	Target Cells	CD8+ Effector Cells	E:T Ratio	Ratio of CD4+ Vβ8+/CD4+ Vβ8− Control	Ratio of CD4+ Vβ8+/CD4+ Vβ8− Plus CD8+ effectors	Percentage of specific deletion (CD4+ Vβ8+)
1	CD4+	CTL-3	2:1	8.20	5.50	28.9
		CTL-4	2:1		6.00	26.8
2	CD4+	CTL-3	2:1	8.49	6.26	26.3
		CTL-4	2:1		6.23	26.8
3	CD4+Vβ8+ plus CD4+ Vβ8−	CTL-3	2:1	1.05	0.71	32.4
			1:1		0.72	31.4
		CTL-4	2:1		0.73	30.5
			1.1		0.74	29.5

The experiments were performed as in Table 4. Target cells were stimulated with SEB only. To obtain different initial Vβ8+/Vβ8− ratios, total purified CD4+ T cells were stimulated with SEB (1 μg/ml) (Experiments 1 and 2) or purified CD4+ Vβ8+ and CD4+ Vβ8− T cells were stimulated with SEB (1 μg/ml) separately and mixed in a 1:1 ratio prior to use as targets (Experiment 3).

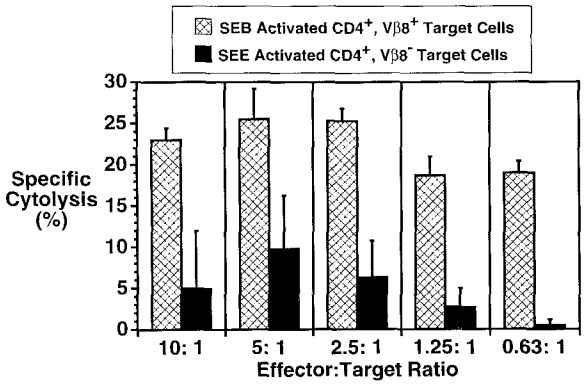

Figure 3. TCR Vβ8-Specific CD8+ T Cells Killed CD4+ Vβ8+ Target T Cells but Not CD4+ Vβ8− Target T Cells

^{51}Cr-release assay was used to detect the killing capacity of TCR Vβ8-specific CD8+ Vβ8− T cell lines. SEB-activated CD4+ Vβ8+ T cells and SEE-activated CD4+ Vβ8− T cells (prepared as described in Experimental Procedures) were used as specific and nonspecific targets. This figure represents data from three separate experiments with four independent CTL lines.

ture, one interpretation of the above data was that the Vβ8+ cells were not killed but merely suppressed in growth. To determine directly whether cell lysis was occurring, we cocultured CD8+ effectors with the CD4+ Vβ8+ or CD4+ Vβ8− targets separately at varying effector:target ratios for 12 hr and assayed cell lysis by measuring specific ^{51}Cr release. As shown in Figure 3, four independent CD8+ T cell lines specifically lysed the CD4+ Vβ8+ T cell targets.

The Vβ8-Specific In Vitro Cytotoxicity Mediated by CD8+ T Cells Is Dependent on β_2m-Associated Molecules

Because CD8+ cytotoxic T cells usually recognize antigens associated with MHC class I molecules, we asked whether CD4+ Vβ8+ target cells derived from $\beta_2m^{-/-}$ mice would be lysed by the Vβ8-specific CD8+ CTL. Thus, Vβ8-specific CTLs derived from B6 mice were added to CD4+ Vβ8+ and CD4+ Vβ8− targets from B6 mice or from $\beta_2m^{-/-}$ mice, and deletion of CD4+ Vβ8+ cells was assayed by FACS. No deletion of CD4+ Vβ8+ cells was observed when targets were derived from the $\beta_2m^{-/-}$ mice (Table 6). These same $\beta_2m^{-/-}$ targets were susceptible to killing in a mitogen (concanavalin A [ConA])-induced MHC class I–independent assay (data not shown). Furthermore, deletion of CD4+ Vβ8+ T cells was readily observed in the control B6 targets (Table 6). Taken together, these data show that the Vβ8-specific in vitro cytotoxicity mediated by CD8+ T cells is dependent on β_2m-associated molecules.

The Vβ8-Specific In Vitro Cytotoxicity Mediated by CD8+ T Cells Is Not Blocked by Antibody to MHC Class Ia Molecules

The next set of experiments were designed to identify which β_2m-associated molecules were involved in the Vβ8-specific in vitro cytotoxicity mediated by CD8+ T cells. Because β_2m molecules are known to be associated with MHC class I, we first asked whether antibodies known to block classical MHC class I–restricted allogeneic CTL responses would also inhibit the CD8+ cells mediating Vβ8-specific killing of syngeneic CD4+ cells. For these experiments, we used the M1/42.39 antibody, which is a rat anti-mouse H-2 monoclonal antibody (MAb) known to be specific for all H-2 haplotypes, including H-2K^d and H-2D^d and H-2L^d MHC class I antigens (Stallcup et al., 1981). In the first set of experiments, we demonstrated the effectiveness of the M1/42.39 antibody in blocking allospecific cytotoxicity. We used L3 cells, which are H-2^b CD8+ allospecific killers and efficiently lyse allogeneic P815 (H-2^d) targets, and tested cytotoxicity in the presence or absence of the M1/42.39 antibody or control normal rat immunoglobulin. These experiments showed that M1/42.39 but not the control rat immunoglobulin markedly inhibited the lysis of P815 allogeneic targets over a range of antibody concentration from 800–25 μg/ml (data not shown). We next tested the effectiveness of the M1/42.39 antibody in blocking the CD8+ cells mediating Vβ8-specific killing of syngeneic CD4+ cells in BALB/c mice (H-2^d). Vβ8-specific CD8+ killers were generated as before, and cytotoxicity of CD4+ Vβ8+

Table 6. $CD4^+$ $V\beta8^+$T Cells from $\beta_2m^{-/-}$ Mice Can Not Be Killed by Syngenic TCR Vβ8-Specific CTLs

Experiment number	$CD4^+$ Target Cells	E:T Ratio	Ratio of $CD4^+$ $V\beta8^+/CD4^+$ $V\beta8^-$ Control	Ratio of $CD4^+$ $V\beta8^+/CD4^+$ $V\beta8^-$ $CD8^+$ effectors	Percentage of specific deletion ($CD4^+$ $V\beta8^+$)
1	C57BL/6	2:1	0.33	0.26	21.2
		1:1		0.27	18.2
	$\beta_2m^{-/-}$	2:1	0.92	0.89	3.3
		1:1		1.00	−8.6
2	C57BL/6	2:1	0.45	0.32	28.9
		1:1		0.33	26.7
	$\beta_2m^{-/-}$	2:1	0.60	0.58	3.3
		1:1		0.59	1.7

$CD4^+$ $V\beta8^+$ and $CD4^+$ $V\beta8^-$ targets from B6 and $\beta_2m^{-/-}$ mice were stimulated with SEB, and served as targets. TCR Vβ8-specific CTLs were prepared from B6 mice as described in Experimental Procedures.

targets tested in the presence or absence of M1/42.39 or control rat immunoglobulin. In four separate experiments, the M1/42.39 antibody, although highly efficient in blocking conventional CTL directed against $H\text{-}2^d$ at 25 μg/ml, does not block the Vβ8-specific killing of $CD4^+$ targets at 200 μg/ml, even though the targets express the $H\text{-}2^d$ MHC class I alleles recognized by the M1/42.39 MAb (data not shown). These data suggest that the MHC class Ia determinants recognized by M1/42.39 antibody are not involved in the cytotoxicity mediated by the Vβ8-specific $CD8^+$ killer cells. These data are consistent with the analysis of Vβ-specific $CD8^+$ killer cells in man, which are not inhibited by the antibody W6/32 known to be reactive with all known MHC class Ia molecules (Ware et al., 1995 [this issue of *Immunity*]).

The Vβ8-Specific In Vitro Cytotoxicity Mediated by $CD8^+$ T Cells Is Efficiently Blocked by Antiserum to the MHC Class Ib Molecule, Qa-1

The above data demonstrated that although the Vβ8-specific in vitro cytotoxicity of $CD4^+$ cells mediated by $CD8^+$ T cells is dependent on β_2m-associated molecules, antibody to the classical MHC class Ia molecules did not block the cytotoxicity. These data raised the possibility that other β_2m-associated molecules, including MHC class Ib molecules, might be involved as target structures for the Vβ-specific killers. Moreover, because the MHC class Ib molecule, Qa-1, has previously been implicated as defining populations of regulatory cells important in immune suppression (Eardley et al., 1978), we reasoned that Qa-1 might be involved as a structure expressed on $CD4^+$ cells in the $CD8^+$ T cell–mediated Vβ-specific killing we were observing. To test this idea, we performed a series of blocking studies using well-characterized sera, which initially defined the Qa-1a and Qa-1b alleles. In initial experiments, we showed that the Qa-1b but not the Qa-1a determinant was expressed on the $CD4^+$ target cells derived from BALB/c mice. We also showed that the antisera to Qa-1a and Qa-1b did not block the killing of P815 target cells by $CD8^+$ anti-MHC class Ia allogeneic killer L3 cells (data not shown). These data indicated that the Qa-1a and Qa-1b antisera were not nonspecifically capable of blocking cell-mediated cytotoxicity. We next tested the effectiveness of the anti-Qa-1b antiserum in blocking the $CD8^+$ T cell–mediated Vβ8-specific killing of syngeneic $CD4^+$ cells in BALB/c mice and used anti-Qa-1a antiserum as a specificity control. Vβ8-specific $CD8^+$ killers were generated as before and cytotoxicity of $CD4^+$ $V\beta8^+$ targets tested in the presence or absence of Qa-1a and Qa-1b antisera (Table 7). In six separate experiments, we showed that Vβ-specific cytotoxicity was blocked by the specific Qa-1b antiserum but not by Qa-1a antiserum.

Discussion

In the present studies, we demonstrated that $CD8^+$ T cells participate in the in vivo regulation of $CD4^+$ $V\beta8^+$ T cells following SEB administration. We initially confirmed the work of others (Kawabe and Ochi, 1991), which has shown a deletion of 30%–40% of $CD4^+$ $V\beta8^+$ cells 7–14 days after a single injection of SEB in mice. Then, we specifically showed that the down-regulation of $CD4^+$ $V\beta8^+$ T cells below baseline is not observed in mice depleted of $CD8^+$ cells by treatment with anti-CD8 MAb or in $CD8^+$ T cell–deficient $\beta_2m^{-/-}$ mice. Moreover, we observed that following SEB administration, splenic and lymph node $CD8^+$ T cells preferentially recognizing $CD4^+$ $V\beta8^+$ T cells are generated and can be specifically expanded in vitro. These $CD8^+$ T cells are cytotoxic to autologous $CD4^+$ $V\beta8^+$ T cells but not to autologous $CD4^+$ $V\beta8^-$ T cells. Furthermore, we demonstrated that this autologous TCR Vβ-specific cytotoxicity is dependent on recognition of β_2m-associated molecules and is inhibited by antiserum specific for Qa-1 molecules but not by antibody to classical MHC class Ia molecules. Together, these data support the idea that the specificity of immune regulation may be mediated in part by specific recognition by $CD8^+$ T cells of TCR Vβ chains or peptides derived from TCR Vβ chains bound to Qa-1 MHC class Ib molecules expressed on the surface of autologous $CD4^+$ T cells.

Table 7. Antimurine Qa-1b but Not Anti-Qa-1a Antisera Blocked TCR Vβ8-Specific Killing in BALB/c Mice*

Experiment Number	Ratio of CD4+ Vβ8+/ CD4+ Vβ8−				Percentage of Vβ-specific deletion	Percentage of blocking	Percentage of αQa-1b specific blocking
		Plus CD8+ effectors					
	Control	Minus Ab	Plus αQa-1[a]	Plus αQa-1[b]			
	0.32	0.25			21.9	—	
1			0.26		19.0	13.2	
				0.33	0	100	86.8
	0.23	0.16			30.4	—	
2			0.17		26.1	14.1	
				0.24	0	100	85.9
	0.88	0.52			40.9	—	
3			0.55		37.5	8.3	
				0.74	15.9	61.1	52.8
	0.38	0.24			36.8	—	
4			0.25		34.2	7.1	
				0.34	10.5	71.5	64.4
	0.58	0.32			44.8	—	
5			0.34		41.4	7.6	
				0.59	0	100	92.4
	0.76	0.54			28.9	—	
6			0.53		30.3	0	
				0.73	3.9	86.5	86.5

* TCR Vβ8-specific CD8+T cells and CD4+ Vβ8+, CD4+ Vβ8− targets were prepared and experiments were performed as described in Table 5. Anti-Qa-1 sera were added at a final dilution of 1:10 to the targets and incubated at room temperature for 30 min before mixed with effector cells. Percentage of blocking of killing was calculated as: {[percentage of killing of control group (without antisera) minus percentage of killing of experimental group (with antisera)] / percentage of killing of control group} × 100%.

The evidence that CD8+ T cells arise that are specific for CD4+ cells expressing particular Vβ TCRs was demonstrated by showing that CD8+ T cells obtained by stimulation with SEB-activated CD4+ Vβ8+ T cells only kill SEB-activated CD4+ Vβ8+ T cells but not SEB-activated CD4+ Vβ8− T cells, even though SEB activates Vβ7+ and Vβ17+ T cells as well as Vβ8+ T cells in BALB/c mice. We assayed the destruction of CD4+ cells by a conventional ^{51}Cr release assay, as well as by measuring the change in the ratio of Vβ8+/Vβ8− cells induced by CD8+ effector cell in mixed T cell culture by FACS. The killing observed is not related to T cell activation alone, because Vβ8+ and Vβ8− targets cells were both activated. Further, the killing is not dependent on the particular superantigen used, because Vβ8− T cells activated either by SEB or SEE were not killed, whereas Vβ8+ cells activated by SEB were killed.

The precise molecular target structure for the Vβ-specific CD8+ T cells demonstrated here is unknown. Our studies do not formally exclude the possibility that the TCR Vβ-specific CD8+ T cells described here recognize the whole TCR Vβ chain in association with MHC class I molecules. However, because CD8+ T cells usually recognize target cells expressing particular MHC class I–peptide complexes, it is more likely that these Vβ-specific CD8+ T cells recognize MHC class I molecules complexed to peptides derived from TCR Vβ chains on target T cells. The hypothesis that the self-TCR antigens must either be associated with or presented by MHC class I molecules is supported by our findings that target cells derived from $\beta_2m^{-/-}$ mice, which are devoid of both MHC class Ia and MHC class Ib molecules, do not function as targets for the Vβ-specific CD8+ effector cells. It is of interest to point out that owing to the limited polymorphism and tissue-restricted expression of MHC class Ib molecules, they may be highly appropriate for the presentation of endogenous self-peptides (Stroynowski, 1990; Joyce et al., 1994; Aldrich et al., 1994). The data obtained from our in vitro experiments that the TCR Vβ-specific killing mediated by the CD8+ T cells could be blocked by anti-Qa-1 antiserum but not anti-MHC class Ia antibody strongly suggested that Qa-1 molecules are the major self-TCR peptide-presenting molecules in this particular T–T cell interaction. Since anti-Qa-1 serum only blocked about 70%–80% of the TCR Vβ-specific killing in our experiments, we could not exclude the possibility that other MHC class Ib molecules are also involved in this cellular event. On the other hand, our finding that a monoclonal pan anti-class Ia antibody, M1/42.39, did not inhibit Vβ-restricted cytotoxicity is against a role for class Ia MHC in our system. The results from the companion paper (Ware et al., 1995) that anti-human MHC class Ia antibody W6/32 did not block Vβ-specific cytotoxicity mediated by human CD8+ T cells further support the hypothesis that nonpolymorphic MHC class Ib molecules are the major presenting molecules involved. Moreover, studies in EAE also suggest that nonpolymorphic MHC class Ib molecules can be important in immune regulation. Sun et al. (1988) isolated a CD8+ T cell line from EAE rats induced by the encephalitogenic CD4+ T cell line S1. This autologous anti-S1 CD8+ T cell line proliferated when cultured with S1 cells, but not when cultured with MBP presented by classical APCs. Moreover, the anti-S1 CD8+ T cells were cytotoxic to S1 cells, and the specific proliferation of the CD8+ T cells to S1 cells could not be blocked by anti-MHC class Ia antibody. In addition, it is interesting that studies of regulatory T suppressors indicated that the CD4+ inducers of CD8+ suppressors expressed high levels of the Qa-1

molecules and, if the inducer population was depleted of Qa-1-expressing T cells, they lost the capacity of inducing T suppressors (Eardley et al., 1978). Our data, viewed in this light, supports the possibility that Qa-1 molecules may play a role in the control of immune responses by presenting self-TCR peptides to regulatory $CD8^+$ T cells.

The in vitro experiments show that the $CD8^+$ effector cells killed the specific $CD4^+$ targets at low E:T ratios. However, the maximum fraction of $CD4^+$ $V\beta8^+$ cells lysed was only approximately 30%. There are several possible explanations for such partial killing of target T cells in vitro. First, the expression of Qa-1/TCR Vβ target structure may not be present in sufficient quantity on all targets and limit the number of cells killed. Second, Qa-1^+ and Qa-1^- $CD4^+$ T cells with differential susceptibility to killing may emerge following antigen activation. Third, it has been shown that most of the $CD4^+$ $V\beta8^+$ T cells that survived 10–14 days after SEB administration in vivo were anergic (Rellahan et al., 1990; Kawabe and Ochi, 1990). This raises the possibility that the state of anergy of $CD4^+$ T cells is correlated to their susceptibility to the lysis. In addition, it is known that the interactions between cytotoxic T cells and their specific targets is not only dependent on cognitive recognition between TCR and specific antigen–MHC complexes, but is also dependent on the interactions of adhesive molecules, including CD2, VLA, LFA1, and LFA3 (Williams and Barclay, 1988; Staunton et al., 1988; Shimizu et al., 1990). The expression of these molecules may also vary with T cell activation and limit killing mediated by $CD8^+$ Vβ-specific effector cells.

It is of interest to relate the in vitro cytotoxicity observed to the SEB-induced delayed $CD8^+$ T cell–dependent deletion (4–21 days after SEB administration) of $CD4^+$ $V\beta8^+$ T cells observed in vivo. (Figure 1; Table 2). The mechanisms of this $CD8^+$ T cell–dependent $CD4^+$ T cell deletion in vivo is most likely complex. We envision that populations of Vβ8-specific $CD8^+$ T cells are initially stimulated by SEB-reactive $CD4^+$ $V\beta8^+$ T cells to grow and differentiate. These Vβ8-specific $CD8^+$ T cells most likely secrete a variety lymphokines, which directly or indirectly may be responsible for the death of activated Vβ8-expressing $CD4^+$ T cells. In addition, the deletion of $CD4^+$ T cells could result from the direct cytotoxic T effector function of the Vβ8-specific $CD8^+$ killer cells we have documented in vitro. The relationship between our in vitro and in vivo data is also highlighted by the present experiments using $\beta_2m^{-/-}$ mice. In these $CD8^+$ T cell–deficient animals, there is no delayed deletion of $CD4^+$ $V\beta8^+$ T cells below baseline (Table 2) and, perhaps as important, $CD4^+$ $V\beta8^+$ targets cells from the $\beta_2m^{-/-}$ mice are not lysed by $CD8^+$ effector cells derived from syngeneic β_2m^+ mice (Table 6).

The idea that regulatory $CD8^+$ T cells that recognize TCR target structures expressed on $CD4^+$ T cells are important in the control of immune responses in vivo is supported by several other lines of investigation. First, in studies of EAE in mice, rats, and humans, it has been shown that encephalitogenic T cells expressing specific Vβ segments can induce autoregulatory T cells (Sun et al., 1988; Cohen and Weiner, 1988; Lider et al., 1988; Lohse et al., 1989; Zhang et al., 1993). Moreover, TCR peptides derived from encephalitogenic $CD4^+$ T cells could protect animals from the development of EAE in vivo (Offner et al., 1991; Vandenbark et al., 1989; Howell et al., 1989). Further, it has been shown that $CD8^+$ CTL emerge in rats that have recovered from EAE induced by an encephalitogenic $CD4^+$ cell line (Sun et al., 1988). These $CD8^+$ CTL are specific for the inducing T cell line and, importantly, efficiently neutralize the encephalitogenic functions of the inducing cells in vivo. In addition, it has recently been shown that in the EAE model in mice, regulatory $CD4^+$ T cells also emerge, which down-regulate the antigen-specific immune response mediated by $CD4^+$ $V\beta8.2^+$ T cells (Kumar and Sercarz, 1993). However, even these regulatory $CD4^+$ cells were shown to be $CD8^+$ T cell dependent. Thus, immunoregulatory networks involving regulatory $CD4^+$ and $CD8^+$ T cells both recognizing TCR Vβ segments may ultimately control the immune response in EAE. Furthermore, more than a decade ago, Kimura and Wilson (1984) showed that during the development of graft-versus-host disease in rats, $CD8^+$ CTL derived from A/B F1 strain rats emerge that specifically kill A strain anti-MHC B $CD4^+$ T blasts but do not kill A strain anti-MHC C $CD4^+$ T blasts. These cytotoxic $CD8^+$ T cells do kill anti-MHC B $CD4^+$ T blasts from a third party, strain C. Thus, like the $CD8^+$ CTL described in this paper, these CTL recognize a common target structure expressed on $CD4^+$ T blasts derived from MHC A or C strain, which is most likely the TCRs specific for MHC B alloantigen. Interestingly, these CTL are not restricted by classical polymorphic MHC class Ia molecules. This kind of CTL might be responsible for the resistance of graft-versus-host disease induced by previous inoculation of T cells from one of the parental strain in F1 rats in vivo.

Because we have only studied SEB-treated mice in this study, we do not know whether the delayed and profound deletion of other Vβ T cell families induced by other superantigens, like the deletion of Vβ3 T cells induced by SEA (McCormack et al., 1993, 1994), are due to a similar mechanism involving $CD8^+$ cells recognizing Qa-1/TCR Vβ structures on $CD4^+$ T cells. It is possible that Vβ8 families, which are known to be involved in EAE and other autoimmune states in mice and rats, are unique. However, we would like to believe that our results are not confined to Vβ8 in mice, and experimental support for this notion is present in the accompanying paper (Ware et al., 1995). There, we demonstrate that human $CD8^+$ T clones specific for TCR Vβs can be induced in vitro by superantigen-activated autologous $CD4^+$ T cell clones. These human $CD8^+$ T clones specifically recognize and kill autologous clones of $CD4^+$ target cells expressing particular Vβ TCRs. This killing is not inhibited by antibody to human MHC class Ia molecules. Thus, the TCR Vβ-specific cytotoxicity mediated by $CD8^+$ T cells reported here appears to represent a more general phenomenon, and may play important physiological roles in immunoregulation and in control of autoimmunity.

Experimental Procedures

Animals

BALB/c mice, C57BL/6J (B6) mice, $\beta_2m^{-/-}$ mice, and 129B6F2/J control

mice (female, 6–12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine), and maintained in our animal facilities.

Antibodies and Antisera

Fluorescein (Fl) or allophycocyanin (APC) 53-6.72 (anti-mouse CD8), Fl–34.1.2 (anti mouse H-2^d), APC–GK1.5 (anti-mouse CD4), biotin–F23.1 (anti-mouse TCR Vβ8.1–3), biotin–KJ-16 (anti-mouse TCR Vβ8.1,2), and biotin–RR3.15 (anti-mouse Vβ11) were purified from the ascites of correspondent hybridomas and conjugated in our laboratory. Biotin–RR4.7 (anti-mouse TCR Vβ6) was purchased from Pharmingen (San Diego, California). M1/42.39 was purified from the supernatant of the hybridoma culture using a protein G column. Anti-Qa-1a and anti-Qa-1b antisera were prepared as described previously (Boyse et al., 1972; Stanton and Boyse, 1979; Eardley et al., 1978).

Cell Lines

L3 cells, an allo-reactive CD8+ CTL line from C57BL/6J strain specific to H-2^d alloantigen, were provided by Dr. G. Siu of Columbia University (Glasebrook and Fitch, 1979). These cells were cultured in Clicks' HEAA medium (Irvine Scientific, Santa Ana, California), supplemented with 10% fetal calf serum, 100 U/ml of penicillin and streptomycin, 2 mM β-mercaptoethanol, and 25 U/ml of human interleukin-2 (IL-2) (DandR Systems, Incorporated, Minneapolis, Minnesota). The L3 line was maintained by stimulation with irradiated (3,000 rads) spleen cells from BALB/c mice at a 1:5 to 1:10 ratio, every 10–14 days. The P815 (H-2^d) and EL4 (H-2^b) murine cell lines were obtained from the American Type Culture Collection, maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin and streptomycin, and 2 mM β-mercaptoethanol.

Generation of TCR Vβ8-Specific CTL Lines

Pooled spleen and lymph node cells from four SEB-primed mice (4–14 days after SEB injection) were first depleted of B cells, by incubating with goat anti-mouse immunoglobulin and goat anti-rat immunoglobulin coated magnetic beads (Advanced Magnetic, Incorporated, Cambridge, Massachusetts) at a ratio of 1×10^7 cells per ml of beads for 30 min at 4°C. The cells were separated from beads by a Bio Mag Separator (Advanced Magnetic, Incorporated, Cambridge, Massachusetts), and the unbound cells were then depleted of Vβ8+ T cells by using the anti-mouse TCR Vβ8 MAb, F23.1 (1×10^7, 10 μg). The cells from the second round of negative selection were positively selected for CD8+ cells by incubating 1×10^7 cells with 0.1 ml of 1:40 ascites of 53–6.72 (anti-mouse CD8), followed by incubation with goat anti-rat coated beads and subsequent magnetic separation. The cells bound to the beads were incubated overnight at 37°C to allow their dissociation from beads. The final purified population consisted of CD8+ Vβ8− T cells (>95%). The CD8+ Vβ8− T cells were then incubated with irradiated (3,000 rads) SEB-activated syngeneic CD4+ Vβ8+ T cell line (5–10 days after SEB stimulation) and APCs (syngeneic spleen cells) in a 1:1:1 ratio, at 37°C, 6% CO_2 for 14 days. IL-2 (10 U/ml) (DandR Systems, Incorporated, Minneapolis, Minnesota) was added on day 3 of the culture.

Generation of SEB and SEE-Induced CD4+ Target T Cell Lines

Spleen and lymph node cells from naive mice were first depleted of CD8+ T cells and then positively selected for TCR Vβ8+ T cells, or negatively selected for TCR Vβ8− T cells. CD4+ Vβ8+ T cells were stimulated with SEB (1 μg/ml) (Toxin Technology, Madison, Wisconsin); CD4+ Vβ8− T cells were stimulated with SEE (0.1 μg/ml) (Toxin Technology); and CD4+ T cells were stimulated with both SEB (1 μg/ml) and SEE (0.025 or 0.1 μg/ml) or SEB (1 μg/ml) alone. In all SEB- or SEE-primed T cell cultures, irradiated (3000 rads) spleen cells were added as APCs. After 3 days, T cell blasts were isolated and cultured in complete IMDM medium supplemented with IL-2 (10 U/ml). The phenotypes of these T cells were determined by staining the cells with APC–GK1.5, Fl–53–6.72, and biotin–F23.1 or biotin–RR-3.15 (anti-Vβ11) and analyzed by FACS.

FACS Analysis for Detecting SEB-Induced TCR Vβ-Specific T Cell Death in Peripheral Blood

Mice were injected in the tail vein on day 1 with either 50 μg SEB in 0.1 ml phosphate-buffered saline (PBS) or PBS alone. One group of mice were CD8+ T cell depleted 7 days before SEB/PBS injections as previously described (Jiang et al., 1992). On days 4, 7, 14, and 21 after priming, the mice were examined for numbers of CD4+ Vβ8.1,2+; CD8+ Vβ8.1,2+, CD4+ Vβ6+, and CD8+ Vβ6+ T cells. At each timepoint, peripheral blood drawn from the tail vein of 4–6 mice in each group was pooled, mononuclear cells were purified on a lymphocyte separation medium gradient (Organon Technicka, Durham, North Carolina), and stained with the following MAbs: Fl–53–6.72 (anti-CD8); APC–GK1.5 (anti-CD4); and biotin–KJ-16 (anti-Vβ8.I,2) or biotin–RR4.7 (anti-Vβ6). Biotin conjugates were revealed with Texas red–avidin. Dead cells were excluded using propidium iodide. Flow cytometric analysis was performed on a dual laser FACStar plus (Becton-Dickinson, San Jose, California) using FACS/DESK data analysis software (Stanford University, Stanford, California). The data are expressed as the percent of total CD4+ or total CD8+ T cells.

FACS Analysis for Measuring Specific CTL Activity

To assay the L3 cell line for H-2^d allospecific killing, we used the H-2^d-expressing target, P815, a CD8− mastocytoma cell line derived from DBA/2 mice as the positive target. El-4, a H-2^b-expressing CD8− T lymphoma line served as the negative target. Graded numbers of L3 effector cells were added to a fixed ratio of H-2^{d+} (specific targets) to H-2^{d-} (nonspecific targets) for 4 hr prior to FACS analysis (see below). The reduction in the ratio of H-2^{d+}/H-2^{d-} cells in the presence of effector cells, compared with the ratio of target cells in the absence of effectors, served as a measure of cell death.

In the TCR Vβ8-specific cytolytic system, SEB-activated CD4+ Vβ8+ T cells were used as specific Vβ8+ targets. In addition, SEB plus SEE or SEB alone were used to activate CD4+ Vβ8− T cells. These CD4+ Vβ8− served as nonspecific Vβ8− targets. In this system, the target T cells used were from day 4–6 superantigen-activated (SEB or SEE) cultures, because we had noted that susceptibility to lysis varied as a function of time following superantigen activation and peaked between days 4 and 6. Graded numbers of putative CD8+ CTL effector populations were then added to Vβ8+ and Vβ8− targets for 24 hr and the ratio of Vβ8+/Vβ8− cells was measured by FACS. The baseline control was the ratio of Vβ8+/Vβ8− after 24 hr in the absence of effector cells.

In both CTL systems, triplicate or duplicate samples were set up for each E:T ratio. Following effector to target cell incubation, two-color fluorescence was used to distinguish targets from effectors, as well as specific from nonspecific targets. Targets and effectors were distinguished by expression of CD8 using the conjugated anti-CD8 antibodies Fl–53–6.72, or APC–53–6.72 antibodies. In the L3 system, cells were stained with Fl-34.1.2 (anti H-2^d) to distinguish P815 (H-2^d) from EL4 (H-2^b). In TCR Vβ-specific cytolytic system, cells were stained with biotin–F23.1 to distinguish Vβ8+ targets from Vβ8− targets, or biotin–RR3.15 to distinguish Vβ11+ targets from Vβ11− targets. Biotin-conjugated reagents were revealed with Texas red–avidin. Dead cells were excluded using propidium iodide. For data analysis, the CD8+ T cells were gated out and the data was expressed as the ratio of the positive stained cells (specific targets) versus negative stained cells (nonspecific targets). The percentage of the specific cytolysis of specific targets was calculated as

$$\{([\text{positive stained cells / negative stained cells of control group}] - [\text{positive stained cells / negative stained cells of experimental group}]) / [\text{positive stained cells / negative stained cells of control group}]\} \times 100\%$$

^{51}Cr Release Assay

P815, EL4, SEB-activated CD4+ Vβ8+ T cells, and SEE-activated CD4+ Vβ8− T cells were labeled with 200 μCi ^{51}Cr (New England Nuclear, Boston, Massachusetts) for 45–60 min, washed three times and used as targets. L3 effector cells and TCR Vβ8-specific CD8+ CTL cells were added to corresponding targets at varying E:T ratio and incubated at 37°C for either 4 hr (standard assay) or 12 hr (special assay). In lectin-induced MHC class I–independent cytolysis, ConA was added at the beginning of the culture (20–40 μg/well). After incubation, the supernatants were harvested, and radioactivity was counted on a γ counter (LKB, Piscataway, New Jersey). The mean of triplicate samples was calculated and the percent specific ^{51}Cr release was determined as follows:

Percentage of specific cytolysis = [(experimental ^{51}Cr release − control ^{51}Cr release) / (maximum ^{51}Cr release − control ^{51}Cr release)] × 100%

Experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from targets exposed to 5% Triton X-100.

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