Selective Anergy of Vβ8+,CD4+ T Cells in Staphylococcus Enterotoxin B-primed Mice

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Summary

The cellular basis of the in vitro and in vivo T cell responses to Staphylococcus enterotoxin B (SEB) has been investigated. The proliferation and cytotoxicity of Vβ8.1,2+,CD4+ and CD8+ T cells were observed in in vitro response to SEB. In primary cytotoxicity assays, CD4+ T cells from control spleens were more active than their CD8+ counterparts, however, in cells derived from SEB-primed mice, CD8+ T cells were dominant in SEB-specific cytotoxicity. In vivo priming with SEB abrogated the response of Vβ8.1,2+,CD4+ T cells despite the fact that these cells exist in significant number. This SEB-specific anergy occurred only in Vβ8.1,2+,CD4+ T cells but not in CD8+ T cells. These findings indicate that the requirement for the induction of antigen-specific anergy is different between CD4+ and CD8+ T cells in post-thymic tolerance, and the existence of coanergic signals for the induction of T cell anergy is suggested.

Staphylococcal enterotoxins (SEs)[1] provoke dramatic T cell responses (1–3). These are extremely potent polyclonal mitogens stimulating a large proportion of both murine and human T cells. Minor lymphocyte stimulating (Mls) antigens have a similar nature to SEs (4–6), but the primary structure of the Mls antigen is not known, as neither the gene nor its product are yet isolated. These antigens are labeled "superantigen." Recent evidence has demonstrated that these antigens are recognized by T cells expressing particular TCR Vβ gene families in the context of class II MHC (Ia) antigen (7–11), and this was further supported by reports showing the affinity of SEs to Ia antigen (12, 13). These antigens are reported to cause the intrathymic negative selection of reactive cells and result in major effects on the TCR repertoire in both CD4+ and CD8+ T cells (10, 14–19). It was also shown that post-thymic tolerance to foreign Mls antigen is achieved by clonal anergy of CD4+ T cells (20). Although superantigens have these strong effects on the formation of the repertoire in both CD4+ and CD8+ T cells, it is not clear whether CD8+ T cells are responsive to these antigens or under similar control in the induction of post-thymic tolerance. Here we demonstrate that both Vβ8.1,2+,CD4+ and CD8+ T cells in vitro respond to Staphylococcus enterotoxin B (SEB), whereas in vivo injection of SEB causes anergy only in CD4+ but not in CD8+ T cells. These findings indicate that the requirement for the induction of antigen-specific anergy is different between CD4+ and CD8+ T cells in post-thymic tolerance. It is suggested that antigen recognition by TCR alone is not sufficient and that supplemental signals, termed here "Coanergic" signals, are necessary to induce anergy.

[1] *Abbreviations used in this paper:* Mls, minor lymphocyte stimulating; RT, room temperature; SE, Staphylococcal enterotoxin.

Materials and Methods

Mice. BALB/c byJ mice (4–6 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME).

mAbs and Mitogens. B cell hybridoma lines producing antibodies directed against murine CD4 (GK1.5), CD8 (3.155) were purchased from American Type Culture Collection (Rockville, MD). Vβ8.1,2 idiotype-specific mAb KJ16-133- (21) and Vβ8.1,2,3 idiotype-specific mAb F23.1- (22) producing hybridoma lines were provided by Drs. P. Marrack, J. Kappler (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), and M.J. Bevan (Research Institute of Scripps Clinic, La Jolla, CA). PE-anti-CD4 antibody and PE-avidin were purchased from Becton Dickinson & Co. (Mountain View, CA). SEA was purchased from Toxin Technologies, Inc. (Madison, WI). SEB was purchased from Sigma Chemical Co. (St. Louis, MO). Con A was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Cell Preparation. Spleen cells were treated with biotinated anti-CD4 (GK1.5) or anti-CD8 (3.155) (both were purified from the supernatant of B cell hybridomas by protein A-Sepharose [Pharmacia Fine Chemicals, Uppsala, Sweden]). Cells were washed and mixed with streptavidin magnetic beads (Dynabeads M280; Dynal A.S., Oslo, Norway) at 10.0 μl/10^5 cells for 30 min at room temperature (RT). The magnetically coated cell were isolated by a magnetic field generated by MACS, (Miltenyi Biotec GmbH., Meitzfeld, FRG). Purification for positively coated cells was performed twice, resulting in >90% purity for the phenotype selected. CD4- spleen cells, CD8- spleen cells, or CD4,CD8 double-

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negative spleen cells were obtained after the above separation protocol.

Flow Microfluorometry. Spleen single cell suspensions of unprimed BALB/c mice were treated with Tris-buffered 0.16 M ammonium chloride to lyse the RBC. These cells were cultured for 72 h in RPMI 1640 medium supplemented with 10% FCS and 5×10^{-5} M 2-ME in a 24-well culture plate at a concentration 2×10^6/ml per well in the presence of 10.0 μg/ml SEB or 5.0 μg/ml of Con A at 37°C. Those cultured or fresh spleen cells were washed and then incubated with KJ16-133 or F23.1 culture supernatant at 10^6/ml for 30 min. These cells were washed three times and treated with fluorescein (green) goat anti-rat or mouse Ig reagent. The cells were then treated with PE (red) anti-CD4 antibody or biotinated anti-CD8 for 30 min. To determine CD8 expression, cells were washed and further incubated with PE-avidin for another 30 min. Cells were washed followed by two-color (red and green) fluorescence analysis by Epics-C fluorocytometry (Coulter Electronics Inc., Hialeah, FL). All incubations during cell staining were done on ice.

Proliferation Assay. Spleen cells were stimulated in vitro with SEA, -B, or Con A in 96-multiwell tissue culture plates at 10^5 cells/100 μl or a reciprocal number (2^{-2}–$2^2 \times 10^4$) of cells in each well. After 40 h of culture, cells were pulsed with 1 μCi/well of [^{3}H]thymidine (Amersham International, Amersham, UK). Cells were collected 8 h later by glass fiber filter mats. Radioactivity was determined in a liquid scintillation beta counter.

IL-2 Production Assay. Single cell spleen suspensions of SEB-primed or control BALB/c mice were stimulated with SEB (10.0 μg/ml) in 24-well plastic culture dishes at 2×10^6/ml per well. Supernatants were collected after 48 h and were tested for the growth promotion of the IL-2-dependent cell line CTLL-1. To do this, CTLL-1 cells were cultured in 96-well U-bottomed culture plates at 5×10^4 cells/well in 200 μl of medium containing varied concentrations of samples. After 24 h, cells were pulsed with 1 μCi/well of [^{3}H]thymidine for 16 h. Cells were collected onto glass fiber filter mats by a microsample harvester (Skatron, Inc., Sterling, VA). Radioactivity was determined in a liquid scintillation beta counter. Data indicated are arithmetic means of triplicate samples.

Cytotoxicity Assay. ^{51}Cr (Amersham International)-labeled murine B lymphoma cells, A20-2J (23) (H-2^d, Ia$^+$) (10^4), are cultured with varied number of effector cells (spleen cells) in the presence of 10 μg/ml of SEB in 96-well U-bottomed tissue culture plates (final volume 200 μl/well). 100 μl of the 16-h culture supernatant was assayed for radioactivity by a gamma counter. We performed a 16-h assay to compare CD4$^+$ T cell cytotoxicity (which is optimum after 10 h; data not shown) and CD8$^+$ T cell cytotoxicity (which has a faster time course and shows significant cytotoxicity after 2 h; data not shown). Maximum release of ^{51}Cr was measured by incubating targets in 1 M HCl. Spontaneous release of ^{51}Cr from A20-2J cells was consistently ~20% after 16 h in culture. The percentage of specific lysis was calculated as 100× [(experimental − spontaneous release)/(maximum − spontaneous release)].

Results

In Vitro Primary Proliferative Response of Spleen T Cells to SEB. We compared the relative responsiveness of CD4$^+$ and CD8$^+$ T cells against SEB. Unfractionated spleen cells were stimulated in vitro with SEB at a concentration of 10.0 μg/ml, and with 5.0 μg/ml of T cell mitogen, Con A (Table 1). Two-color fluorescence analysis showed that SEB stimulation dramatically increased the proportion of both CD4$^+$ and CD8$^+$ T cells expressing Vβ8.1,2, whereas Con A did not change the proportion of these T cells compared with unstimulated fresh spleen cells. The Vβ8.1,2$^+$ cells increased more than twofold among CD4$^+$ T cells, while Vβ8.1,2$^+$, CD8$^+$ cells had a lower, but still significant increase. Thus, it was demonstrated that Vβ8.1,2$^+$ cells are selectively expanded in both the CD4$^+$ and CD8$^+$ T cell population by SEB stimulation.

Proliferative Response of SEB-primed Spleen Cells. We now had the unique opportunity to perform a comparative study of the specific immunoresponse of CD4$^+$ and CD8$^+$ T cells by a single antigen. Therefore, we further examined the secondary in vitro response using spleen cells of BALB/c mice primed with SEB 7 d before the experiment. The size of the spleen was usually larger in the SEB-primed mice than control mice and contained two- to threefold more cells than control spleens, but the ratio of Thy-1$^+$ cells and surface Ig-positive cells was unchanged (data not shown). Single cell suspensions from the spleens of these mice were stimulated in vitro with SEB to examine the functional capacity in proliferative response in comparison with that of PBS-injected control mice (Fig. 1). Spleen cells of control mice showed a strong response at $>3 \times 10^4$ cpm at 10.0 μg/ml SEB in a [^{3}H]thymidine incorporation assay (Fig. 1 *a*). The spleen cells derived from SEB-primed mice, however, showed reduced levels of proliferation at $<10^4$ cpm. Reduction of proliferation was dependent on the priming dose of SEB, and >80% of the response was suppressed in 50.0 μg SEB-primed mice. The suppressed proliferation of SEB-primed spleen cells was further studied by the cell dose titration experiment (Fig. 1 *b*). The results indicated a poor proliferation of 50 μg SEB-primed spleen cells in response to SEB stimulation, and the proliferative efficacy was ~25% compared with the control cells. These SEB-unresponsive cells and control cells, however, showed comparative responses in a proliferation assay to SEA that stimulates Vβ1, -3, -11, and -12 TCR$^+$ cells (24) (Fig. 1 *c*), indicating that the functional unresponsiveness occurred in a SEB-specific manner.

IL-2 Production of SEB-primed Spleen Cells. The results presented in Fig. 2 showed unresponsiveness in IL-2 production of SEB-primed spleen cells. In this experiment, IL-2 in 10.0 μg/ml SEB-stimulated spleen supernatant was quantitated by testing growth-promoting activity on the indicator cell line, CTLL-1. In SEB-primed mice, the production of IL-2 in response to in vitro SEB stimulation was suppressed in a primed dose-dependent fashion. The IL-2 production was already reduced at 1.0 μg per injection, and [^{3}H]thymidine uptake was ~30% of the control. The entire IL-2 production was almost abrogated at 50.0 μg of injection.

Proliferative Unresponsiveness of CD4$^+$ T Cells in SEB-primed Spleen. Spleen cells from 50.0 μg SEB-primed mice were further studied by purifying CD4$^+$ T cells and CD8$^+$ T cells to determine if both populations were equally unresponsive to SEB (Fig. 3). In control mice, both CD4$^+$ and CD8$^+$ T cells responded to SEB (10.0 μg/ml) in the presence of CD4$^-$, CD8$^-$ spleen cells as the source of Ia$^+$ cells. The response of CD4$^+$ T cells was significantly reduced when cells purified from SEB-primed mice were used. However, CD8$^+$

Table 1. *In Vitro Primary Proliferative Response of Vβ8.1,2⁺,CD4⁺ and CD8⁺ Spleen T Cells to SEB*

Phenotype of T cells	Percent of positive cells		
	Fresh spleen (n = 4)	SEB (10.0 μg/ml) (n = 3)	Con A (5.0 μg/ml) (n = 3)
CD4⁺	28.4 ± 2.1	44.2 ± 2.6	45.9 ± 2.3
CD8⁺	10.6 ± 1.6	46.6 ± 5.9	35.9 ± 7.3
KJ16⁺, CD4⁺	6.0 ± 0.9	23.6 ± 0.2	12.3 ± 0.4
KJ16⁺, CD8⁺	3.0 ± 0.6	19.3 ± 1.9	8.5 ± 0.3
KJ16⁺, CD4⁺/CD4⁺*	19.9 ± 1.7	56.7 ± 2.2	26.8 ± 1.3
KJ16⁺, CD8⁺/CD8⁺‡	27.6 ± 2.0	42.6 ± 10.4	24.7 ± 4.9

* Proportion of Vβ8.1,2⁺ cells among CD4⁺ T cells.
‡ Proportion of Vβ8.1,2⁺ cells among CD8⁺ T cells.

T cells from these mice proliferated in response to SEB to a level comparable with that seen in nonprimed control cells.

Proportional Study of Vβ8⁺ T Cells in SEB-primed Spleen. Previous studies have shown that the response of murine T cells to SEB is specific for the expression of Vβ3- or Vβ8-bearing TCR on the responding cell (10). BALB/c is a strain that has nondetectable levels of Vβ3⁺ T cells in the periphery because the Mls allotype is "2" (8). Therefore, Vβ8⁺ T cells represent the only SEB-reactive T cells in this mouse strain. We therefore examined whether the reduction in proliferative response was due to the deletion or anergy of Vβ8⁺ T cells. As shown in Table 2, the proportion of Vβ8.1,2⁺ cells was ~20% in CD4⁺ T cells and ~30% in CD8⁺ T cells in control mice. The proportion of Vβ8.1,2⁺ T cells decreased to ~15% in CD4⁺ T cells in SEB-primed mice, while the effect of SEB injection on the proportion of Vβ8.1,2⁺ cells was minimal in CD8⁺ T cells (but slightly decreased at a SEB dose of 50.0 μg). The study of F23.1⁺ cells (detects all Vβ8.1⁺, Vβ8.2⁺, and Vβ8.3⁺ cells) also showed similar results in 50 μg SEB-primed mice. These results show that Vβ8⁺ T cells remain in SEB-primed mice, and thus, CD4⁺ T cells seem to be in anergy.

Cytotoxic Response of SEB-primed Spleen Cells to SEB. In another set of experiments, we examined the cytotoxicity of spleen cells primed with SEB 7 d prior (Fig. 4). The spleen cells from control mice showed cytotoxicity against an Ia⁺

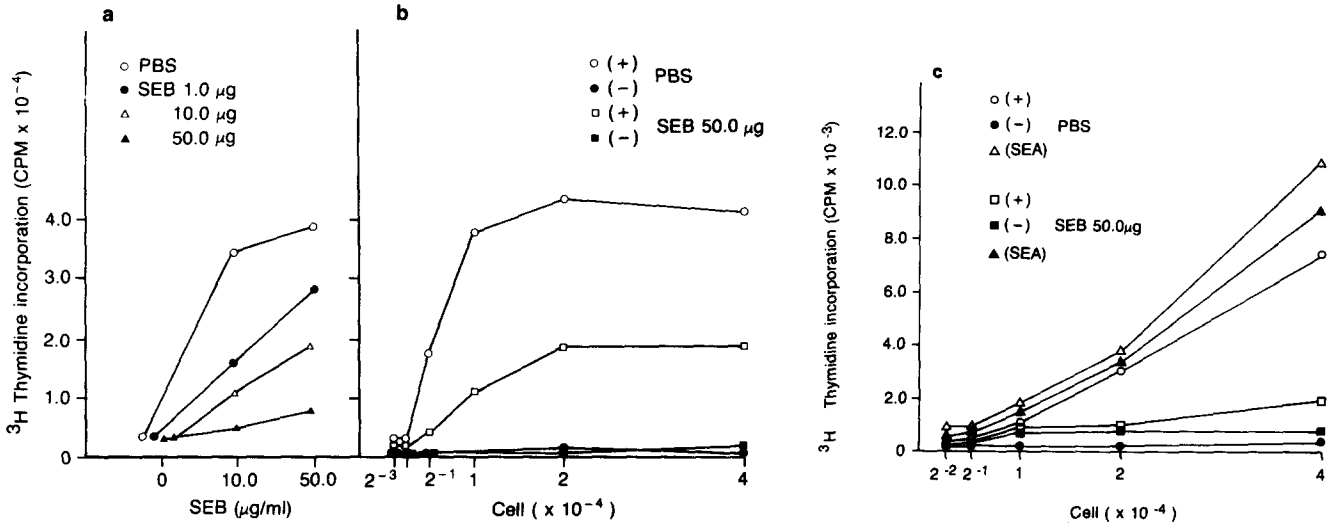

Figure 1. Suppression of SEB-specific proliferative response of SEB-primed spleen cells to SEB. BALB/c byJ mice were given one intravenous injection (by tail vein) of 1.0, 10.0, and 50.0 μg of SEB in 0.2 ml PBS. 1 wk later, spleen cells were in vitro stimulated with 10.0 and 50.0 μg/ml of SEB (*a*). In the second experiment, reciprocal numbers of 1-wk 50 μg SEB-primed spleen cells were stimulated with SEB at a concentration of 10.0 μg/ml (*b*). Reciprocal numbers of 1-wk 50 μg SEB-primed spleen cells were stimulated with SEB or SEA at a concentration of 10.0 μg/ml (*c*). Data are indicated as the arithmetic mean of triplicate samples. SD was normally <10%. Data shown is representative of three experiments. (−) SEB not added; (+) 10.0 μg/ml SEB added. (SEA) 10 μg/ml SEA added.

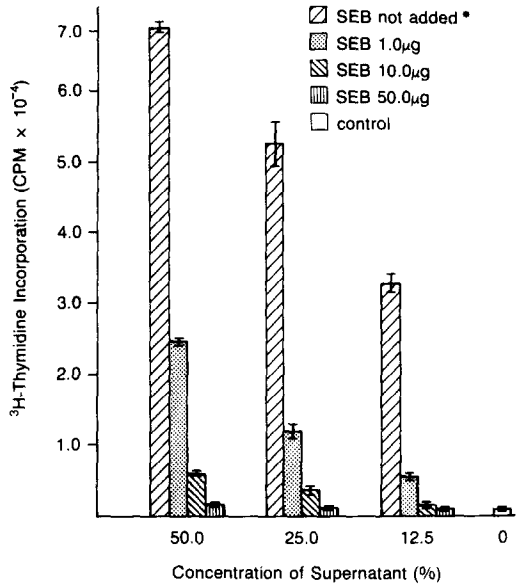

Figure 2. Unresponsiveness of IL-2 production in SEB-primed spleen. Single cell spleen suspensions of SEB-primed or control mice were stimulated with SEB (10.0 μg/ml). Supernatants were collected after 48 h and were tested for growth promotion of the IL-2-dependent cell line CTLL-1. Data are indicated as arithmetic means of triplicate samples.

Table 2. *Proportional Study of Vβ8$^+$ T Cells in CD4$^+$ and CD8$^+$ T Cells of SEB-primed Mice*

Reagents primed	T cells	Origin	Percent expressing Vβ8	*n*
PBS	KJ16$^+$,* CD4$^+$	CD4$^+$	20.3 ± 1.6	5
	F23.1$^+$,‡ CD4$^+$	CD4$^+$	31.0 ± 1.2	3
	KJ16$^+$, CD8$^+$	CD8$^+$	28.5 ± 1.3	3
	F23.1$^+$, CD8$^+$	CD8$^+$	36.6 ± 0.6	3
SEB (1.0 μg)	KJ16$^+$, CD4$^+$	CD4$^+$	16.3 ± 0.7	5
	KJ16$^+$, CD8$^+$	CD8$^+$	27.5 ± 0.8	3
SEB (10.0 μg)	KJ16$^+$, CD4$^+$	CD4$^+$	14.5 ± 1.3	5
	KJ16$^+$, CD8$^+$	CD8$^+$	25.3 ± 5.9	3
SEB (50.0 μg)	KJ16$^+$, CD4$^+$	CD4$^+$	14.2 ± 2.8	4
	F23.1$^+$, CD4$^+$	CD4$^+$	24.1 ± 1.0	3
	KJ16$^+$, CD8$^+$	CD8$^+$	20.7 ± 3.0	3
	F23.1$^+$, CD8$^+$	CD8$^+$	34.3 ± 2.3	3

* The cells expressing Vβ8.1,2$^+$ TCRs.
‡ The cells expressing Vβ8.1,2,3$^+$ TCRs.

B cell lymphoma (A20-2J) when 10.0 μg/ml of SEB was added to the culture. The observed cytotoxicity was cell dose dependent, and rose to >40% at an E/T ratio of 80 (Fig. 4 *a*). Cytotoxicity from SEB-primed spleen cells was as high as the control group and was SEB dose dependent. The CD phenotypes of these SEB-specific cytotoxic T cells, in both control and SEB-primed spleen, were determined by cytotoxic assays on T cells positively selected for CD4 or CD8 expression. Also, the negative fraction obtained from the selection of CD4$^+$ or CD8$^+$ T cells was tested for SEB-specific killing (shown in Fig. 4, *b* and *c*). In control mice, cytotoxicity was dominantly demonstrated by the CD4$^+$ and the CD8$^-$ cell fractions. The profile of cytotoxic effector T cell phenotype was inverted in 50.0 μg SEB-primed mice, where the CD8$^+$ and the CD4$^-$ cell fractions are dominantly cytotoxic. Inhibition studies by anti-CD4 mAb blocked the cytotoxicity of the control spleen cells, but the cytotoxicity of SEB-primed spleen cells was not inhibited. In contrast, anti-CD8 antibody was not effective in inhibition of cytotoxicity in either case (data not shown).

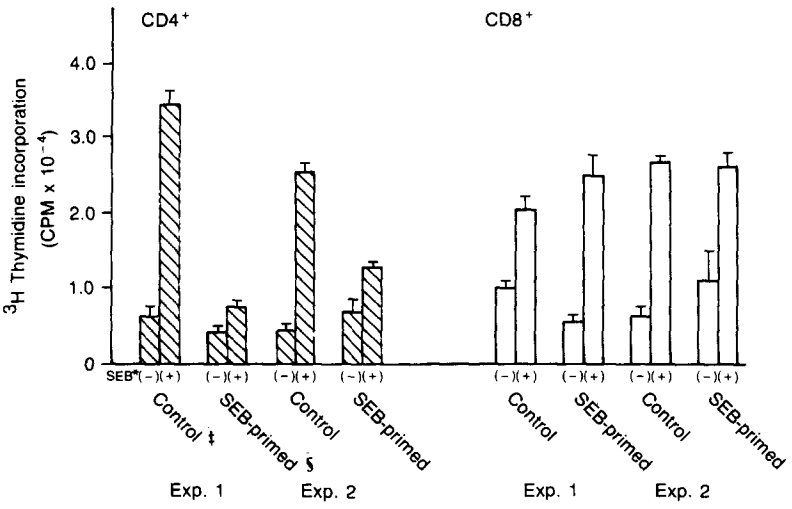

Figure 3. Proliferative unresponsiveness of CD4$^+$ T cells in SEB-primed spleen. BALB/c byJ mice were given one intravenous injection of 50.0 μg of SEB in 0.2 ml PBS. 1 wk later, the separated CD4$^+$ and CD8$^+$ spleen T cells were mixed (1:1) with the CD4$^-$, CD8$^-$ fraction of spleen cells to examine the SEB-specific proliferation. Data from two separate experiments are presented. (*) 10.0 μg/ml SEB; (‡) PBS pre-injected; (§) SEB 50.0 μg pre-injected; (−) SEB not added; (+) 10.0 μg/ml SEB added.

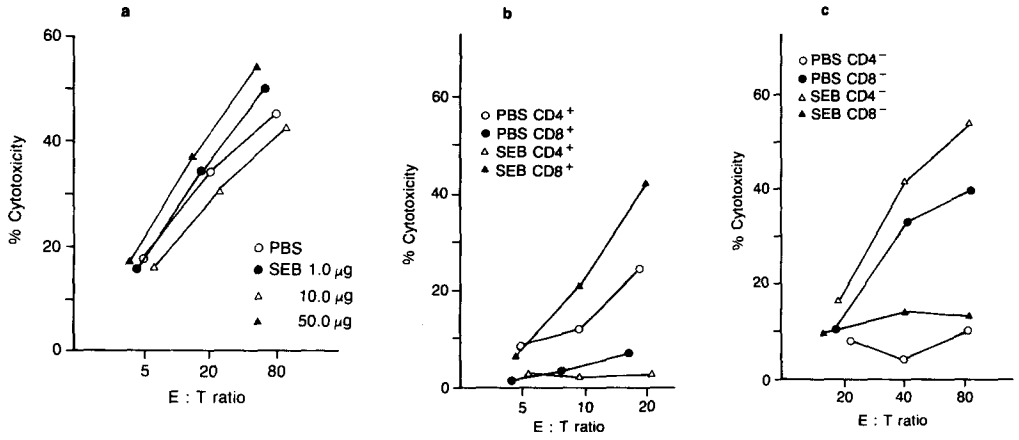

Figure 4. Cytotoxic response of SEB-primed spleen cells to SEB. Unfractionated spleen cells (*a*), purified CD4+ and CD8+ T cells (*b*), and CD4− or CD8− fractions (*c*) were cultured with 10^4 ^{51}Cr-labeled A20-2J and 10.0 μg/ml SEB at varied effector (spleen cells)/target (A20-2J) ratios. (*c*) Specific lysis was calculated by the same formula, except that the nonspecific release from the coculture of effector and target cells in the absence of SEB for each E/T ratio was used instead of spontaneous release. This is due to the elevated background cytotoxicity (~20%) from CD4− or CD8− spleen cells. Presented data are the means of triplicate wells. SD was normally <10%. Data shown represent three separate experiments that consistently gave similar results. In *a*, SEB 1, 10, and 50 μg, indicates the dose of SEB-primed 7 d previous to the experiment.

Discussion

In the studies cited above, we have observed the in vitro proliferation and cytotoxicity of CD4+ and CD8+ T cells in response to SEB. In vivo priming with SEB abrogates the response of Vβ8+,CD4+ T cells despite the fact that these cells exist in significant number. This supports the previous observation of anergy occurring in Vβ6+,CD4+ T cells in Mls-1^b mice immunized with Mls-1^a-expressing cells reported by Rammensee et al. (20). We also determined that IL-2 production was suppressed in SEB-primed spleen cells, which further supports SEB-specific anergy, since it parallels the results of Rammensee. However, in contrast to Mls-specific anergy and our in vitro results, we consistently observed a decreased proportion of Vβ8+, CD4+ T cells in vivo. This occurred in lymph nodes as well as in spleen (data not shown). This observation may reflect an in vivo regulatory mechanism to prevent the expansion of particular Vβ+ T cells by an idiotypic network. Detailed studies of this reduction of Vβ8+ T cells in SEB-primed spleen and lymph nodes are in progress (Kawabe, Y. and A. Ochi, manuscript submitted for publication).

In primary cytotoxicity assays, CD4+ T cells from control spleens were more active than their CD8+ counterparts, but in cells derived from SEB-primed mice, CD8+ T cells were dominant in cytotoxicity. This may reflect the need of CD8+ cytotoxic T cells for "help" from CD4+ T cells to mature functionally (25). It is an intriguing question whether these functionally mature CD8+ T cells have any connection with the SEB-induced suppressor T cells that have been previously reported (2, 26). While no significant suppressor activity has been observed from these cells with regard to SEB-specific primary proliferation, the effect may be B cell specific.

The major question on how CD4+ T cells can be induced to anergy while CD8+ T cells remain active in vivo remains to be answered. The observation that CD4+ T cells recognize SEB and Ia in association with the CD4 molecule (27, 28) may indicate that the induction of anergy requires the recognition of the antigen-MHC complex by the TCR and the interaction of supporting elements such as CD4 and CD8. These molecules may indeed mediate "coanergic" signals since it has been reported that they are capable of transducing both positive and negative signals (29–31). Therefore, since CD8+ T cells fail to generate these putative coanergic signals, due to the lack of the CD4-Ia interaction, this may then explain the observed tolerance to anergy of CD8+ T cells.

In summary, an experimental system using a soluble, well-characterized TCR Vβ-specific antigen that stimulates both CD4+ and CD8+ T cells in vitro will provide us with an unique opportunity to elucidate the mechanism of positive and negative regulation of T cell response in the post-thymic environment.

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