In Vivo Induction of Anergy in Peripheral Vβ8+ T Cells by Staphylococcal Enterotoxin B

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Summary

We have developed a model of peripheral in vivo T cell tolerance that is induced by administration of the protein superantigen staphylococcal enterotoxin B (SEB). Rather than activating Vβ8+ T cells, in vivo administration of SEB induced in them a profound state of anergy. This was shown by their failure to proliferate to subsequent in vitro restimulation with SEB or to anti-Vβ8 antibodies. This unresponsiveness was Vβ8 specific since T cells from SEB-immunized mice responded normally to other antigens. 8 d after SEB administration, there was no reduction in the number of Vβ8+ T cells or in the intensity of Vβ8 T cell receptor (TCR) expression. Although a portion of the Vβ8+ T cells from SEB-primed mice were able to express interleukin 2 receptors (IL-2Rs), they failed to proliferate in response to exogenous IL-2, indicating they were defective in their IL-2 responsiveness. 2–4 wk after SEB administration, there was a reduction of ~50% in the number of Vβ8+ cells in immunized compared with control animals. There was, however, no reduction in the level of TCR expression on the remaining Vβ8+ cells. These data demonstrate that proteins that activate T cells in vitro in a Vβ-specific manner can induce a state of anergy in peripheral T cells in vivo and may possibly further mediate clonal deletion in a portion of the tolerized cells.

During the development of a functional immune repertoire, antigen-specific components of the immune system must learn to discriminate self from non-self. Recent studies have shown that to establish self tolerance many potentially self-reactive T lymphocytes are clonally deleted intrathymically (1–5). This has been demonstrated in systems wherein the expression of particular TCR Vβ regions correlates with specificity to self antigens such as I-E and minor lymphocyte stimulatory (Mls)[1] antigens (1–3), as well as in TCR transgenic mouse models (4, 5). Additionally, it was demonstrated that tolerance in the developing immune system can be acquired by functional inactivation (anergy) in the absence of clonal deletion, notably to self antigens expressed only on thymic epithelium (6) or only extrathymically (7–9).

Mature peripheral T cells can also be rendered tolerant in vitro (10, 11) and in vivo (10, 12, 13). The studies on in vivo tolerance induction demonstrated that it is possible to induce tolerance in mature T cells to minor or class I histocompatibility antigens (9, 12), to Mls antigens (12, 13), and to protein antigens that were coupled to chemically modified APC (10). Furthermore, tolerance can be induced in mature T cells by soluble protein antigens after both in vivo or in vitro priming (14–19). Tolerance induction in these situations was critically dependent upon factors such as the route of administration (either intravenously or oral), and the amount and chemical state of the antigen (monomeric rather than aggregated). Because TCR usage in these responses was undetermined, it was not possible to distinguish among anergy, clonal deletion, or regulation by suppressor cells as the mechanism of tolerance.

The purpose of the current investigation was to examine the mechanisms of the induction of tolerance in vivo in mature peripheral T cells. In the current study, we demonstrate that T cells from BALB/c mice primed with the superantigen staphylococcal enterotoxin B (SEB) displayed a dramatically diminished response to this antigen upon subsequent in vitro activation. Because SEB has been shown to specifically activate T cells that express Vβ8+ TCRs (20), we were able to directly assay the activity of Vβ8+ T cells using Vβ8-specific antibodies. In vivo administration of SEB (either subcutaneously in adjuvant or intravenously) induces a state of profound unresponsiveness in Vβ8+ T cells, yet these cells are present in normal numbers, with no apparent downregulation of the level of TCR expression. The observed unresponsiveness is specific because SEB-immunized animals respond

[1] *Abbreviations used in this paper:* Mls, minor lymphocyte stimulatory; PPD, purified protein derivative; SE, staphylococcal enterotoxin.

normally to in vitro stimulation with other antigens. Thus, these data demonstrate that a soluble protein that activates T cells in a Vβ-specific manner can induce tolerance in peripheral T cells by the induction of clonal anergy.

Materials and Methods

Animals. BALB/c and B10.BR mice (4–8 wk old) were purchased from either the National Cancer Institute Frederick Animal Research Facility (Frederick, MD) or The Jackson Laboratory (Bar Harbor, ME). All other inbred strains of mice were purchased from The Jackson Laboratory.

Reagents. SEB was purchased from Sigma Chemical Co. (St. Louis, MO). SEA was purchased from Toxin Technologies (Madison, WI).

Fluorescence Staining (FACS). Cells were suspended in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide (FACS buffer). Cells (10^6/100 μl buffer) were incubated for 30 min on ice with 10 μl of the appropriate FITC-conjugated antibody. For two-color analysis, after washing, cells were incubated with biotinylated mAb to CD4 or CD8, and then allophycocyanin-labeled avidin (Caltag Laboratories, Inc., San Francisco, CA). Control staining of cells with irrelevant antibody was used to obtain background fluorescence values. Single-color analysis was done on a FACSCAN (Becton Dickinson & Co., Mountain View, CA) interfaced to a PDP 11/24 computer. Reagents used for direct staining were FITC- or biotin-conjugated anti-: α/β (21), CD8 (22), CD4 (23), Vβ3 (24), Vβ6 (25), Vβ8 (26), and IL-2R (27).

T Cell Proliferation Assays. Mice were immunized in the hind footpad with SEB (50 or 100 μg/hind foot pad) emulsified in CFA H37Ra (Difco Laboratories Inc., Detroit, MI) or CFA alone. In some experiments, animals were injected with SEB in PBS intravenously through the tail vein. 8, 14, or 30 d later, T cells pooled from either the spleens or lymph nodes of three to five mice/group were isolated by passage over nylon wool. Cultures were performed in flat-bottomed microtiter wells (antigen-induced proliferation assays) or round-bottomed microtiter wells (mAb-induced proliferation assays) in a 1:1 mixture of Eagle's Hanks Amino Acids medium and RPMI 1640 supplemented with 10% FCS, 2-ME (5×10^{-5} M), Hepes buffer, glutamine, penicillin, and gentamycin. For antigen-induced proliferation, 10^5 T cells/well were stimulated with 5×10^5 irradiated (2,000 rad) Thy-1.2$^-$ (New England Nuclear, Boston, MA) syngeneic spleen cells as a source of APC in the presence of various concentrations of SEB, SEA, or 20 μg/ml purified protein derivative (PPD). After 3 d, cultures were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear) and harvested 12–18 h later. For the experiments that assessed the effect of exogenous IL-2 addition, 100 U/ml of rIL-2 (Cetus Corp., Emeryville, CA) was added per well at the initiation of the culture period. Thereafter, cell cultures were treated as described above.

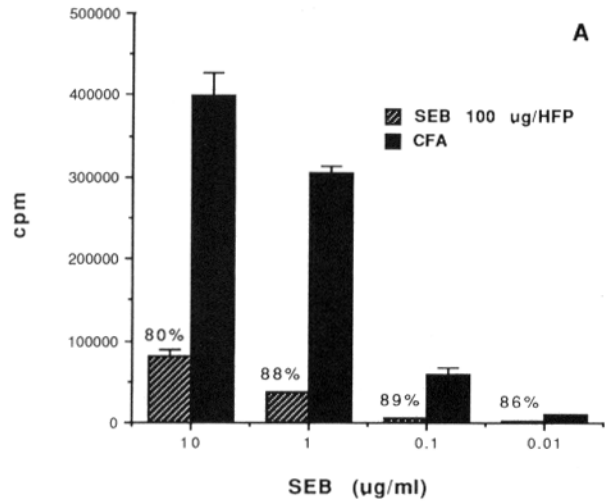
To assay Vβ-specific proliferation, purified mAbs were diluted to 10 μg/ml with PBS, and 30 μl was added per microtiter well. Plates were incubated at 37°C for 2–3 h and then washed three times with PBS before use. Various numbers of T cells were added in 100 μl medium (see above) and were cultured in a total of 150 μl. After 3 d, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 12–18 h later. For all the proliferation assays, the experimental values shown represent the arithmetic means of determinations performed in triplicate wells. SE were invariably <20% of the mean.

Analysis of IL-2R Expression. Nylon wool–purified lymph node T cells (5×10^5/ml) from BALB/c mice immunized with either SEB in adjuvant or CFA alone were stimulated with irradiated (2,000 rad) Thy-1.2$^-$ syngeneic spleen cells (10^6/ml) as a source of APC in the presence of 10 μg/ml SEB for 48 h. The culture medium was as described above for the T cell proliferation assays. After 48 h, aliquots of each culture (SEB vs. CFA) were removed and assessed for proliferation by the addition of 1 μCi of [^{3}H]thymidine. Pulsed cells were harvested 12–18 h later. The remainder of the cells were collected, washed once, and purified by density centrifugation over Ficoll-Hypaque (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ) to remove dead cells. The cells were then stained and analyzed by FACS for their expression of the TCR-α/β, TCR Vβ8, TCR Vβ6, and the IL-2R α chain.

Results

BALB/c mice (three/group) were immunized with either SEB emulsified in CFA or with CFA alone. Mice administered SEB by this protocol displayed no discernible ill effects and appeared healthy throughout the post-immunization period. 8 d after in vivo priming with SEB, lymph nodes or splenic T cells were assayed for proliferation to SEB, SEA, PPD, and F23.1 (anti-Vβ8). SEA was used as a control because it is a superantigen that activates Vβ1$^+$, Vβ3$^+$, and Vβ11$^+$, but not Vβ8$^+$ T cells (20). Fig. 1 shows the in vitro proliferation of lymph node T cells from SEB- vs. CFA-primed BALB/c mice after stimulation with SEB and SEA. The proliferation of T cells from SEB-primed mice to SEB was dramatically reduced in comparison with the SEB-induced proliferation of T cells from mice primed with CFA alone (Fig. 1 *A*). In contrast, there was no significant difference between the SEA- and PPD-induced proliferative responses of T cells from SEB/CFA- vs. CFA-immunized mice (Fig. 1 *B*). These results were highly reproducible in several experiments. Because Vβ8$^+$ T cells predominate in the response to SEB, it was possible that the diminished SEB response occurred because SEB administration had eliminated Vβ8$^+$ T cells (SEB also activates Vβ3$^+$ T cells [20], but BALB/c mice express Mls^c and thus have eliminated all Vβ3$^+$ T cells by clonal deletion [28, 29]). Therefore, we examined the level of Vβ8 expression in the SEB- vs. CFA-primed mice. As shown in Fig. 2 *A*, the percentage of T cells expressing Vβ8 was comparable in SEB- and CFA-primed animals. There appeared to be no downregulation of the level of TCR on the Vβ8$^+$ cells in SEB-primed animals. Moreover, the total number of T cells present in the lymph nodes and spleens of SEB/CFA- and CFA-primed mice were also comparable (Fig. 2). In four separate experiments, the mean percentages of α/β$^+$ T cells expressing Vβ8 8 d after immunization were 27.5 ± 1.47% for SEB/CFA-primed animals and 28.0 ± 2.2% for CFA-primed animals. Significantly, both CD4$^+$ and CD8$^+$ T cells were well represented among the Vβ8$^+$ T cells from the SEB-primed mice (Fig. 2 *B*).

We next examined the proliferative capacity of Vβ8$^+$ T cells in each group by directly activating T cells with the Vβ8-specific mAb, F23.1 (Fig. 3). The data in Fig. 3 *A* show a >90% reduction in the F23.1-induced proliferation in the SEB-primed mice compared with the CFA-primed controls. Because the degree of inhibition of Vβ8$^+$ T cells was ~90%



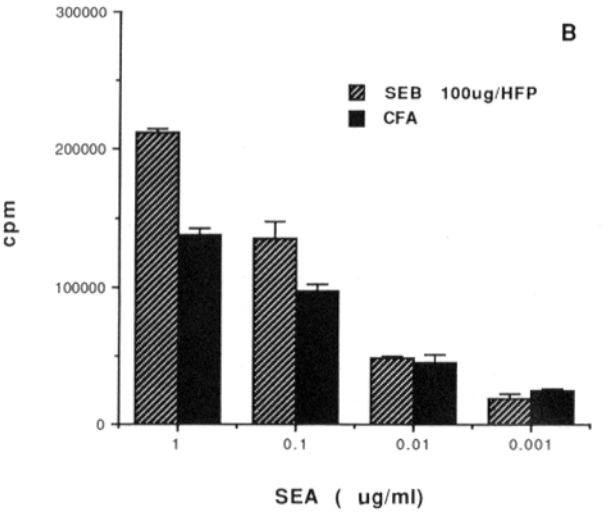
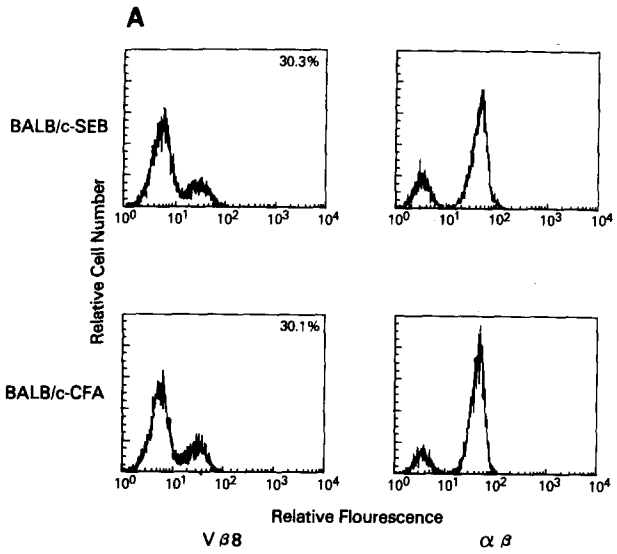


Figure 1. Lymph node T cells from BALB/c mice immunized subcutaneously with SEB are profoundly unresponsive to subsequent in vitro restimulation with SEB, but respond normally to SEA. Pooled nylon wool-purified T cells from three mice per group immunized with either SEB emulsified in CFA or CFA alone were stimulated in vitro (10^5 T cells/well) with 5 × 10^5 irradiated (2,000 Cy) Thy-1-depleted syngeneic spleen cells as a source of APC and various concentrations of SEB (*A*), SEA (*B*), PPD at 20 μg/ml, or medium alone. Counts per minute induced by PPD were 21,502 for SEB-primed T cells and 19,645 for CFA-primed T cells. The percent reductions in SEB-induced proliferation by the SEB/CFA- compared with the CFA-primed T cells are shown for each in vitro dose of SEB (*A*). In three independent experiments identical to the one shown here, the mean percent reductions in in vitro SEB-induced proliferation of SEB/CFA vs. CFA-primed T cells were 10 μg/ml SEB, 81.2 ± 1.4%; 1 μg/ml SEB, 92.1 ± 2.1%; 0.1 μg/ml SEB, 92.4 ± 1.6%; 0.01 μg/ml SEB, 83.8 ± 5.3%. The error bars indicate the SEM of triplicate determinations.



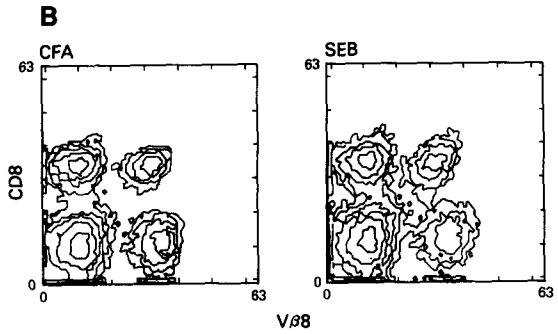


Figure 2. Flow cytometry analysis of cell surface phenotype of T lymphocytes from BALB/c mice immunized 8 d previously with either SEB or CFA. (*A*) Analysis of total Vβ8$^+$ cells nylon wool-purified lymph nodes from mice (three/group) immunized with either SEB/CFA (*top*) or CFA (*bottom*). Percentages in the upper right hand corner of the Vβ8 FACS profiles represent percent of Vβ8$^+$ cells as a fraction of total α/β$^+$ cells. The mean fluorescence intensity for Vβ8$^+$ cells from SEB/CFA mice was 389, and for Vβ8$^+$ cells from CFA mice was 395. The mean overall T cell yields from these mice, calculated from four separate experiments were (no. of T cells per group; three mice in each group): lymph nodes, CFA-primed mice: 3.68 ± 1.1 × 10^7; lymph nodes, SEB/CFA-primed mice: 3.39 ± 0.52 × 10^7; spleens, CFA-primed mice: 4.25 ± 0.08 × 10^7; spleens, SEB/CFA-primed mice: 4.95 ± 2.2 × 10^7. (*B*) Two-color flow cytometry analysis of cell surface Vβ8 vs. CD8 expression on T lymphocytes from BALB/c mice immunized 8 d before with either SEB/CFA (*right*) or CFA (*left*). The percent of cells that were TCR-α/β$^+$ was 74% for SEB/CFA mice, and 83% for CFA mice. The percent of Vβ8$^+$ cells that were CD8$^+$ was 50% for SEB/CFA mice and 38% for CFA mice, while the percent of Vβ8$^+$ T cells that were CD4$^+$ was 50% for SEB/CFA mice and 60% for CFA mice (not shown).

and Vβ8$^+$ T cells were well distributed among the CD4$^+$ and CD8$^+$ subsets (Fig. 2 *B*), it can be inferred that SEB has induced anergy in both subsets because both CD4$^+$ and CD8$^+$ T cells can normally respond to TCR-specific antibodies (30). Moreover, the proliferation of Vβ6$^+$ T cells,

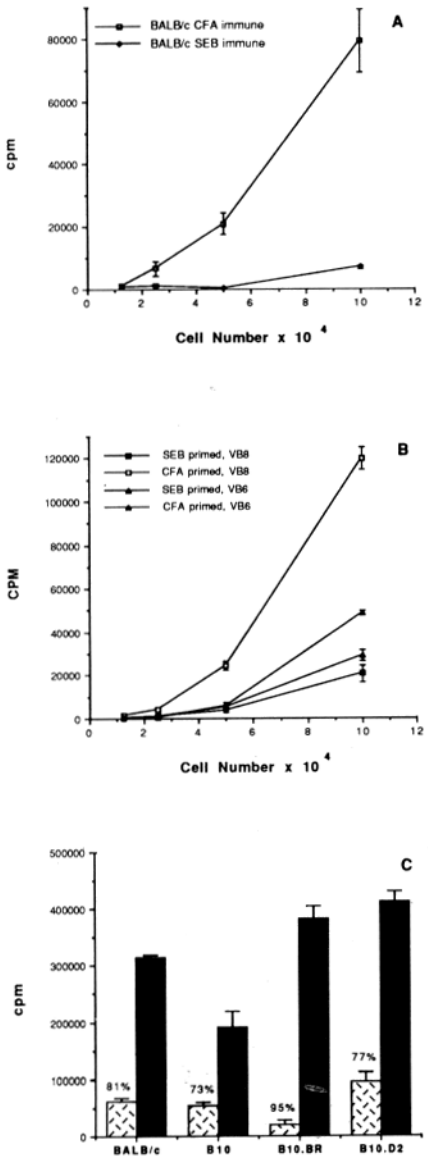


Figure 3. In vivo administration of SEB induces a state of unresponsiveness in Vβ8$^+$ T cells. Shown are the proliferative responses of nylon wool–purified lymph node T cells from mice immunized subcutaneously with either SEB/CFA or CFA to in vitro stimulation with plate-bound anti-Vβ8 mAb (10 μg/ml) or anti-Vβ6 mAb (10 μg/ml). (*A*) Proliferative response of BALB/c T cells to anti-Vβ8 antibodies; (⊡) CFA immune; (◆) SEB/CFA immune. In three independent experiments, the mean percent inhibition of Vβ8-specific mAb-induced proliferation of SEB-primed T cells (10^5/well) was 85.8 ± 3.7%. (*B*) Proliferative response of BALB/c T cells to Vβ8- and Vβ6-specific antibodies; (■) SEB/CFA immune with anti-Vβ8; (□) CFA immune with anti-Vβ8; (▲) SEB/CFA immune with anti-Vβ6; (△) CFA immune with anti-Vβ6. (*C*) The proliferation of T cells from BALB/c, B10, B10.BR, and B10.D2 to anti-Vβ8. The solid bars represent proliferation of T cells from CFA-immunized mice; and the hatched bars represent the proliferation of T cells from SEB-immunized mice. The percent reduction in proliferation of SEB-primed T cells is shown above each bar. Proliferation is shown as Vβ8-induced cpm minus medium alone cpm.

Table 1. *Priming with SEB Inhibits Proliferative Response of Splenic T Cells*

	Percent inhibition of SEB at:			
Exp.	10 μg	1.0 μg	0.1 μg	0.001 μg
1	84	96	99	91
2	90	91	78	91

Nylon wool–purified splenic T cells (10^5) from BALB/c mice immunized subcutaneously with either SEB/CFA or CFA alone were stimulated with 5 × 10^5 irradiated (2,000 rad) Thy-1.2$^-$ syngeneic spleen cells as a source of APC in the presence of varying doses of SEB or SEA, as described in Materials and Methods. Shown is the percent reduction in SEB-induced proliferation of SEB/CFA- vs. CFA-primed T cells. Responses were normalized, based on the SEB response relative to the SEA response in SEB/CFA- vs. CFA-primed animals to account for different percentages of TCR-α/β^+ cells in the spleen cell populations.

which are not reactive with SEB (20), to stimulation with anti-Vβ6 antibodies was comparable in the SEB- and CFA-immunized groups (Fig. 3 *B*). These data show that the unresponsiveness induced by in vivo SEB administration was specific and not due to a generalized T cell paralysis. The Vβ8-specific anergy was also not strain or MHC specific, such that similar inhibition of the Vβ8 proliferative response was observed in B10, B10.BR, B10.D2, and C3H/HeJ mice primed with SEB (Fig. 3 *C* and data not shown).

To test whether inhibition was regionally confined to draining lymph nodes, we also examined the proliferative response of splenic T cells from SEB-immunized mice to in vitro SEB stimulation. As shown in Table 1, there was also a dramatic decrease in the ability of splenic T cells from SEB-immunized animals to proliferate after in vitro restimulation with SEB- relative to CFA-primed controls. These data show that the anergy induced by subcutaneous administration of SEB is not solely a regional effect but exists in T cells removed from the site of SEB administration.

Because one of the classical means of generating peripheral T cell tolerance has been to inject proteins or cells intravenously, we next determined whether SEB would also induce tolerance after intravenous injection. SEB diluted in PBS, or PBS alone, was injected intravenously, and 8 d later, the responses of splenic T cells to stimulation with SEB, SEA, and anti-Vβ8 antibodies were assayed for both groups. The response of T cells from intravenously SEB-immunized animals to in vitro restimulation with both SEB and anti-Vβ8 was significantly reduced in comparison with the PBS controls (Fig. 4, *A* and *C*). In contrast, the responses of both groups to stimulation with SEA were comparable (Fig. 4 *B*). As with the subcutaneous administration of SEB, 8 d after intravenous administration, there appeared to be no difference in either the percentage of Vβ8$^+$ T cells or in the receptor density on Vβ8$^+$ cells between mice administered SEB/PBS vs.

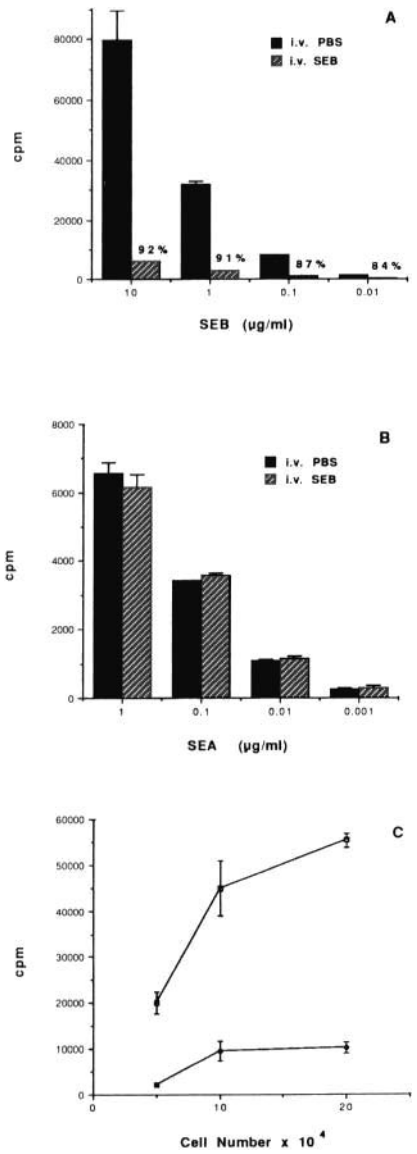


Figure 4. SEB-administered intravenously induces anergy in Vβ8$^+$ T cells. Nylon wool–purified BALB/c splenic T cells (10^5) from mice (four/group) immunized intravenously with SEB in PBS (1,000 μg/mouse) (*hatched bars*) or PBS alone (*solid bars*) were stimulated with 5×10^5 irradiated Thy-1.2$^-$ syngeneic spleen cells as a source of APC in the presence of various doses of SEB (*A*) or SEA (*B*). The percent reduction in SEB-induced proliferation is shown above each bar for the SEB-immunized T cells in *A*. Mice administered SEB intravenously appeared to be sluggish for 2–3 d after injection but appeared entirely normal thereafter with no discernible abnormalities. There were no deaths among mice given SEB intravenously. Also, a virtually identical degree of anergy was induced after administration of half the intravenous dose (500 μg) of SEB given in this experiment (data not shown). (*C*) Varying numbers of the same T cells were stimulated with plate-bound anti-Vβ8 mAb. The open squares represent the proliferation of T cells from mice given PBS intravenously, and the closed diamonds represent the proliferation of T cells from mice given SEB intravenously.

PBS alone (data not shown). Also, there was no significant difference between the two groups in the number of splenic T cells recovered (data not shown). Therefore, these data indicate that the intravenous administration of SEB also results in the specific induction of anergy in Vβ8$^+$ peripheral T cells.

We next examined the duration of the SEB-induced tolerance. Profound inhibition persisted for at least 4 wk after in vivo SEB administration (Fig. 5). Interestingly, however, 2 wk after SEB administration, and thereafter, there was a highly reproducible 50–60% decrease in the number of Vβ8$^+$ T cells in SEB- as compared with CFA-primed mice (Fig. 5). The remaining Vβ8$^+$ T cells expressed normal receptor levels (data not shown). Because the SEB- and anti-Vβ8-induced proliferation of T cells from SEB-primed animals was reduced by 80–90%, the decrease in absolute numbers of Vβ8$^+$ cells can not fully account for the absence of the Vβ8$^+$ T cell response seen in SEB-primed mice. Thus, these data suggest that a proportion of the Vβ8$^+$ T cells from SEB-primed mice that are anergic 8 d after priming are deleted by day 14 post-priming, and that the remaining Vβ8$^+$ cells persist in an unresponsive state.

To begin to examine the mechanism of SEB-induced unresponsiveness, we studied IL-2R expression and the IL-2 responsiveness of SEB-tolerized T cells. In the experiment shown (Fig. 6), 2 d after in vitro restimulation with SEB, Vβ8$^+$ T cells from SEB-primed mice were able to express IL-2R, although this expression was reduced somewhat (50% vs. 76%) when compared with CFA controls. Also, after 2 d of in vitro stimulation with SEB, 42% of the T cells from CFA-primed animals were Vβ8$^+$, as compared with only 24% for SEB-primed animals, consistent with the failure of the Vβ8$^+$ T cells from these latter mice to proliferate in response to SEB in vitro. The addition of exogenous IL-2 at the initiation of culture had little effect on the ability of Vβ8 cells to proliferate in response to restimulation with SEB (Fig. 6). This suggests that the anergized T cells are defective in both their ability to express normal levels of IL-2R, and to proliferate in response to IL-2.

In the course of our studies, it was found that, although all strains assayed showed Vβ8-specific SEB-induced anergy, only BALB/c and C3H/HeJ mice responded with a consistent decrease to in vitro restimulation with SEB (data not shown). T cells from B10, B10.D2, and B10.BR mice responded inconsistently to in vitro restimulation with SEB. BALB/c and C3H/HeJ mice are Mls^c and therefore delete Vβ3$^+$ T cells (28, 29), whereas B10, B10.D2, and B10.BR mice are Mls^b and have peripheral Vβ3$^+$ cells. Since SEB is known to activate Vβ3$^+$ as well as Vβ8$^+$ T cells in vitro (20), it was possible that in vivo SEB administration was not affecting Vβ3$^+$ and Vβ8$^+$ T cells equally. To address this issue, lymph node T cells from SEB-immunized B10 mice (Mls^b) were assayed for proliferation to both anti-Vβ8 and anti-Vβ3 plate-bound antibodies. As shown in Fig. 7, there was a significant reduction in the proliferation of T cells from SEB-immunized animals to anti-Vβ8 stimulation in comparison with control T cells, while their response to anti-Vβ3 stimulation was identical to that of the controls. These data indicate that in vivo administration of SEB induces anergy in a Vβ-specific manner, such that not all Vβ-expressing T cells capable of being activated by SEB in vitro are tolerized after in vivo SEB administration.

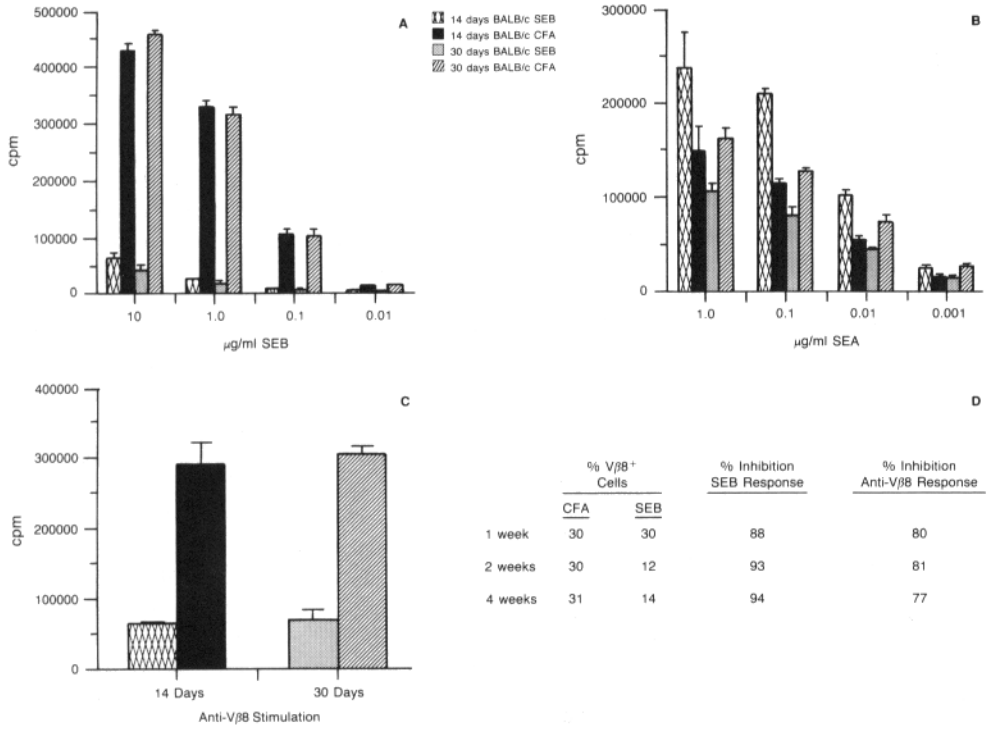


Figure 5. SEB-induced anergy is long lived. Nylon wool–purified T cells from BALB/c mice immunized 14 or 30 d earlier with SEB or CFA were assayed for proliferation to SEB (*A*), SEA (*B*), or PPD, as in Fig. 1. Proliferation to anti-Vβ8 (*C*) was assayed as in Fig. 2. Cross-hatched and dotted bars represent anti-Vβ8-induced proliferation of T cells from SEB-primed mice, solid and hatched bars represent the anti-Vβ8-induced proliferation of T cells from CFA-primed mice. PPD controls were: 21,083 cpm, SEB, 14 d; 11,542 cpm, SEB 30 d; 17,083 cpm, CFA, 14 d; and 14,482 cpm, CFA, 30 d. In three experiments, the mean percent decrease in SEB (1 μg/ ml)-induced proliferation of SEB-primed T cells compared with controls was 84.8 ± 4.4% at 2 wk after immunization and 79.5 ± 14% after 1 mo. (*D*) Summary of percent Vβ8+ T cells, percent inhibition of the SEB response, and percent inhibition of the response to Vβ8-specific antibodies, 1, 2, and 4 wk after SEB immunization. The mean percent (± SD) Vβ8+ lymph node T cells at 14 and 30 d after SEB/CFA vs. CFA immunization were: 14 d, CFA, 30.4 ± 0.58%; 14 d, SEB/CFA, 13.3 ± 2.6%; 30 d, CFA, 34.2 ± 4.0%; 30 d, SEB/CFA, 16.0 ± 2.8%. These values were determined from two separate experiments, with six mice/group.

Discussion

The advent of transgenic mice and specific anti-TCR reagents has made possible the direct demonstration of intrathymic clonal deletion (1–5), and the intrathymic induction of clonal anergy during development (6). Tolerance of mature peripheral T cells to MHC antigens (12) and Mls antigens (13) has been demonstrated under conditions where T cells expressing TCRs known to be reactive for these antigens were not deleted (were present in normal numbers). These results suggested that the peripheral tolerance was due to clonal anergy, although direct stimulation with TCR-specific antibodies was not investigated. In this report, we have shown directly the in vivo induction, by a Vβ-specific mitogen, of T cell tolerance due to clonal anergy. We have found that after the in vivo administration of SEB (either subcutaneously emulsified in CFA, or intravenously in PBS), Vβ8+ T cells become unresponsive to subsequent restimulation with either SEB or anti-Vβ8 antibodies (F23.1). Though previous studies have reported nonspecific immune suppression after in vivo administration of SE (31–33), the unresponsiveness we describe in this report is specific since the same cell population will respond normally to restimulation with SEA, PPD, or Vβ3- and Vβ6-specific antibodies. Other mechanisms to account for this tolerance were ruled out. First, the SEB-induced unresponsiveness does not appear to be due to residual SEB blocking of Vβ8+ TCRs because F23.1 was able to stain Vβ8+ cells from SEB-primed mice with the same intensity as Vβ8+ cells from CFA-primed mice, suggesting that SEB is not blocking the binding of F23.1 to Vβ8+ TCRs. In addition, mixing experiments with cells from CFA- and SEB-primed animals eliminated the possibility that suppressor cells were responsible for the tolerance (data not shown).

The fact that SEB belongs to the group of superantigens that stimulate T cells on the basis of Vβ expression alone suggests a possible mechanism to explain tolerance induction in this system. Recent studies have shown a rise in intracellular Ca^{2+} levels and modulation of the TCR complex after T cell clones were incubated with SEB in the absence of APC (34). This suggests that SEB can directly bind and induce signaling through the TCR. Because an increase in intracellular Ca^{2+} levels in the absence of other accessory signals has been suggested as one mechanism for tolerance induction in vitro (10, 35, 36), it is possible that SEB induces anergy in Vβ8+ cells because it is able to directly bind and induce signaling through the TCR in the absence of accessory signals provided by APC. The failure of SEB to tolerize Vβ3+ T cells in vivo while it does tolerize Vβ8+ T cells may reflect differences in the relative affinity that SEB has for Vβ3 vs. Vβ8 TCRs.

SEs have also been shown to bind directly to class II molecules with high affinity (37, 38), and the in vitro stimulatory effect of SEs is believed to be based on their ability to cross-link the TCRs on T cells with class II molecules on APC (34, 39) in the absence of antigen processing. Thus, tolerance induction by SEB could also result from a very high affinity interaction between the SE/Ia complex and the TCR,

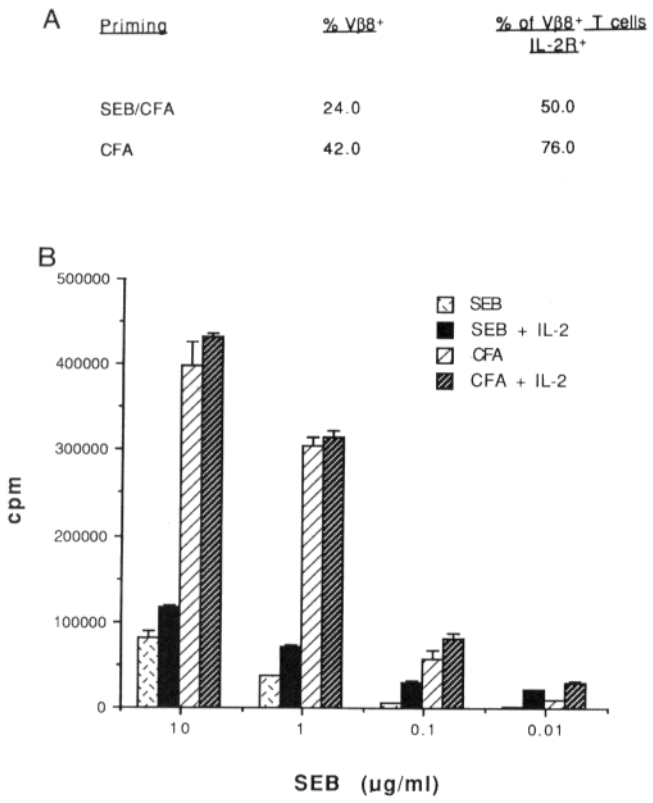


Figure 6. Exogenous IL-2 does not overcome SEB-induced anergy. (*A*) The IL-2R expression of Vβ8+ T cells from SEB/CFA- and CFA-primed mice. Nylon wool–purified lymph node T cells from SEB/CFA- or CFA-primed mice were cultured in 10 µg/ml SEB with Thy-1.2− APC for 48 h before staining for IL-2R expression. The overall percentage of Vβ8+ T cells and the percentage of Vβ8+ T cells expressing IL-2R were determined by two-color flow microfluorometry. (*B*) The in vitro proliferative response of T cells from SEB/CFA- and CFA-primed mice to SEB with and without the addition of exogenous IL-2. (▨) SEB/CFA primed, without IL-2; (■) SEB/CFA primed, with IL-2; (▨) CFA primed, without IL-2; (▨) CFA primed, with IL-2. Nylon wool–purified lymph node T cells from SEB/CFA- or CFA-immunized mice (four/group) were stimulated as in Fig. 1 with or without the addition of IL-2 (10^2 U/ml). IL-2 was added at the initiation of the culture period.

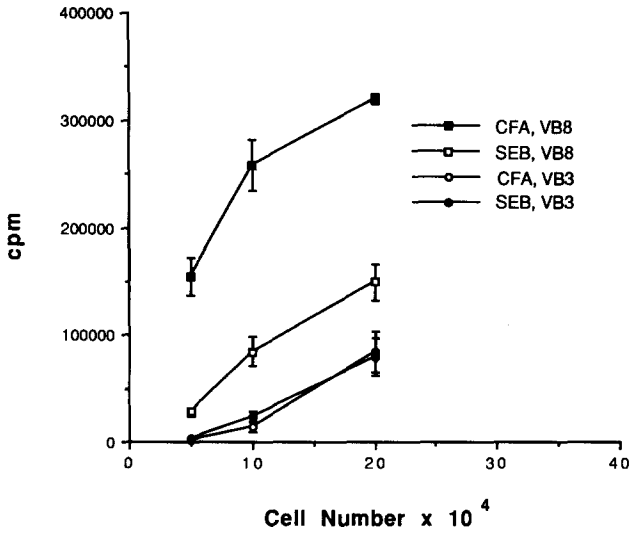


Figure 7. In vitro administration of SEB does not inhibit the response of Vβ3+ T cells. Nylon wool–purified lymph node T cells from B10 mice immunized subcutaneously with either SEB/CFA or CFA alone (three/group) were stimulated as in Fig. 2 with anti-Vβ8: (□) SEB/CFA primed; (■) CFA primed; and anti-Vβ3 mAbs (●) SEB/CFA primed; (○) CFA primed.

perhaps involving predominant presentation by B lymphocytes, which are relatively poor at providing accessory signals (40–42). In this context, it was recently shown that the relative potency of peptide antigens in vitro correlated directly with their capacity to induce tolerance in vivo (43). Moreover, in the murine experimental allergic encephalomyelitis model, analogues of the encephalitogenic MBP-1-9 peptide that were more potent than the native peptide at activating encephalitogenic T cell clones in vitro paradoxically were protective against disease when administered in vivo (44). It was suggested that the in vivo administration of such peptides could result in the tolerization of the encephalitogenic T cell clones by clonal anergy (44). SEs have also been shown to activate T cells in vivo, and it has been demonstrated that T cell activation and lymphokine release may be responsible for some of the manifestations of systemic staphylococcal infection (33). It would be of interest to define the parameters of toxin administration that lead alternatively to T cell activation or the induction of tolerance.

2 wk after SEB administration, Vβ8+ T cells were diminished in number, although the remainder were still anergic. The intensity of Vβ8 expression on these cells appears to be normal, indicating that the decrease in Vβ8 cells is not due to receptor downregulation. It is also doubtful that the diminished number of Vβ8+ T cells after 2 wk is due to SEB-induced intrathymic deletion because the decrease consistently occurs between day 8 and 14 post-immunization, and it is unlikely that 40–50% of all peripheral Vβ8+ cells are normally replaced during a 6-d period. In addition, preliminary experiments indicate that there is minimal downregulation of Vβ8 on cortisone-resistant thymocytes 8 d after subcutaneous SEB administration (unpublished data). Therefore, these data suggest that in vivo administration of SEB may result in the clonal deletion of some peripheral Vβ8+ T cells.

The fact that some Vβ8+ cells first appear to be anergized and then proceed to clonal deletion suggests that the clonal anergy observed and the subsequent deletion of a portion of the anergized cells may be discrete stages of a continuing process. Recent studies demonstrate some similarities between the intracellular mechanisms leading to anergy and to clonal deletion, such as dependence on an early TCR-CD3-mediated Ca^{2+} flux, and sensitivity to cyclosporine A (45–50). However, clonal deletion is not an inevitable consequence of anergy induction because at least 50% of the Vβ8+ cells were able to persist in a state of anergy. In addition, in other reported examples of T cell tolerance due to anergy, the anergized cells were not clonally deleted and persisted for extended periods of time (6, 13). The decision to progress from anergy to deletion could depend on factors such as the maturation state

of the T cell, or the intensity and duration of the stimulus a cell receives through its TCR (51).

In vitro models of T cell anergy suggest that a major defect in these cells is their inability to produce IL-2. In the case of in vitro anergized cells, the anergized T cell clones were still fully responsive to exogenously added IL-2 (50). However, our model of in vivo induced anergy, as well as that of others (6, 13), suggest the defect is more generalized than this. Upon in vitro restimulation with SEB, ~40–50% of the $V\beta8^+$ T cells from SEB-primed mice express normal levels of IL-2Rs. The remainder either do not express IL-2Rs at all or do so at undetectable levels. Even so, all these cells are only minimally responsive to exogenous IL-2. The fact that 50% of the $V\beta8^+$ cells can be $IL\text{-}2R^+$ but are nevertheless unresponsive to exogenous IL-2 suggests that stages of activation in addition to the production of IL-2 are blocked in peripherally anergized T cells. These additional defects may be at the level of IL-2R expression (i.e., an ability to express high affinity IL-2 receptors), or an inability to transmit the signal normally delivered by IL-2. Although other models of in vivo T cell anergy also find that anergized cells are only minimally responsive to exogenous IL-2 (6, 13), in the case of Rammensee et al. (13), the anergic $V\beta6^+$ cells rendered tolerant after the in vivo administration of Mls^a spleen cells expressed normal levels of IL-2Rs after activation.

Although soluble protein antigens have long been used to study the induction of tolerance in both T and B cells, they were ineffective as tolerogens if administered subcutaneously, particularly if emulsified in adjuvant. Most proteins studied as tolerogens were administered intravenously or orally, in an unaggregated form, or at very high doses or very low doses (15–19). SEB thus differs from conventional protein antigens in that it induces anergy when administered subcutaneously in CFA as well as intravenously. This may reflect the fact that the trimolecular interaction between SE, the Ia molecule, and the TCR is very likely to be distinct from that of conventional protein antigens (52, 53). Superantigens may bind directly to Ia molecules outside the putative antigen binding groove, and in view of their activation of T cells based on TCR $V\beta$ expression alone, they probably interact with a region of the $V\beta$ protein not involving the third complementarity determining region formed by VDJ recombination (52, 53). If this is true, it raises the possibility that if the mechanism by which SEs bind to specific $V\beta$ TCRs can be understood, it may be possible to design peptides that mimic this binding and that can be used to suppress or amplify immune responses in a T cell $V\beta$-specific manner. Such peptides could be useful to specifically control autoimmune diseases that are mediated by a limited set of $V\beta^+$ T cells (54–56).

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