Brief Definitive Report

Memory T Cells Are Anergic to the Superantigen Staphylococcal Enterotoxin B

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Summary

We have used staphylococcal enterotoxin B (SEB) to study the role of naive and memory T cells in the induction of peripheral tolerance. After administration of SEB to mice, the numbers of naive and memory T cells increase, as does the proportion of memory T cells, which are unresponsive to further stimulation with SEB in vitro. In addition, memory T cells generated in response to conventional antigen, which proliferate and provide help to B cells in the presence of the conventional antigen, fail to respond to superantigen. Hence, memory T cells, in general, are anergized by SEB. These results suggest that SEB-induced activation and anergy reflect the combined responses of naive and memory T cells. The differential activation vs. anergy of naive and memory T cells by superantigen may be related to cytokine production and may play an important role in the etiology of autoimmune diseases or immunodeficiency diseases such as acquired immune deficiency syndrome.

Peripheral tolerance, due to either T cell deletion or inactivation (anergy), plays a major role in the suppression of autoimmunity (1). The mechanisms underlying induction of tolerance in peripheral T cells are incompletely understood, but significant progress has been made using model systems that use superantigens (2, 3), such as Mls antigens or bacterial enterotoxins. Superantigens react with both class II antigens on APCs and external domains of specific TCR Vβ regions (2). In studies of peripheral tolerance using either Mls antigens or bacterial exotoxins, such as staphylococcal enterotoxin B (SEB), both T cell deletion and anergy follow an immune response (4, 5). Hence, after administration of the superantigen, specific TCR Vβ-containing T cells rapidly proliferate, reflecting the superantigen-mediated mitogenic effects that are seen in vitro (4, 5). After this expansion phase, T cells decline in number, presumably reflecting a deletion phase. Analyses of the remaining T cells indicate that they are unresponsive to restimulation by the superantigen in vitro (T cell anergy) (6, 7).

We have used the SEB superantigen to examine the role of memory T (T_m) cells during the induction of tolerance. Our data suggest that superantigen-induced T cell anergy may be due to the differentiation of SEB-reactive virgin T cells into anergic memory cells and/or the activation and subsequent deletion of virgin T cells. We favor the former possibility since all the anergic T cells displayed a memory phenotype as indicated by changes in expression of the CD45R isoform. Unexpectedly, our data also demonstrate that T_m cells are unresponsive to SEB, regardless of whether they are generated by prior exposure to SEB or to conventional antigens. Based on our results we suggest that: (*a*) T cell tolerance can be induced after memory cells have been generated (i.e., during a secondary response), and (*b*) naive and memory T cells are differentially stimulated by superantigens. The latter observation suggests the use of alternative signaling pathways by the T cells or, more likely, a different requirement for accessory signals by the APC.

Materials and Methods

Animals. Female BALB/c mice were bred and maintained in the mouse colony in the Department of Microbiology, University of Texas Southwestern Medical Center. All mice were used at 6–8 wk of age.

Antigens and Immunizations. KLH was obtained from Calbiochem-Behring Corp. (La Jolla, CA). For the generation of T_m cells, mice were primed by intraperitoneal injection with 100 μg of KLH in CFA 4–8 wk before experimentation. To induce T cell anergy, mice were primed by intravenous injection with 20 μg of SEB (Sigma Chemical Co., St. Louis, MO) in PBS 7 d before experimentation.

Lymphokines and Antibodies. Murine IL-2 was purchased from Genzyme Corp. (Boston, MA). Rat anti-murine CD45RB (23G2) (8) was purchased from Tex-Star Monoclonals (Dallas, TX). Mouse anti-rat κ (Mark-1) (9) was obtained from Zymed Laboratories (South San Francisco, CA). Rat anti-murine Thy-1.2 (HO13.4) (10), anti-CD8 (3.155) (11), and anti-CD4 (GK1.5) (12) were obtained from American Type Culture Collection (Rockville, MD). Rat anti-murine CD4 (2B6) (13) was a gift from Dr. J. Forman (University of Texas Southwestern Medical Center), hamster anti-murine CD3 (145-2C11) (14) was a gift from Dr. R. Noelle

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(Dartmouth College, Hanover, NH), and biotinylated mouse anti-murine Vβ8 (F23.1) (15) was a gift from Dr. T. Waldschmidt (University of Iowa, Iowa City, IA).

Preparation of Cells. In all experiments $CD4^+$ T cells from BALB/c mice (female, 4–8 wk) were prepared as previously described (16). Resulting populations were >90% $CD4^+$. Purified populations of T_m or virgin T (T_v) cells were prepared from $CD4^+$ splenic T cells by cell sorting using mAb 23G2 (anti-CD45RB) to separate $CD45RB^{hi}$ (T_v) cells from $CD45RB^{lo}$ (T_m) cells. APCs were prepared by T cell depletion of splenocytes using anti-Thy-1.2 and baby rabbit complement (BRC) (Pel-Freeze Biologicals, Rogers, AR) followed by anti-CD4 (mAb 2B6) plus anti-CD8, and by BRC. APCs were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO) (25 μg/ml) for 20 min at 37°C.

Flow Cytometric Analyses. Single cell suspensions were stained with FITC-GK 1.5 (anti-CD4) or biotinylated F23.1 (anti-Vβ8) and 23G2 (anti-CD45RB) followed by PE-strepavidin (Tago Inc., Burlingame, CA) and FITC-MARK-1, respectively. Flow cytometric analyses were performed using a FACStar® (Becton Dickinson & Co., Mountain View, CA).

Culture Conditions. Cells were cultured in 96-well, U-bottomed plates (Falcon Labware, Oxnard, CA) for 3 d followed by a 12-h pulse with [^{3}H]TdR (1 μCi/well). Cells were harvested using an automated cell harvester (Skatron, Norway). Unless otherwise indicated, $CD4^+$ T cells, T_v cells, or T_m cells were cultured at 10^5/well in 0.2 ml RPMI 1640 containing 10% FCS, glutamine, and 50 mM 2-ME. All wells contained 10^5 mitomycin C–treated APCs. Where indicated, SEB (15 μg/ml) (Sigma Chemical Co.), Con A (5 μg/ml) (Sigma Chemical Co.), KLH (15 μg/ml) (Calbiochem-Behring Corp., La Jolla, CA), or human rIL-2 (20 U/ml) (Amgen Biologicals, Thousand Oaks, CA) were added to the cultures. In some experiments wells were coated with mAb 145-2C11 (anti-CD3) (100 μg/ml) for 5–12 h at 37°C before experimentation.

Immunoglobulin Secretion Assays. The preparation of trinitrophenyl (TNP)-specific antigen-binding B cells (TNP-ABCs) and the T/B cognate interaction assay has been described in detail previously (16). Purified $CD4^+$ splenic T cells from KLH-primed mice were sorted into $CD45RB^{hi}$ (T_v) and $CD45RB^{lo}$ (T_m) cell populations using mAb 23G2 and FITC-MARK-1. T cells (10^5/well) were cultured with TNP-ABCs (5×10^4/well) and the indicated stimuli for 7 d. Levels of secreted Ig were determined by radioimmunoassay (16).

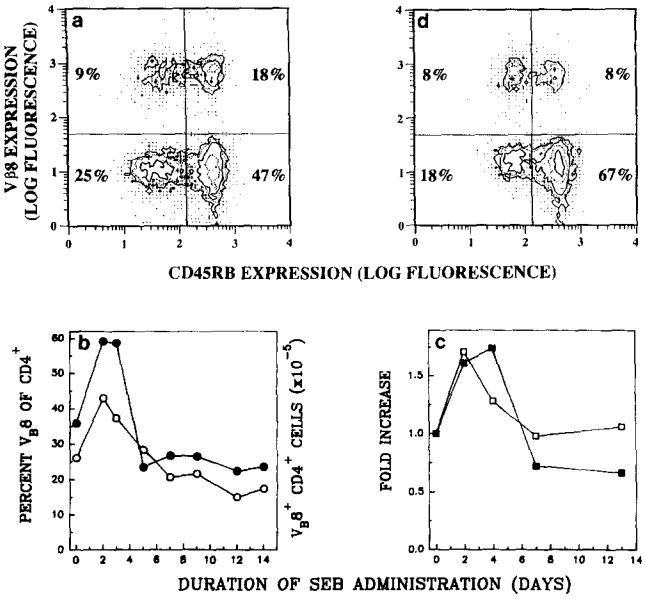

Figure 1. Immunization with SEB causes a relative increase in the proportion of $V\beta8^+$ T_m cells. The staining pattern of $CD4^+$ splenic T cells from mice that were (*a*) unprimed or (*d*) immunized with 20 μg of SEB (intravenously) 7 d before isolation. Quadrants were set to bracket cells stained with negative control (secondary only) reagents. Percentage of cells in each quadrant are indicated in the panels. Calculations of percentages indicate that in the $CD45R^{lo}$ or $CD45R^{hi}$ population there were (*a*) 27% and 28% $V\beta8^+$ cells, respectively, or (*d*) 31% and 10%, respectively. Alternatively, splenocytes from mice primed for different intervals with SEB were stained with anti-CD4 and anti-Vβ8 or anti-CD45RB and anti-Vβ8 antibodies to enumerate the (*b*) numbers of $V\beta8^+CD4^+$ cells (per two pooled spleens) (●), the percent of $V\beta8^+$ cells of $CD4^+$ (○), and (*c*) changes in the levels of $CD4^+$ $V\beta8^+$ T_v (■) and T_m (□) cells. The T_v/T_m ratio of the $V\beta8^-$ cells remained at 2–4:1 throughout the experiment. The T_v/T_m ratio of $V\beta8^+$ cells became 1:1 within 5 d after immunization. In all panels the data are representative of three separate experiments using values from the pooled spleens of two mice.

Results and Discussion

SEB specifically interacts with Vβ8, 7, 11, and 17 (2). We have studied SEB-induced anergy by monitoring the expression of Vβ8 on virgin $CD45RB^{hi}$ (T_v) and memory $CD45RB^{lo}$ (T_m) $CD4^+$ cells after in vivo administration of SEB. In normal mice, similar percentages of splenic T_v and T_m cells expressed Vβ8 (T_v = 28%; T_m = 27%), and there was a normal distribution of T_v and T_m cells within the $V\beta8^+$ cell population (T_v/T_m = 2:1 [Fig. 1 *a*]). After immunization with SEB, $V\beta8^+$ T cells rapidly proliferated and then disappeared (Fig. 1 *b*). This is reflected in both the $V\beta8^+$ T_v and T_m populations (Fig. 1 *c*), and could be due to either activation and deletion of both T cell types or to the expansion of T_v cells followed by their differentiation into T_m cells, which are then deleted. Regardless of the mechanism involved, there was an increase in the proportion of $V\beta8^+$ T_m cells after immunization with SEB ($V\beta8^+$ T_v/T_m = 1:1 [Fig. 1 *d*]). It should be noted that there was also an increase in the proportion of $V\beta8^-$ T_v cells after administration of SEB ($V\beta8^-$ T_v/T_m = 3.7:1 [Fig. 1 *d*]). Although there were modest variations in the $V\beta8^-$ population from experiment to experiment, the values always fell within the range observed in normal mice. The $V\beta8^-$ population shown in Fig. 1 *d* falls within this range. However, in all experiments the $V\beta8^+$ T_v/T_m ratio became 1:1 within 5 d after SEB administration.

We next studied the T_v and T_m cells in functional assays. In accord with previous reports (5, 7), $CD4^+$ cells from SEB-immunized mice were unresponsive to in vitro restimulation with SEB in the presence of mitomycin C–treated APCs, although these cells still responded to the polyclonal activators Con A and immobilized anti-CD3, albeit to a lesser degree than cells from control mice (Fig. 2 *a*). Based on a previous report, polyclonally activated T cells contain the same proportion of $V\beta8^+$ cells as do resting cells (17), suggesting that SEB-responsive $V\beta8^+$ cells respond to Con A. When

T_v and T_m cells from these mice were separated on the basis of CD45RB expression (18), the T_m cells were unresponsive to SEB and the residual response of the unfractionated T cells could be attributed to the T_v cells (Fig. 2 *b*). In comparing Fig. 2, *a* with *b*, it should be noted that the number of cells was two fold greater in the latter and that there was enrichment of the SEB-responsive (T_v) cells in the experiment shown in Fig. 2 *b*. Primary stimulation with SEB (as opposed to conventional antigens) was not required for SEB-mediated anergy of T_m cells. Thus, when $CD4^+$ T_v and T_m cells from KLH-primed mice were examined for their ability to proliferate in response to conventional antigen (KLH) or to superantigen (SEB), the T_m cells were stimulated by KLH in a manner consistent with a recall response, but they proliferated poorly in response to SEB (Fig. 2 *c*). Since the frequency of KLH-responsive T_m cells is much lower than the frequency of SEB-reactive cells (0.025–0.05% vs. 20–30%) (3, 19), the absolute response to KLH is significantly greater. The poor proliferative response of T_m cells to SEB was restored by the addition of IL-2, suggesting that T_m cells are deficient in their ability to produce IL-2 after stimulation with SEB. Since previous studies have shown that conventionally activated T_m cells secrete IL-2 (16, 18), the failure of T_m cells to respond to SEB is not due to their inherent inability to secrete IL-2. In this regard, in all experiments, the SEB-induced proliferative response of T_m cells from KLH-primed animals was slightly higher than that of T_m cells from normal mice (Fig. 2, *c* and *d*), although it was markedly lower than that of T_v cells (Fig. 2 *c*). This enhanced proliferative response may be due to endogenous IL-2 production by T_m cells previously activated by KLH or adjuvant. In addition, the SEB-mediated response of activated T_m cells may explain why cultured $CD45RB^{lo}$ $V\beta8^+$ T cell clones can proliferate when stimulated by SEB (20).

We next examined the ability of T_v and T_m cells to provide help to B cells that present SEB. We have shown previously that T_m cells help hapten-specific B cells to secrete Ig in the presence of the appropriate antigen (16). In contrast, T_v cells, which do proliferate in response to antigen, do not provide help to B cells unless they differentiate into T_m cells (16). We have extended these observations using SEB as the stimulating antigen. As shown in Fig. 3, T_v cells proliferate but cannot help B cells to secrete Ig in the presence of either

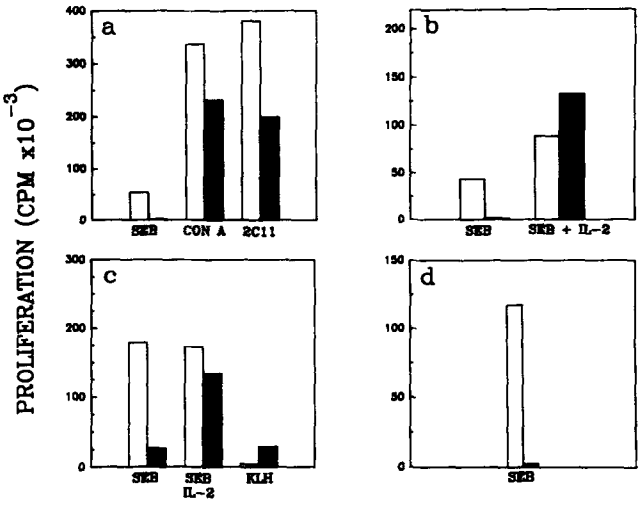

Figure 2. Memory T cells from SEB-primed or normal animals are unresponsive to in vitro stimulation with SEB. BALB/c mice were primed with KLH (intraperitoneally) in CFA 4–6 wk before use or were primed with 20 μg of SEB (intravenously) and used 7 d later. Purified splenic $CD4^+$ T cells sorted into $CD45RB^{hi}$ (T_v) and $CD45RB^{lo}$ (T_m) populations on the basis of CD45RB expression. Cells were cultured at 10^5/well (except for *b*, where 2×10^5/well were used). All wells contained 5×10^4/well APCs. After 3 d the wells were pulsed with [^{3}H]TdR (1 μCi/well) and harvested 12 h later. Where indicated, SEB (15 μg/ml), Con A (5 μg/ml, KLH (15 μg/ml), or human rIL-2 (20 U/ml) were added to the cultures. Proliferation analyses of (*a*) $CD4^+$ splenic T cells from normal (□) or SEB-primed (■) mice that were stimulated with SEB, Con A, or immobilized anti-CD3. Proliferation analyses of purified T_v (□) or T_m (■) cells from (*b*) SEB-primed, (*c*) KLH-primed, or (*d*) unprimed mice that were stimulated with the indicated agents. In the absence of stimulating agents or T cells (APC only), the total proliferation was <1,000 cpm/well. In all panels, the data are an average cpm from duplicate wells from a representative experiment of four separate experiments. Although only a single data point is shown in these panels, broad cell and SEB dose-range experiments were performed with similar results. The data points that are shown reflect these data.

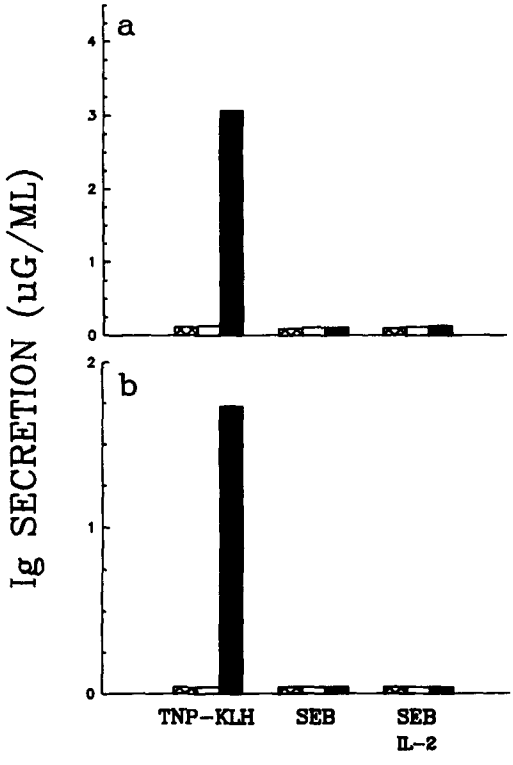

Figure 3. SEB is unable to stimulate either virgin or memory T cells to provide help for an antibody response. T_v or T_m cells were cultured with TNP-ABCs and either conventional antigen (TNP-KLH) (0.1 μg/ml), SEB (10 μg/ml), or SEB plus IL-2 (20 U/ml) for 7 d before analysis of the culture supernatants for secreted (*a*) IgM or (*b*) IgG_1. The values represent cultures containing (▩) B cells only, or B cells plus either (□) T_v cells or (■) T_m cells.

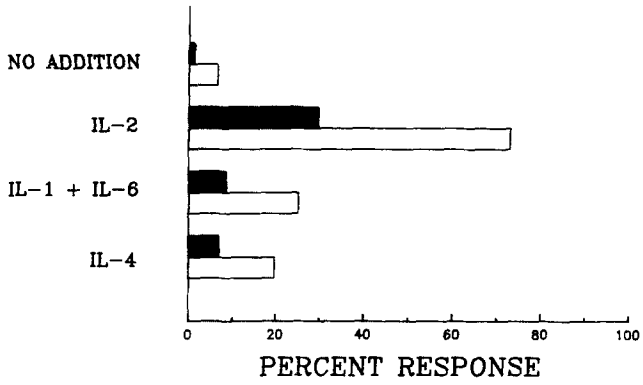

Figure 4. IL-2, -4, and -1 + 6, partially restore the response of memory T cells to SEB. T_v cells were cultured with SEB (15 μg/ml). T_m cells were cultured with the indicated cytokines: IL-2 (20 U/ml), IL-4 (50 U/ml), IL-1 (1 U/ml), and IL-6 (2 ng/ml) in the presence (□) or absence (■) of SEB (15 μg/ml). The results represent the proliferation of T_m cells expressed as a percent of the proliferative response of T_v cells plus SEB (193,734 cpm). The data are from one experiment that is representative of four separate experiments.

SEB or conventional antigen (TNP-KLH). T_m cells (from KLH-primed mice), which do provide help when stimulated with TNP-KLH, do not induce Ig secretion when stimulated with SEB, even when proliferation is restored by the addition of IL-2. Thus, proliferation of SEB-reactive T_m cells is insufficient to generate helper activity, either because SEB, unlike TNP-KLH, does not stimulate the secretion of the appropriate cytokines, or because it does not signal the B cells via its sIg receptor. We consider the latter hypothesis unlikely since it has been shown that activated T cells can help B cells in the absence of a signal mediated by sIg (21).

Our observations suggest that SEB-induced T cell anergy follows primary stimulation of T_v cells and their differentiation into T_m cells. T_m cells then fail to proliferate or provide help to B cells in the presence of SEB. It is possible that the activation requirements of T_m cells are more stringent than those of T_v cells. A foreign antigen, such as KLH, which requires processing and presentation in a conventional fashion, elicits an immune response by T_m cells, whereas SEB, which requires MHC class II^+ APCs, but not antigen processing (3), does not elicit a response. Unlike KLH, presentation of SEB may fail to induce APC-mediated costimulatory signals that are required by T_m but not T_v cells. In this regard anergy could be partially reversed in T_m cells by the addition of either IL-2, IL-4, or IL-1 plus IL-6 (Fig. 4). These cytokines can promote T cell proliferation (22), and both IL-1 and IL-6 are important cytokines typically produced by APCs that may act as "second signals" (23) in the activation of T_m cells. We suggest that the lack of such accessory signals may lead to tolerance even though T_m cells have already been generated by prior activation.

The processing of foreign antigen by APCs may elicit accessory signals that self-peptides do not. If this were the case, then one might speculate as to why T_m cells would be selectively nonresponsive or tolerized. Receptors on naive T cells that bind avidly to self-antigens are deleted in the thymus so that peripheral T_v cells do not recognize self-antigens with high avidity (24). In addition, T_v cells are relatively nonfunctional but must differentiate into T_m cells before they can provide help to B cells. As T_m cells develop, they also express higher levels of adhesion molecules (25), which facilitate interactions with APCs. In a situation where an increase in adhesion leads to the increased binding of T_m cells to self-antigen on APCs, in the absence of appropriate accessory signals the T_m cells might be tolerized. Hence, a possible reason that the activation of T_m cells is more stringently regulated than that of T_v cells is to assure that low affinity binding of T_m cells to self-antigen prevents an autoimmune response.

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