Brief Definitive Report

Proliferation Is a Prerequisite for Bacterial Superantigen-induced T Cell Apoptosis In Vivo

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Summary

Staphylococcal enterotoxin B (SEB) is a bacterial superantigen that binds to major histocompatibility complex class II molecules and selectively interacts with T cells that bear certain T cell receptor (TCR) Vβ domains. Administration of SEB in adult mice results in initial proliferation of Vβ8$^+$ T cells followed by a state of unresponsiveness resulting from a combination of clonal deletion and clonal anergy in the SEB-reactive population. At this time, it is unclear what relationship exists between the T cells that have proliferated and those that have been deleted or have become anergic. Here we show that only a fraction of the potentially reactive Vβ8$^+$ T cells proliferate in response to SEB in vivo, and that all the cells that have proliferated eventually undergo apoptosis. Virtually no apoptosis can be detected in the nonproliferating Vβ8$^+$ T cells. These data demonstrate a causal relationship between proliferation and apoptosis in response to SEB in vivo, and they further indicate that T cells bearing the same TCR Vβ segment can respond differently to the same superantigen. The implications of this differential responsiveness in terms of activation and tolerance are discussed.

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Superantigens are operationally defined as molecules that bind class II MHC and stimulate T cells that bear a given TCR β-chain variable region (1, 2). For instance, staphylococcal enterotoxin B (SEB) predominantly interacts with Vβ8$^+$ T cells (3, 4). In vivo treatment of mice with SEB leads to an initial increase in the percentage of Vβ8$^+$ T cells in both the CD4 and CD8 subsets (5). Substantial numbers of cycling Vβ8$^+$ T cells are observed at this stage (5). This proliferation is followed by a decrease in the numbers of Vβ8$^+$ cells, eventually reaching lower levels than those found before treatment with SEB. The remaining Vβ8$^+$ cells are anergic since they fail to proliferate in vitro in response to SEB or anti-Vβ8 mAb (3, 4, 6).

At this time, it is unclear what relationship exists between cells undergoing proliferation, apoptosis, or anergy in response to SEB. We had previously suggested two models that could account for the different fates of potentially SEB-reactive T cells in vivo (7). In the first model, all Vβ8$^+$ T cells proliferate, and depending on yet undefined (stochastic or environmental) factors, they may progress to either apoptosis or anergy. In the second model, only T cells that react strongly to SEB undergo clonal expansion, which inevitably leads to cell death. In contrast, weakly SEB-reactive Vβ8$^+$ T cells do not receive a strong enough signal to undergo proliferation and death. Rather, they become anergic.

To discriminate between these two possibilities, we studied the response to SEB in Vβ8.2 TCR transgenic mice that had been exposed to the thymidine analogue bromodeoxyuridine (BrdU). We find that only a fraction of Vβ8$^+$ T cells incorporate BrdU in response to SEB in vivo. All T cells that incorporated BrdU eventually undergo apoptosis, whereas virtually no apoptosis could be detected in Vβ8$^+$ cells that did not proliferate. Similar results were observed in both the CD4 and CD8 subsets. The remaining Vβ8$^+$ cells, which we have shown previously to be anergic in these transgenic mice (8), were exclusively BrdU negative, indicating that they did not undergo clonal expansion. These data directly demonstrate a lineage relationship between proliferating and apoptotic T cells that respond to SEB in vivo.

Materials and Methods

Animals and Treatments. 8–10-wk-old BALB/c mice were obtained from Harlan Olac (Bicester, UK). The TCR Vβ8.2 transgenic mouse line (C57BL6.Transgenic93/lbm spf) (9) was kindly provided by Dr H. Bluethmann (Hoffmann-La Roche AG, Basel, Switzerland). These mice were back-crossed at least five times onto the BALB/c background. SEB (10 μg) from Toxin Technology (Saratoga, FL) was injected into each of the hind footpads. BrdU (Sigma Immunochemicals, St. Louis, MO) was added at 1 mg/ml to drinking water that contained 5% glucose 3 d before treatment with SEB and was changed every 3 d throughout the experiment.

Antibodies. The following directly labeled mAbs were used:

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/06/2283/05 $2.00
Volume 181 June 1995 2283–2287

anti–BrdU-FITC and anti–CD4-PE (Becton Dickinson & Co., Palo Alto, CA), anti-CD8-PE (Boehringer Mannheim GmbH, Mannheim, Germany), anti-Vβ8 (F23.1) (10) was directly coupled to FITC in our laboratory.

FACS™ Sorting. Draining LN cells were stained with the relevant antibodies and sorted using a Becton Dickinson FACStar Plus™ (Becton Dickinson & Co., Mountain View, CA). Sorted cells were then incubated at 37°C for 1 h (to allow apoptosis to occur [3, 4]) before further manipulation.

Staining and Flow Cytometry. Simultaneous staining for BrdU incorporation and DNA content was performed on sorted CD4+ or CD8+ LN cells as described (11). Briefly, cells were fixed in 70% ethanol for at least 30 min and the DNA was partially denatured in 3 N HCl, then neutralized with 0.1 M $Na_2B_4O_7$. The cells were subsequently stained with FITC-coupled anti-BrdU, resuspended in 100 μl PBS containing 2.5% FCS, and added to 200 μl PBS/FCS containing 3 μg/ml propidium iodide (PI), 50 μg/ml RNAse A (Sigma), 50 mM Tris base, 50 mM NaCl, and 5 mM EDTA, pH 7.5. Cells were then incubated 5 min at 37°C and analyzed on a FACScan™ equipped with a doublet discrimination module.

Results and Discussion

To confirm that the increase in Vβ8+ cells observed after injection of SEB is a consequence of proliferation rather than selective migration, we evaluated BrdU incorporation in CD4+ or CD8+ cells that were isolated from the draining LN of Vβ8.2 TCR transgenic mice that were fed BrdU starting 3 d before superantigen administration. More than 99% of T cells in these mice bear the transgenic TCR β chain (data not shown and reference 12). Fig. 1 shows that there is an increase in the percentage of BrdU-labeled CD4+ and CD8+ cells as soon as 2 d after immunization, peaking at around day 7, then dropping to background levels by day 14. CD8+ cells had a reproducibly higher BrdU incorporation rate, especially early during the response (day 2). This difference may be caused by the faster doubling time of CD8+ cells compared to CD4+ cells, as observed in vitro (13). Alternatively, more CD8+Vβ8+ T cells may have initially responded to SEB than CD4+Vβ8+ T cells.

It is of interest that only a fraction (50–60%) of the potentially reactive Vβ8+ cells incorporated BrdU (Fig. 1), suggesting that although they bear the same TCR β chain, T cells respond differentially to SEB. This differential response to SEB was not restricted to Vβ8.2 TCR transgenic mice since a similar fraction of Vβ8+ T cells (52%) incorporated BrdU at the peak of the SEB response (day 7) in normal BALB/c mice (data not shown). It could be argued that a higher proportion of Vβ8+ T cells would incorporate BrdU with a higher dose of SEB. Although this is formally possible, we were unable to increase the dose of superantigen beyond 20 μg in our Vβ8.2+ transgenic mice because of high mortality. It has been previously shown that SEB can induce T cell–mediated weight loss in mice (14). This toxicity is mediated by T cell cytokines such as TNF (15). SEB can even be lethal when injected into BALB/c mice in combination with agents that increase the animal's susceptibility to hyperacute cytokine release syndrome, such as the glucocorticoid receptor antagonist RU-38486 (15) or the amino-

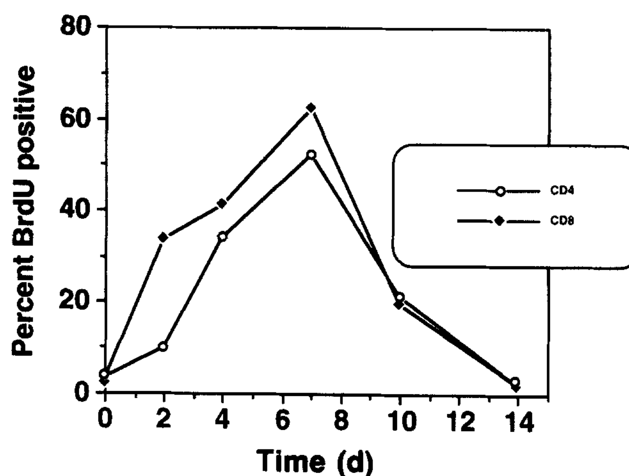

Figure 1. BrdU incorporation by Vβ8+ T cells in response to SEB in vivo. Vβ8.2 TCR transgenic mice were fed BrdU continuously and the incorporation of the nucleotide analogue was determined in sorted LN CD4+ and CD8+ T cells by flow cytometry at the indicated time points following SEB injection. Time 0 d indicates mice that were fed BrdU for at least 7 d but did not receive SEB. One of two comparable experiments is shown here.

sugar GalN (16). The fact that SEB, alone and at doses that were not lethal to nontransgenic littermates, was able to cause death in our Vβ8.2+ transgenic mice is likely to be caused by the higher numbers of SEB-reactive, potentially cytokine-secreting cells generated in these mice.

Since virtually all BrdU-labeled cells from our SEB-treated Vβ8.2 transgenic mice disappeared by 2 wk after immunization, and since some Vβ8+ T cells have been shown to die by apoptosis in vitro in response to SEB in normal mice (3, 4), we asked whether selective apoptosis of proliferating T cells could account for our observations. To address this issue, CD4+ or CD8+ cells were sorted from LN of BrdU-treated, SEB-injected Vβ8.2 transgenic mice and were double stained with anti-BrdU mAb and the DNA-binding dye PI (Fig. 2). 2 d after immunization with SEB, a fraction of the T cells was in cell cycle, and as expected, this fraction was almost exclusively in the BrdU+ population. In contrast, no cycling cells were detected 7 d after SEB administration; however, subdiploid (apoptotic) cells with low PI staining were clearly visible at this time point. Importantly, virtually all subdiploid cells were found in the BrdU+ population.

This observation was extended when PI uptake was evaluated on sorted CD4+ or CD8+ cells that were electronically gated based on BrdU staining (Fig. 3). Subdiploid BrdU+ cells first appeared on day 4 and increased in number to reach their maximum levels at around day 10, after which time they were lost (presumably cleared by scavenger cells). There were no striking differences between the CD4 and CD8 subsets, except possibly a faster initial rate of cell death in the CD8 compartment. Because of the extremely low percentage of BrdU+ cells recovered from mice that did not receive SEB or from mice that were analyzed 14 d after treatment with SEB (Fig. 1), no estimate for subdiploid cell content in these populations could be obtained.

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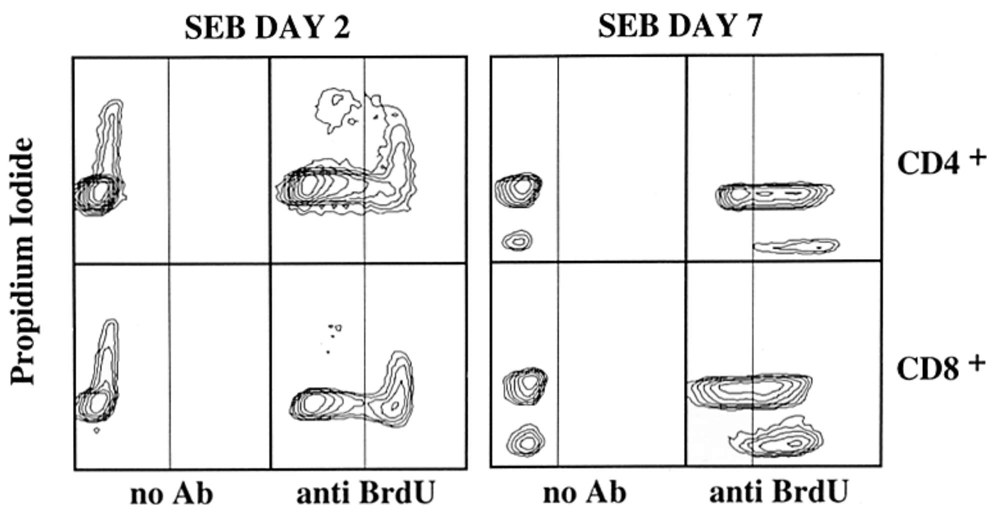

Figure 2. Correlation of DNA content and BrdU uptake in SEB-reactive T cells. BrdU labeling and SEB injections were carried out as described in Materials and Methods. CD4+ and CD8+ T cells were sorted from LN of Vβ8.2 TCR transgenic mice and subjected to double staining with anti-BrdU mAb and PI. Shown here are representative contour plots of sorted cells for 2 and 7 d after injection with SEB.

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This work shows for the first time that in a superantigen response, previous proliferation is a prerequisite for T cell deletion in vivo. A previous study (15) demonstrated that BrdU could be detected in fragmented DNA from T cell populations recovered from SEB-injected, BrdU-treated mice. However, no quantitative analysis at the single cell level was carried out. These findings are consistent with in vitro studies showing that mature T cells in the G1 phase of the cell cycle are refractory to receptor-induced apoptosis, whereas T cells that have progressed to the S phase are susceptible (17). In this context, it is not surprising that growth factors that drive T cells into cycle (such as IL-2) promote apoptotic death of T cells after their subsequent activation by SEB (18), high doses of antigen (19), or TCR ligation (20, 21).

Why do certain Vβ8+ T cells proliferate and die as a result of contact with SEB in vivo whereas others become anergic? One possible explanation may relate to differential affinity of TCR–superantigen interactions, depending on usage of TCR Vα chains. Indeed, we and others have previously shown a skewing in TCR Vα usage among superantigen-reactive T cells (22–24), and two recent reports suggest that this bias may reflect a requirement for efficient TCR–MHC class II interactions during superantigen activation (25, 26). Alternatively, given that a fraction of peripheral T cells in normal mice that bear activation markers such as low surface levels of CD45RB are refractory to further stimulation with superantigens (27) and that specific antigen-primed T cells are resistant to superantigen-induced cell death (28), it is conceivable that at least some of the Vβ8+ T cells that did not incorporate BrdU in response to SEB in normal mice correspond to cells that had been previously activated by environmental antigens. Finally, it is possible that the anergic T cells are those that interacted with SEB on the surface of non-professional APC (those lacking costimulatory signals).

The mechanism that underlies SEB-induced apoptosis of Vβ8+ cells in vivo remains to be clarified. In vitro activation of T cells or T cell clones with anti-CD3 mAbs, Con A, or PMA plus ionomycin leads to the induction of Fas-ligand expression (29, 30), which is a prerequisite for Fas-mediated killing (30). We now have evidence that both Fas and Fas-ligand are rapidly induced on apoptotic Vβ8+ T cells from SEB-injected mice, suggesting the Fas pathway is at least partly responsible for the deletion of SEB-reactive T cells in vivo (Renno, T., M. Hahne, J. Tschopp, and H. R. MacDonald, manuscript submitted for publication). It can be speculated that proliferating (BrdU+) SEB-reactive T cells eventually upregulate their expression of Fas and Fas-ligand, thus predisposing them to Fas-mediated killing.

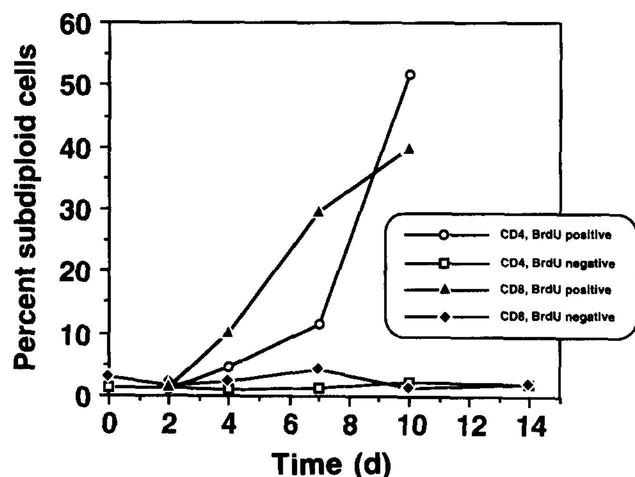

Figure 3. Kinetics of appearance of apoptotic cells in sorted CD4+ and CD8+ populations from BrdU-fed Vβ8.2 TCR transgenic mice after SEB injection. Curves represent the percentage subdiploid cells in electronically gated BrdU-positive or -negative populations. Because of the extremely low percentage of BrdU+ cells on day 14 (see Fig. 1), no estimate for subdiploid cell content could be obtained.

We wish to thank Mr. Pierre Zaech for FACS® sorting and Dr. Hans Acha-Orbea for critical reading of the manuscript.

T. Renno is supported in part by the Medical Research Council of Canada.

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Received for publication 6 January 1995 and in revised form 22 February 1995.

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