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*J Immunol* 2000; 165:1171-1174; doi: 10.4049/jimmunol.165.3.1171

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Cutting Edge: Cell Autonomous Rather Than Environmental Factors Control Bacterial Superantigen-Induced T Cell Anergy In Vivo

Antoine Attinger,* Hans Acha-Orbea,*† and H. Robson MacDonald2*  

Anergic T cells display a marked decrease in their ability to produce IL-2 and to proliferate in the presence of an appropriate antigenic signal. Two nonmutually exclusive classes of models have been proposed to explain the persistence of T cell anergy in vivo. While some reports indicate that anergic T cells have intrinsic defects in signaling pathways or transcriptional activities, other studies suggest that anergy is maintained by environmental “suppressor” factors such as cytokines or Abs. To distinguish between these conflicting hypotheses, we employed the well-characterized bacterial superantigen model system to evaluate in vivo the ability of a trace population of adoptively transferred naive or anergized T cells to proliferate in a naive vs anergic environment upon subsequent challenge. Our data clearly demonstrate that bacterial superantigen-induced T cell anergy is cell autonomous and independent of environmental factors. The Journal of Immunology, 2000, 165: 1171–1174.


dadministration of bacterial superantigens such as staphylococcal enterotoxins (SE)3 to mice induces rapid production of a panel of cytokines and subsequent expansion of the SE-reactive T cell population (1, 2). After the initial phase of SE-induced activation in vivo, the majority of the reactive cells that have proliferated are eliminated by apoptosis and the remaining T cell population fails to proliferate to a subsequent exposure to SE in vitro (3–6). This phenomenon, referred to as anergy, is specific for the SE-reactive T cells and persists for several weeks (7).

Two distinct (and nonmutually exclusive) classes of models have been proposed to explain the phenomenon of SE-induced T cell anergy. On the one hand, several studies described molecular alterations in TCR signaling in anergic cells. In vivo SE-anergized T cells exhibited impaired protein phosphorylation (8) and defective expression of the AP-1 and NF-κB transcription factors (9, 10). In addition, anergic cells displayed altered signaling via the common γ-chain of the IL-2 receptor, which consequently resulted in diminished phosphorylation of several downstream proteins (11). Collectively, these studies suggest that SE-induced anergy is caused by cell autonomous molecular alterations in TCR signaling.

On the other hand, a role for environmental factors in the maintenance of SE-induced T cell anergy has been documented. In vitro, regulatory CD8+ T cells (12) as well as apoptotic bodies (13) have been shown to induce T cell anergy. In vivo, IL-10 and TGF-β produced by SE-primed T cells as well as IFN-γ produced by a population of CD4+CD8− T cells have been reported to be involved in the persistence of SE-induced T cell anergy (14–16). Moreover, both regulatory T cells (17) and mAbs (18) directed against SE-reactive T cells have been implicated in the maintenance of T cell anergy. Taken together, these observations suggest that persistence of T cell anergy could be mediated by environmental factors.

To distinguish the relative contribution of cell autonomous and environmental factors in the persistence of SE-induced T cell anergy in vivo, we took advantage of a recently described adoptive transfer system that allows us to follow the proliferation of a trace population of 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled staphylococcal enterotoxin B (SEB)-reactive T cells in recipient mice (19). By using criss-cross combinations of naive or anergic donor and recipient mice, we were able to show that SEB-induced T cell anergy is cell autonomous and apparently independent of environmental factors.

Materials and Methods

Mice and treatment

Four- to 8-wk-old BALB/c mice were obtained from Harlan Olac (Bicester, U.K.). SEB (20 μg; purchased from Toxin Technology, Saragota, FL) was injected into the hind footpads (10 μg in each footpad).

CFSE staining and cell transfer

Labeling of naive or anergic splenocytes with CFSE was performed as described previously (19, 20). A total of 5 × 10^7 CFSE-labeled splenocytes were transferred i.v. into the tail vein of naive or anergic syngeneic recipients. One day after the transfer, mice received one single footpad injection 0022-1767/00/$02.00
of SEB or PBS, and T cell proliferation was monitored 2 or 7 days later in the draining popliteal lymph node. Chimerism of CFSE<sup>+</sup> cells in the lymph nodes of recipient mice varied between 1 and 3% in all instances.

**Abs and flow cytometry**

Single-cell suspensions were prepared from popliteal lymph nodes and incubated with anti-Fc receptor mAb 2.4G2 to prevent nonspecific staining. Cells were stained with the following Abs: PE-anti CD25 (PC61), PE-anti CD69 (H1.2F3), and APC-anti CD4 (RM4-5) (PharMingen, San Diego, CA) and PE-anti CD44 (1M.781) (Caltag, San Francisco, CA). FITC-conjugated and biotinylated anti-V<sub>β</sub>8 (F23.1) (21) were generated in our laboratory. Biotinylated anti-V<sub>β</sub>8 was revealed with streptavidin-CyChrome (PharMingen). Stained cells were analyzed on a FACSscan or FACSscalibur (Becton Dickinson, San Jose, CA) using CellQuest software.

**Quantitation of undivided cells**

After SEB injection, we evaluated the fraction of undivided CFSE<sup>+</sup> cells using the previously described formula<sup>a</sup>: percent undivided = \( \frac{a(1 - b)}{(b(1 - a)) \times 100} \), where \( a \) is the fraction of V<sub>β</sub>8<sup>+</sup> among undivided CD4<sup>+</sup> cells in SEB-treated animals and \( b \) the fraction of V<sub>β</sub>8<sup>+</sup> among undivided CD4<sup>+</sup> cells in control PBS-treated animals.

**Results and Discussion**

The bacterial superantigen SEB reacts with a polyclonal population of V<sub>β</sub>8 expressing T cells. Because in BALB/c mice V<sub>β</sub>8<sup>+</sup> T cells represent 30% of the peripheral T cell pool, this fraction is sufficiently high to be followed in an adoptive transfer experiment without purifying the cells. We restricted our analysis to the CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> T cell subset because anergy has been intensively studied in this T cell population. Several reports show that the extent of anergy can differ when monitored in an in vitro or in an in vivo essay (1, 22). To be as close as possible to the physiological situation, we evaluated anergy in vivo.

One single injection with 20 µg SEB in the footpads of a naive animal induces a strong T cell activation (evaluated by increases in forward scatter (FSC) as well as CD25, CD69, and CD44 expression) 1 day after the injection (Fig. 1B) in the draining popliteal lymph node. This activation is restricted to the V<sub>β</sub>8-expressing T cells (data not shown). T cell proliferation was measured as described previously (19) by transferring a CFSE-labeled naive splenocyte population into naive syngeneic recipients. The amplitude of proliferation in the CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> or CD4<sup>+</sup>V<sub>β</sub>8<sup>-</sup> T cell populations (evaluated by the decrease in CFSE intensity) was measured 2 days (Fig. 1A) or 7 days (Fig. 1C) after SEB administration. As shown in Fig. 1A, most proliferating cells on day 2 were found in division peaks 2 and 3, which is consistent with our previous observations (19). Because the majority of SEB-stimulated CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> T cells that have undergone more than three rounds of division are eliminated by apoptosis (19), the remaining CFSE<sup>+</sup> cells on day 7 after SEB injection are either undivided or have undergone one or two cell divisions (Fig. 1C). These residual CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> T cells displayed a phenotype similar to naive cells (Fig. 1D).

The extent of T cell anergy in mice that had received a single dose of SEB 7 days previously was evaluated by the administration of a second dose of SEB. T cell activation phenotype and proliferation were evaluated 1 day and 2 days, respectively, after the second SEB administration. Anergic T cells could still become blasts (FSC<sup>high</sup>) and re-express activation markers such as CD25, CD69, or CD44 upon a second contact with SEB (Fig. 1F). However, compared with T cells after a single SEB injection (Fig. 1B), this activation was reduced. The influence of the second SEB injection on T cell proliferation is depicted in Fig. 1E. Two days after the second SEB challenge, there is no striking difference in the distribution of proliferating cells in the different division peaks as compared with cells that have been activated once with SEB 7 days before (c.f. Fig. 1E and 1C, respectively). In both cases, the majority of the cells are either undivided or have cycled one or two times. Therefore, upon a second SEB challenge, the absence of significant proliferation and diminished expression of activation markers among CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> T cells demonstrate that these cells are anergic. These data are consistent with a previous report showing that anergic CD4<sup>+</sup>V<sub>β</sub>8<sup>+</sup> T cells displayed impaired cell cycle progression and IL-2 production in vivo upon SEB stimulation as compared with naive T cells (1).

To gain insight into the role of the environment in the maintenance of anergy, we performed adoptive transfers of CFSE-labeled anergic or naive cells in different environments. A series of BALB/c mice were injected with SEB. After 7 days, half of them were sacrificed and their splenocytes were used as donor anergic cells for adoptive transfers. The remaining SEB-injected animals were used as anergic recipients. Similarly, control BALB/c animals were used to provide either naive cells for transfer or naive recipients. Anergic or naive splenocytes were labeled with CFSE and transferred in either naive or anergic recipient mice. Then,

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<sup>a</sup> A. Attinger, H. R. MacDonald, and H. Acha-Orbea. Submitted for publication.
obtained are unequivocal; naive cells in an anergic environment proliferated normally, whereas anergic cells in a naive environment were strongly impaired in their capacity to proliferate. Analysis of CFSE profiles (Fig. 2) as well as the percentage of undivided cells (Fig. 3) show that proliferation of naive cells in an anergic environment is comparable to naive cells in a naive environment. Reciprocally, when anergic cells were transferred in naive or anergic recipient mice the percentage of undivided cells was very similar (Fig. 3) and the few cells that were engaged in proliferation underwent only one cell cycle (Fig. 2). If anything, a slightly higher proportion of naive or anergic donor cells entered in cycle in anergic recipients compared with naive recipients (Fig. 3), although this difference was only marginally statistically significant ($p = 0.05$).

In conclusion, we show that in vivo SEB-induced persisting T cell anergy is independent of inhibitory environmental factors, such as cytokines or Abs. This conclusion is supported by two observations. First, naive T cells, when transferred in an anergic environment, are still able to proliferate normally upon SEB stimulation despite the hypothetical presence of suppressor factors. Second, anergic cells remain unresponsive when transferred in a naive and therefore presumably suppressor factor-free environment. It should be emphasized that the average chimerism of CFSE-labeled cells was $\sim 2\%$ in these criss-cross transfer experiments. Hence the cell autonomous behavior of naive and anergic T cells was achieved even under conditions where such cells were outnumbered by a ratio of $\sim 50:1$ in their respective environments. Therefore, we favor a model where T cell anergy would be maintained by a cell autonomous mechanism. Interestingly, using T cell clones, accumulating evidence suggests that anergy in vitro could be maintained by a dominant-acting repressor molecule that inhibits IL-2 signal transduction (23–27). Whether a similar mechanism is responsible for SEB-induced T cell anergy in vivo remains to be investigated.

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