**Brief Definitive Report**

Clonal Anergy Blocks In Vivo Growth of Mature T Cells and Can Be Reversed in the Absence of Antigen

By Benedita Rocha,* Corinne Tanchot,* and Harald Von Boehmer†

From *U345 INSERM, Centre Hospitalier Universitaire Necker-Enfants Malades, 75015 Paris, France; and the †Basel Institute for Immunology, CH-4005 Basel, Switzerland

Summary

Experiments in various models have indicated that immunological tolerance can result from the physical elimination (deletion) of reactive lymphocytes as well as from anergy. We have previously reported that mature CD4+CD8+ T cells when confronted with their antigen can proliferate extensively but are finally eliminated or become intrinsically anergic such that remaining cells are refractory to stimulation by any T cell receptor ligands, even in the presence of exogenous interleukin 2. Here we show that in vivo the anergy can be reversed in the absence of antigen, such that the cells are then able to proliferate extensively in vivo to a new challenge with the antigen in question.

Lymphocytes are susceptible to different mechanisms of tolerance induction at different stages of maturation (1-10). In the presence of self-antigens, immature thymocytes do not divide and are deleted by apoptosis (2). Mature T cells may become anergic, i.e., nonreactive to further stimulation with or without prior proliferation (3-6). Deletion of mature self-reactive T cells was also reported, but it is often incomplete such that some anergic T cells do persist (3-6).

The fate of anergic T cells in vivo is not clear. They may become irreversibly committed to nonresponsiveness (7-9). Alternatively, anergy may represent a temporary unresponsiveness that under appropriate conditions can be reverted (11). In the latter case, the consequences of anergy induction will, by necessity, differ from those of clonal deletion in the thymus. Studies concerned with this topic have so far given ambiguous results. In the absence of antigen, CD4 (8) or CD8 T cells (7) tolerant to conventional antigens did not regain reactivity. In contrast to virgin or activated male-specific cells, these tolerant cells had downregulated surface expression of TCR and CD8 (6), and were intrinsically anergic to various stimuli transduced through their TCR (6, 12). Here we study the in vivo behavior of male-reactive versus anergic TG cells, upon adoptive secondary transfer into nu+ mice possessing or lacking the male antigen.

Materials and Methods

**Mice.** The TCR α/β transgenic mice were described previously (13). These mice, as well as syngeneic C57Bl/6 µ+ mice, were obtained from the Centre de Sélection et Elevage d'Animaux de Laboratoire (Orléans, France).

**Adoptive Transfers.** Spleen cells, from pools of female TG mice or intermediate nude recipients, were depleted of B cells by magnetic sorting with anti-Ig-coated Dynabeads (Dynal, A.S., Oslo, Norway), and injected intravenously into either female or male nu+ hosts.

**Cell Labeling.** For surface staining (6) we used the anti-TCR antibodies F23.2 (13) (anti-TCR β TG chain, βT) and T3.70 (14; anti-TCR α TG chain, αT), coupled to biotin, and revealed with streptavidin-PE (Southern Biotechnology Associates, Inc., Birmingham, AL) together with the FITC-labeled anti-CD8β chain mAb, H35-17-2. Cells were analyzed on a FACScan®, using the Lysis II program (Becton Dickinson & Co., Mountain View, CA).

**Identification of TG Populations in the Spleen of Recipient Mice.** In these studies, we identified CD8+ populations from female TG mice using an anti-CD8 mAb that recognizes the β chain of the CD8 molecule (15). This later antibody was used rather than the anti-CD8α chain antibody, since its expression is restricted to CD8 populations generated in the thymus. CD8α expression, in contrast, can be induced in multiple cell types, by activation events.
in the periphery (12, 16), and may be expressed in nu+ B6 hosts T cells (16).

The absolute number of TCR γ TG CD8+ lymphocytes expressing (CD8αγ−), or lacking the TG-TCR α chain (CD8αγ+), was evaluated as described (16). Briefly, a standard procedure was used to obtain as many cells as possible from the spleen. Cells were counted before washing. Cell suspensions were stained with FITC-anti-CD8β and anti-CD8α mAbs. CD8+ cells of female TG origin were identified by the coexpression of the TG TCR β (βT), expressed in all TG cells, as well as high surface levels of CD8β chain. The total number of TG CD8+ cells was calculated from the percentage of CD8+ TG lymphocytes and the total number of cells recovered from the spleen.

To determine the percentage of CD8αγ− and CD8αγ+ lymphocytes, spleen cell suspensions were depleted of B cells by magnetic sorting and stained with the anti-TCR αT mAb T3.70 and anti-CD8β mAb. Due to the experimental protocol used, donor and recipient mice could not be analyzed simultaneously and, as such, slight fluctuations between experiments of the labeling intensity were unavoidable. Therefore, in each experiment, the T cell population from female TG mice were analyzed. CD8αγ+ cells in recipient nu+ mice were those expressing similar levels of TCR αT and CD8β.

In Vitro Proliferation. 5 x 10⁶ spleen cells depleted of B cells or S1g- spleen cells were cultured in the presence of 1 μg/ml of T3.70 mAb as previously described (12). 3 d later, cultures were supplemented with 10 U/ml IL-2 and expanded for an additional 2 d.

The methodology used for limiting dilution analysis has been described elsewhere (17). In brief, limiting dilution analysis cultures contained limiting numbers of responder cells (24 wells/group, four to six groups) and nude peritoneal cells as feeders were set up in round-bottomed microplates in a final volume of 20 μl of culture medium, containing 1 μg/ml of T3.70 mAb. 3 d after culture, wells were supplemented with 10 U/ml IL-2, and cells expanded for 4 d. At the end of this period, cell growth was evaluated by [3H]thymidine incorporation (1 μCi/culture). Cultures were scored as positive when cpm/culture was higher than the mean plus three times the standard deviation of background cultures without responder cells. Frequency estimates were obtained with a software developed by L. Gastinel, using the χ² minimization according to the method of Taswell (18).

Results and Discussion

T Cell Populations in Female TG Mice. The peripheral T cells from female TG mice used in these studies (14) express TCR α/β receptors encoded by a TG-TCR β transgene (βT) expressed in all T cells, which permits the identification of TG cells upon adoptive transfer. The βT may be associated with the TCR α transgene (αT) or with endogenous TCR α chains (αE). The response to H-Y requires the coexpression of high cell surface levels of the TCR β-βTγ- and CD8 receptors. Peripheral T cells from female TG mice thus contain a male-reactive TCR-βTγ-CD8- population (CD8αγ−) as...
well as TCR-βαε cells with other specificities (CD4αε+, CD8αε+). Few cells expressing a TCR-βαε but not CD4 or CD8 coreceptors can also be detected in the spleen and nodes of female TG mice. These cells, representing 2% or less, do not proliferate very slowly if at all in the presence of the male antigen and are rapidly outgrown by CD4 and CD8 cells when adoptively transferred into male or female recipients.

Virgin T Cells and Activated T Cells Are Able to Proliferate upon Adoptive Secondary Transfer, whereas Anergic T Cells Are Not. Transfer of TG populations into female and male nu+ mice showed that the expansion of male-specific T cells required the presence of the male antigen: CD8αε+ T cells did not expand after transfer into female nu+ recipients, while they proliferated vigorously after transfer into male nu+ hosts (6). 4 d after transfer, CD8αε+ T cells were the dominant donor CD8+ population in male nu+ mice (Fig. 1), and were activated, as shown by increased size and higher CD44 expression (12), when compared with the same population recovered from female nu+ hosts. Both virgin and activated male-specific cells then recovered were able to proliferate in the presence of the male Ag in vitro (6, 12), and expanded in vivo when transferred into secondary male nu+ recipients (Fig. 1).

This contrasts with the behavior of male-specific cells, recovered in male nu+ hosts at later times in point where anergy has been shown. CD8αε+ T cells had then downregulated levels of TCR and CD8, and the T cells recovered from these mice did not proliferate in the presence of the male antigen (6), or the anti-ε mAb T3.70 (12). Investigation of the frequency of T lymphocytes able to proliferate to stimulation with the anti-ε antibody revealed no significant response when compared with that found in the absence of the anti-ε antibody (Table 1). CD8αε+ anergic cells transferred into nu+ male recipients did not expand. The number of CD8αε+ T cells recovered was always inferior to the number of CD8αε+ injected, and corresponded to the fraction of donor cells expected to home to peripheral lymphoid organs after intravenous injection (19). On the contrary, CD8+ T cells with other specificities (CD8αε+) expanded in these mice (Fig. 2). Because of the preferential expansion of CD8αε+ T cells, CD8αε+ became progressively diluted from the total population and could no longer be detected in significant proportion in recipient organs from 2 wk after transfer (Fig. 1). Thus, both virgin or activated, but not anergic, CD8αε+ T cells are able to expand in vivo after transfer. These results suggest that anergy induction has similar effects in vivo and in vitro: it blocks the capacity of T lymphocytes to respond with proliferation to stimulation by anti-TCR ligands.

Anergic CD8αε+ T Cells Maintained in the Absence of Antigen In Vivo Regain Responsiveness. We investigated whether anergy could be reversed, by “parking” anergic cells for different periods of time in female nu+ mice. The inoculum contained 5 x 105 CD8αε+ anergic T cells, and similar numbers of CD8αε+ lymphocytes and, as far as we could detect, no cells able to proliferate in the presence of anti-ε mAb (Table 1) or in the presence of the male antigen (not shown). Anergic T cell populations were “parked” from 4 d up to 8 wk in female nu+ mice. The parked population was subsequently injected into a third set of male nu+ recipients (Fig. 1).

Whether anergy was reversed or not during parking in female nu+ recipients, we did not expect to recover a high proportion of CD8αε+ T cells in female nu+ hosts, since growth of male-specific cells was shown to require the presence of the male Ag (6). Indeed, the CD8αε+ T lymphocytes could be detected within the CD8+ TG population in female hosts at early time points after transfer (not shown), but were rapidly diluted because of the expansion of T cells with endogenous TCR αβ chains. After 3 wk of parking, CD8αε+ cells represented <2% of the CD8+ TG cells,

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Number of donor cells/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anergic T3.70 + IL-2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Anergic IL-2</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>TG mouse T3.70 + IL-2</td>
<td>57.3 ± 16</td>
</tr>
</tbody>
</table>

Anergic, the slg- T cell population, recovered from male nu+ mice 1 mo after the injection of T cells from female TG mice (shown in Fig. 1). The frequency of CD8αε+ cells in this cell suspension, as determined by cell surface labeling, was 69/106 cells. TG mouse, the spleen cells from a female TG mouse, which were studied simultaneously. The frequency of CD8αε+ cells in this cell suspension, as determined by cell surface labeling, was 55.4/106 cells, i.e., all CD8αε+ virgin T cells were able to proliferate in response to T3.70 mAb. Similar results were obtained in another experiment.
Table 2. Reverted CD8α+ T Cells Are Able to Proliferate in the Presence of T3.70 mAb

<table>
<thead>
<tr>
<th>Condition</th>
<th>[3H]Thymidine uptake (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells alone</td>
<td>600 ± 200</td>
</tr>
<tr>
<td>Cells + T3.70</td>
<td>9,300 ± 2,500</td>
</tr>
<tr>
<td><strong>5 × 10^5 αCD8+</strong></td>
<td></td>
</tr>
</tbody>
</table>

- **5 × 10^5 αCD8+** anergic cells were parked for 26 d in female nu+ mice, and sIg- spleen cells from these mice were injected intravenously into male nu+ mice. Each mouse received 3.5 × 10^6 CD8+ TG cells. Recipient mice were studied from 2 wk up to 4 mo after transfer. sIg- spleen cells from each mouse were cultured with the anti-αTG mAb T3.70. Each column represents the response of an individual mouse. Results represent the mean [3H]thymidine uptake of triplicate cultures.

* In the presence of IL-2.
We are grateful to C. Tucek for reading this manuscript, to N. Dautigny and A.-M. Joret for technical assistance, and to S. Hamon for typing.

This work was supported by a grant from DRET.

Address correspondence to B. Rocha, U.345 INSERM, CHU Necker-Enfants Malades, 156 rue de Vaugirard, 75015 Paris, France.

Received for publication 9 February 1993.

References