CLONAL DELETION AND CLONAL ANERGY IN THE THYMUS INDUCED BY CELLULAR ELEMENTS WITH DIFFERENT RADIATION SENSITIVITIES

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Self-tolerance is an essential characteristic of the T cell repertoire, and results initially from negative selection processes that developing T cells undergo during differentiation in the thymus. Potentially autoreactive thymocytes expressing anti-self TCR specificities encounter self antigens in the thymus, and the signals resulting from engagement of their TCRs are thought to abort their differentiation, either by signaling the cells to die (clonal deletion) or by signaling the cells to become anergic (clonal inactivation). Using anti-Vβ mAbs specific for TCRs reactive against various self antigens, it has been possible to determine the fate of potentially autoreactive T cells as they differentiate in the thymus. Thus, it was found that TCR$^{hi}$ Vβ17$a^+$ thymocytes are deleted in IE$^+$ mice (1), and Vβ6$^+$ thymocytes are deleted in Mlsa$^a$ mice (2). In the present study, we show that clonal deletion is not the only mechanism by which Mlsa$^a$ and IE-specific tolerance can be induced in the thymus, as clonal inactivation of developing Vβ6$^+$ and Vβ17$a^+$ T cells can also be induced, but the alternative tolerance mechanisms are mediated by cellular elements with different sensitivities to γ irradiation.

Material and Methods

Experimental Animals. Radiation bone marrow chimeras are designated as bone marrow donor → irradiated recipient, and were constructed by injecting 1.5 × 10$^7$ T-depleted bone marrow cells into 950-rad γ-irradiated recipients. Chimeras were examined no earlier than 5 wk after irradiation and bone marrow reconstitution. At that time, no cells of host origin were detectable in the thymi of these chimeras, but spleen cell populations did contain <5% radiation-resistant host cells that were essentially all T cells, so that purified populations of spleen T cells were contaminated with, on average, 33.6% host cells.

Flow Cytometry (FCM). Cells were stained as indicated and samples were analyzed on a modified dual laser (488 nm, 590 nm) FACS II (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Fluorescence data were collected using three-decade logarithmic amplification on viable cells as determined by forward light scatter intensity and propidium iodide exclusion.

T Cell Populations. Purified spleen T cells for FCM were obtained by passage over either nylon wool columns or anti-Ig plates. For functional studies, purified SJL (Ly5.1$^+$) spleen

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T cells were obtained from chimeric animals by coating spleen cells with anti-Ly5.2 mAb, incubating them on anti-Ig plates, and collecting the nonadherent cells. The resultant cell populations were >80% Thy-1.2* and >99.7% Ly5.1*.

**Proliferation Assays.** Triplicate cultures of responder T cells were stimulated with either mitomycin C-treated cells or anti-TCR mAb as indicated, and then pulsed with 1 μCi [3H]thymidine for 8–12 h before harvesting.

**Results and Discussion**

As previously reported, thymus and spleen T cell populations in SJL mice contain significant numbers of TCR hi Vβ6+ and Vβ17a+ T cells (1), whereas T cell populations from SJL × CBA/J mice expressing Mlsα and IEk determinants do not (Fig. 1, Table I). To track the fate of developing Vβ6+ and Vβ17a+ thymocytes encountering Mlsα and IEk determinants on either radiation-resistant or radiation-sensitive cellular elements, we constructed experimental animals by injecting SJL bone marrow stem cells into irradiated hosts of various genotypes (Fig. 1, Table I). Because only SJL cells express the Ly5.1 allelic marker, we were able to focus exclusively on SJL T cells in each experimental animal. TCR hi Vβ6+ and Vβ17a+ SJL T cells were present in both the thymus and spleen of SJL → B10 chimeras in which neither Mlsα nor IE determinants were expressed. Differences in frequency of Vβ17a+ SJL T cells maturing in a normal SJL thymus vs. the chimeric B10 thymus (Table I) are consistent with the role of Kk as a positive selecting element for Vβ17a+ T cells (3). In contrast, few if any TCR hi Vβ6+ and Vβ17a+ SJL T cells were present in SJL + B6 × CBA/J → B10 experimental animals that were identical to SJL → B10 animals, except for the injection of additional unirradiated B6 × CBA/J (MlsαIEk) bone marrow cells, demonstrating that expression of Mlsα and IEk de-

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**Table I**

*Development of Vβ6+ and Vβ17a+ T Cells in Experimental Mice*

| Experimental animal | T cell source | Percent TCR hi SJL T cells expressing*: |
|---------------------|--------------|--------------------------------------|
| SJL                 | Thymus       | TCR hi SJL T cells expressing*: |
|                     | Spleen       | Vβ6        | Vβ17a        |
| SJL × CBA/J        | Thymus       | 0.9 ± 0.05 | 0.4 ± 0.1   |
|                     | Spleen       | 0.2 ± 0.03 | 0.8 ± 0.6   |
| SJL → B10          | Thymus       | 13.5 ± 0.3 | 5.2 ± 0.2   |
|                     | Spleen       | 13.1 ± 1.3 | 5.7 ± 0.2   |
| SJL → B6 × CBA/J   | Thymus       | 11.6 ± 0.8 | 6.3 ± 0.5   |
|                     | Spleen       | 4.5 ± 1.0  | 3.2 ± 0.6   |
| SJL + B6 × CBA/J → B10 | Thymus | 1.8 ± 0.2  | 1.5 ± 0.2   |
|                     | Spleen       | 1.0 ± 0.2  | 1.0 ± 0.3   |

* Two-color immunofluorescence staining with anti-Ly5.1 mAb in red and anti-TCR (CD3, Vβ6, and Vβ17a) was used to identify T cells of donor SJL origin. Values are Vβ6+ SJL T cells as a percentage of CD3+ SJL T cells ± SE for no fewer than four animals. T cells were defined as TCR hi as described in Fig. 1.
terminants on unirradiated bone marrow–derived cells (e.g., dendritic cells) is able to delete developing \( \text{V}\beta 6^+ \) and \( \text{V}\beta 17a^+ \) T cells (1). We next examined T cell populations from SJL \( \rightarrow \) B6 \( \times \) CBA/J animals in which Mls\(^a\) and IE\(^k\) determinants were expressed only on radiation-resistant host elements. Surprisingly, \( \text{V}\beta 6^+ \) and \( \text{V}\beta 17a^+ \) SJL T cells were present in SJL \( \rightarrow \) B6 \( \times \) CBA/J animals, indicating that radiation-resistant cellular elements such as thymic epithelium fail to delete developing \( \text{V}\beta 6^+ \) and \( \text{V}\beta 17a^+ \) thymocytes. Table I summarizes the frequencies of \( \text{V}\beta 6^+ \) and \( \text{V}\beta 17a^+ \) T cells observed in the thymi and spleens of all the experimental mice tested.

The failure of radiation-resistant B6 \( \times \) CBA/J host cells to delete \( \text{V}\beta 6^+ \) and \( \text{V}\beta 17a^+ \) SJL T cells in SJL \( \rightarrow \) B6 \( \times \) CBA/J animals might have resulted in a failure...
of the irradiated host to induce Mls\(^a\)- and IE\(^k\)-specific tolerance. To assess this possibility, purified Ly5.1\(^+\) (SJL) T cell populations from experimental animals were assessed for their proliferative responses against stimulator cells expressing third-party (DBA/2), IE\(^k\) (B10.BR), or Mls\(^a\)IE\(^k\) (CBA/J) alloantigens. It can be seen in Table III that SJL T cell populations from SJL → B6 × CBA/J as well as SJL + B6 × CBA/J → B10 animals were functionally tolerant to both Mls\(^a\) and IE\(^k\), even though V\(\beta\)6\(^+\) and V\(\beta\)17a\(^+\) T cells were present in the former mice. To understand how V\(\beta\)6\(^+\) and V\(\beta\)17a\(^+\) T cells present in SJL → B6 × CBA/J mice could fail to react against Mls\(^a\)- and IE\(^k\)-bearing stimulator cells, we attempted to stimulate them by crosslinking their TCRs directly with anti-V\(\beta\)6 and anti-V\(\beta\)17a mAbs (Table III). To maximize responses, the cultures contained syngeneic accessory cells as well as exogenous T cell growth factors (Table III). In contrast to Mls\(^a\)- and IE\(^k\)-responsive V\(\beta\)6\(^+\) and V\(\beta\)17a\(^+\) SJL T cells from normal mice, tolerant V\(\beta\)6\(^+\) and V\(\beta\)17a\(^+\) SJL

| Experimental animal | T cell source\(^a\) | Strain of stimulator cells | SJL (H-2\(^b\), Mls\(^a\)) | DBA/2 (H-2\(^b\), Mls\(^a\)) | B10.BR (H-2\(^k\), Mls\(^b\)) | CBA/J (H-2\(^k\), Mls\(^b\)) |
|---------------------|---------------------|---------------------------|---------------------------|-----------------------------|-----------------------------|-----------------------------|
| SJL → B10          | Thymus              | 0 ± 0.1                   | 61.9 ± 1.4                | 16.5 ± 2.2                  | 29.8 ± 0.9                  |
|                     | Spleen              | 0.2 ± 0.1                 | 32.5 ± 0.8                | 2.0 ± 0.1                   | 76.5 ± 7.2                  |
| SJL → B6 × CBA/J    | Thymus              | 0 ± 1.5                   | 18.2 ± 1.2                | 0 ± 1.3                     | 0 ± 1.0                     |
|                     | Spleen              | 1.7 ± 0.2                 | 18.0 ± 1.7                | 0.5 ± 0.1                   | 4.0 ± 0.5                   |
| SJL + B6 × CBA/J → B10 | Thymus          | 0 ± 0.2                   | 23.4 ± 1.3                | 0 ± 0.2                     | 1.4 ± 0.4                   |
|                     | Spleen              | 0.8 ± 0.2                 | 11.3 ± 1.5                | 0.1 ± 0.2                   | 1.9 ± 0.5                   |
| SJL                 | Thymus              | 2.5 ± 1.3                 | 41.6 ± 2.6                | 24.5 ± 0.8                  | 27.2 ± 1.6                  |
|                     | Spleen              | 1.1 ± 0.1                 | 39.9 ± 2.4                | 8.6 ± 2.1                   | 86.3 ± 1.4                  |
| B10.BR              | Thymus              | 7.8 ± 0.6                 | 31.1 ± 2.5                | 0 ± 0.1                     | 28.9 ± 3.1                  |
|                     | Spleen              | 14.5 ± 1.7                | 54.9 ± 2.6                | 0.3 ± 0.2                   | 83.3 ± 8.8                  |

| T cell source\(^a\) | Strain of stimulator cells | Specificity of stimulating mAb\(^b\) |
|---------------------|---------------------------|-------------------------------|
| SJL → B6 × CBA/J    |                             | V\(\beta\)6 | V\(\beta\)17a | \(\alpha/\beta\) | CD3 |
| Thymus              | 1.8 ± 0.5                 | 0.4 ± 0.5               | 189.5 ± 33.1               |
| Spleen              | 0.1 ± 0.1                 | 0 ± 1.0                 | 45.0 ± 5.2                 |
| SJL                 | 14.9 ± 1.1                | 16.5 ± 1.6               | 142.2 ± 3.5                |
| Spleen              | 30.9 ± 2.7                | 34.7 ± 2.6               | 22.1 ± 1.9                 |
| CBA/J               | 0.5 ± 3.3                 | 0 ± 2.2                 | 371.0 ± 22.4               |
| Spleen              | 0 ± 2.1                   | 0 ± 1.4                 | 15.5 ± 2.3                 |

\(^a\) Responder thymocytes (10\(^5\)) and spleen T cells (5 × 10\(^5\)) from individual animals were cultured with 5 × 10\(^5\) stimulator cells. Values are the mean ± SE of cultures containing stimulator cells minus the mean ± SE of cultures without stimulator cells. Results are representative of three experiments.

\(^b\) 5 × 10\(^5\) responder thymocytes or 10\(^5\) responder Ly5.1\(^+\) spleen T cells were cultured with 25% culture supernatant from RRA-7 anti-V\(\beta\)6 (7), KJ23a anti-V\(\beta\)17a (1), H57-597 anti-TCR-\(\alpha/\beta\) (8), or 145-2C11 anti-CD3. Mitomycin C-treated syngeneic spleen cells were added as FCR+ accessory cells. Cultures also were supplemented with exogenous T cell growth factors in the form of 25% Con A supernatant. Values represent the mean ± SE of cultures containing stimulating mAb minus the mean ± SE of cultures without stimulating mAb. Results are representative of three experiments.
T cells from SJL → B6 × CBA/J mice failed to proliferate in response to direct TCR engagement by either anti-Vβ6 or anti-Vβ17a mAbs, indicating that the undeleted T cells were anergic (Table III). Indeed, clonal anergy of undeleted but tolerant T cells developing in SJL → B6 × CBA/J mice could explain why it is only in these mice that the relative frequency of Vβ6+ and Vβ17a+ T cells is significantly lower among spleen T cells than thymic T cells (Table I), since anergic T cells would fail to clonally expand in the periphery in response to environmental antigens with a resultant decrease in their relative frequency.

We next assessed how far anergic Vβ6+ and Vβ17a+ thymocytes could differentiate in SJL → B6 × CBA/J thymi by phenotyping them for CD4/CD8 expression using three-color FCM. We found that the distribution among various CD4/CD8 thymus subpopulations of anergic SJL → B6 × CBA/J thymocytes resembled that of normal SJL thymocytes, with normal numbers of phenotypically mature CD4+8- and CD4-8+ cells (Table II). Nevertheless, because Qa2 has been reported to be expressed only on functionally competent single-positive thymocytes and peripheral T cells (4), we thought that anergic Vβ6+ thymocytes from SJL → B6 × CBA/J mice might have failed to differentiate into Qa2+ cells, but such was not the case (Table II). Thus, there was no identifiable differentiation step that the anergic thymocytes had failed to undergo, even though they apparently could not proliferate in response to TCR engagement. While the role of TCR signaling in driving either thymocyte maturation or proliferation is uncertain, it is likely to be involved in selective events occurring in the thymus. Consequently, we wanted to determine if the anergic thymocytes were at all responsive to TCR-mediated signals. Indeed, as has been observed in anergic T cell clones (5), we observed that anergic Vβ6+ thymocytes do respond to TCR crosslinking by increasing their expression of IL-2-Rs (Fig. 2).

From the present study, it is clear that TCR engagement on developing thymocytes does not necessarily lead to clonal deletion. In fact, clonal deletion seems to require TCR engagement by immature T cells of self antigens on a specialized subpopulation of radiation-sensitive bone marrow-derived cells (probably dendritic cells), whereas TCR engagement of self antigens on other cells, possibly including thymic epithelium, induces clonal anergy. These results should help clarify conflicting reports in which T cell tolerance induced during development variably led to clonal deletion (1, 2, 6). Thus, TCR engagement without a competent second signal from bone

| IL-2R ON Vβ6+ THYMOCYTES |
|--------------------------------------------------|
| CELL NUMBER                                      |
| 0 1 2 3 4 5                                    |
| LOG10 FLUORESCENCE                              |
| a anti-CD3 stimulation                         |
| b anti-αβ stimulation                          |
| c no stimulation                               |

Figure 2. Increased IL-2-R expression on Vβ6+ thymocytes from SJL → B6 × CBA/J after anti-TCR stimulation. Thymocytes were cultured for 16 h without exogenous growth factors at 37°C in plates coated with 5 μg/ml purified anti-CD3 (a), 50 μg/ml anti-αβ (H57-597) (b), or no mAb (c). Cells were assessed by two-color FCM by staining for Vβ6 and counterstaining for IL-2-R with 7D4 mAb. Software gating was used to select Vβ6+ cells for analysis of IL-2-R expression. Because TCR stimulation causes downmodulation of cell surface TCR expression such that stimulated TCRαβ cells appear TCRβ-, the gates included both Vβ6αβ and Vβ6ββ cells. Single-color histograms depict the level of IL-2-R expression on stimulated Vβ6+ SJL thymocytes from SJL → B6 × CBA/J mice.
marrow-derived APC may lead to clonal anergy in developing T cells as it does in mature T cell populations (5). The signals inducing clonal deletion in immature thymocytes, as well as the signals driving the differentiation of anergic thymocytes into phenotypic maturity, remain to be identified.

Summary

The present study demonstrates that immune tolerance can be achieved in the thymus both by clonal deletion and by clonal inactivation, but that the two tolerant states are induced by cellular elements with different radiation sensitivities. TCR engagement of self antigens on bone marrow-derived, radiation-sensitive (presumably dendritic) cells induces clonal deletion of developing thymocytes, whereas TCR engagement of self antigens on radiation-resistant cellular elements, such as thymic epithelium, induces clonal anergy. The nondeleted, anergic thymocytes can express IL-2-Rs but are unable to proliferate in response to either specific antigen or anti-TCR antibodies, and do develop into phenotypically mature cells that emigrate out of the thymus and into the periphery.

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