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References

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Mechanisms of Acquired Thymic Tolerance In Vivo: Intrathymic Injection of Antigen Induces Apoptosis of Thymocytes and Peripheral T Cell Anergy

Wanjun Chen, Mohamed H. Sayegh, and Samia J. Khoury

Intrathymic injection of Ag induces Ag-specific tolerance in several clinically relevant experimental autoimmune and transplantation models. However, the exact mechanisms of acquired thymic tolerance in vivo remain unclear. We investigated the mechanisms of acquired thymic tolerance in mice that are transgenic for the TCR specific for peptide 323-339 of OVA. Intrathymic injection of OVA leads to apoptosis of thymocytes starting as early as 3 h after injection and persisting up to 7 days. Double positive thymocytes undergo apoptosis earlier than single positive thymocytes, and significantly higher percentages of double positive thymocytes ultimately die as compared with single positive cells. Apoptotic cells show decreased surface expression of CD4. In the periphery, T cells from intrathymically injected animals had suppressed proliferation and IL-2 production to OVA compared with T cells from control Ag-injected mice. We conclude that intrathymic injection of Ag induces apoptosis of immature thymocytes and a subpopulation of mature thymocytes and induces prolonged anergy in peripheral T cells in vivo. Understanding the mechanisms of acquired thymic tolerance may lead to development of novel clinical strategies to prevent autoimmune disease and transplant rejection. The Journal of Immunology, 1998, 160: 1504–1508.

Materials and Methods

Animals

The DO11.10 transgenic mice expressing TCR with specificity for chicken OVA peptide 323-339 were kindly provided by Dr. D.Y. Loh (Washington University School of Medicine, St. Louis, MO) (14). The mice were extensively backcrossed onto BALB/c background and were bred and screened for expression of TCR transgenes by mAb KJ1-26 or mAb to Vβ 8, as previously described (14); virtually all thymocytes in these mice express the transgene and all peripheral T cells are CD4+ KJ1-26+. Mice were maintained under specific pathogen-free conditions at Brigham and Women’s Hospital animal facility and were used at 8 to 12 wk of age.

Abs and reagents

FITC-conjugated CD8 (rat IgG2a), FITC or phycoerythrin (PE)-conjugated CD4 (rat IgG2a), and their respective isotype control anti-murine Abs were purchased from Caltag Laboratory (San Francisco, CA). Chicken OVA and hen egg lysozyme (HEL) were purchased from Sigma (St. Louis, MO). 7-Amino-actinomycin D (7-AAD), purchased from Calbiochem (La Jolla, CA), was dissolved at 5 mg/ml with 100% ethanol and stored at −20°C.

Intrathymic injection

Mice were injected intrathymically with 100 µg of chicken OVA or HEL dissolved in 50 µl of sterile PBS, as previously described (10). The optimal dose of Ag required to induce tolerance when injected into the thymus was determined in BALB/c mice by a dose-response experiment and found to be 100 µg of OVA (data not shown).

Preparation of thymocytes and staining for surface molecules

For staining of surface Ags, 5 × 10^6 of thymocytes suspended in PBS without Ca²⁺ and Mg²⁺ (Biowhittaker, Walkersville, MD) containing 1% BSA (Irvine, Santa Ana, CA) and 0.02% sodium azide (Sigma) were incubated with FITC- or PE-conjugated mAbs as indicated for 30 min on ice. After washing twice with 1 ml of PBS-sodium azide, the cells were resuspended in 0.5 ml of PBS-sodium azide.

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Staining DNA of apoptotic cells with 7-AAD and flow cytometry analysis

Staining of apoptotic cells with 7-AAD was performed by the method described by Schmid et al. (16). Briefly, the thymocytes were prestained for surface Ag expression as above and then were incubated with 20 μg/ml of 7-AAD in PBS-sodium azide for 20 min at 4°C and protected from light. Cells were then analyzed on FACSort (Becton Dickinson, San Jose, CA) without further washing. A minimum of 10,000 events were collected and analyzed using Lysis II software (Becton Dickinson). Staining with 7-AAD without further washing. A minimum of 10,000 events were collected and analyzed using Lysis II software (Becton Dickinson). Staining with 7-AAD in PBS-sodium azide for 20 min at 4°C and protected from light. Thymocytes irradiated with 1200 rad and then incubated overnight at 37°C were used as positive controls for 7-AAD staining (16).

Cell cultures

Splenocytes (4 x 10^5 cells in 200 μl per well) were cultured in round-bottom microtiter plates (Costar, Cambridge, MA) and stimulated with OVA. Proliferation was measured by the standard 72-h lymphocyte proliferation assay. For cytokine production, thymocytes (8 x 10^5 cells in 200 μl per well) or splenocytes (4 x 10^5 cells in 200 μl per well) were cultured in X-Vivo serum-free medium (Biowhittaker). Cell-free supernatants were collected after 48 h for measurement of IL-2 and IFN-γ production using both paired mAbs specific for the corresponding cytokine and purified recombinant murine cytokine according to the manufacturer’s recommendations (PharMingen, San Diego, CA). For IL-2 production by thymocytes, the measured IL-2 level was adjusted to the number of CD4^+ cells as determined by staining with anti-CD4 and anti-CD8 Abs as above. In some experiments, rIL-2 (Boehringer Mannheim, Indianapolis, IN) was added to the cell cultures at a concentration of 5 U/ml to study reversal of unresponsiveness (10).

Results

Intrathymic injection of protein Ag induces apoptosis of thymocytes

Results

To investigate whether acquired thymic tolerance is associated with apoptosis of Ag-specific T cells in the thymus, we used a method of staining with 7-AAD that labels apoptotic cells (16). First, thymocytes of OVA TCR-transgenic mice were collected 24 h after intrathymic injection of OVA or HEL, a nonspecific Ag control, and were stained with anti-CD4 and anti-CD8 mAbs followed by staining with 7-AAD. Thymocytes from naive mice served as a negative control, and irradiated cultured thymocytes served as a positive apoptosis control. Double positive (DP) or single positive (SP) cells were gated, and the percentage of 7-AAD positive cells in each population was determined (Fig. 1). In thymocytes from naive mice, 1 to 3% of DP cells and 1 to 5% of SP cells were 7-AAD+. This represents about 0.05 to 1% of total thymocytes that are undergoing apoptosis, consistent with previous reports (17). Intrathymic injection of control protein HEL did not alter the percentage of 7-AAD+ cells in either the DP or SP thymocytes compared with naive animals, although the total number of thymocytes and the number of DP cells were decreased, most likely because of the stress of surgery (Table I). On the other hand, intrathymic injection of OVA resulted in a marked increase of apoptotic SP cells to 17.5 ± 2.5% 7-AAD+ cells of total SP cells.

Table I. Change in numbers of thymocytes of OVA TCR-transgenic mice 24 h (A) and 7 days (B) after intrathymic Ag injection

<table>
<thead>
<tr>
<th></th>
<th>Total No. (x10^6)</th>
<th>CD4+ CD8+ (x10^6)</th>
<th>CD4+ CD8- (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>76.5 ± 14</td>
<td>62.7 ± 10</td>
<td>7.7 ± 1.5</td>
</tr>
<tr>
<td>HEL IT</td>
<td>59.7 ± 7</td>
<td>44.3 ± 3</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>OVA IT</td>
<td>25.7 ± 13</td>
<td>12.1 ± 6d</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>100</td>
<td>84</td>
<td>10.4</td>
</tr>
<tr>
<td>HEL IT</td>
<td>89 ± 3</td>
<td>65 ± 0.5</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>OVA IT</td>
<td>37 ± 2</td>
<td>22 ± 1.8e</td>
<td>5.8 ± 0.4d</td>
</tr>
</tbody>
</table>

a There were three to four mice per group except in the naive group (section B) (one mouse).

b IT, intrathymic.

c p < 0.05 compared with HEL group.

d p < 0.005 compared with HEL group.

e p < 0.0001 compared with HEL group.

The data shown in the table are representative of four independent experiments.
cells, and of DP thymocytes to 10.6 ± 3% 7-AAD− of total DP cells (Fig. 1). Most of the DP thymocytes in the OVA-injected animals that stained positive with 7-AAD showed bright staining (7-AADbright), indicating that they were in the later stages of apoptosis, while the 7-AAD− SP thymocytes showed mostly dim staining for 7-AAD (7-AADDim), suggesting an earlier stage of apoptosis in this population (Fig. 1). When the number of thymocytes was determined, we found that by 24 h after intrathymic injection of OVA, the total number of DP T cells was dramatically decreased (by 73% compared with HEL-injected controls), whereas the number of SP T cells was only moderately reduced (by 30% compared with HEL-injected controls) (Table IA). Significant thymocyte apoptosis was not observed in animals injected systemically with the same dose of OVA (100 μg), but was detected in animals injected with a large dose of OVA (5 mg) (data not shown), as has been previously demonstrated (14, 15).

**Surface staining of surface molecules on apoptotic thymocytes**

Thymocytes from mice injected intrathymically with OVA were stained for CD4 and CD8 surface expression in combination with 7-AAD. CD4 expression of almost all early apoptotic 7-AADDim SP cells was significantly reduced. The mean fluorescence intensity of CD4 was markedly decreased compared with the 7-AAD− SP cells (Fig. 2). Similarly, 7-AADDim DP cells down-regulated their CD4 and CD8 expression (Fig. 2). These results indicate that apoptotic SP thymocytes down-regulate their CD4 expression in the process of apoptosis as previously reported for apoptotic DP thymocytes (18).

**Kinetics of apoptosis induced by intrathymic injection of OVA**

The decrease in number of DP cells 24 h after intrathymic injection of OVA (Table IA) suggested that apoptosis of this population may have been initiated earlier. Thus, we collected thymocytes at different time points after OVA injection. Positive staining with 7-AAD was detectable in DP thymocytes as early as 3 h after OVA injection. By 15 h after OVA injection, many DP cells were deleted and the remaining cells displayed a high percentage of 7-AADDim DP cells. By 24 h after OVA injection, more DP cells were 7-AADbright than 7-AADDim (Fig. 3), and this was associated with a remarkable decrease in live DP cells within the thymus (Table IA). In contrast, the SP thymocytes had no positive staining with 7-ADD until 15 h after intrathymic OVA injection. The peak of apoptosis, as determined by the highest percentage of 7-AADDim cells, occurred 24 h after intrathymic injection of OVA. By 48 h, the percentage of cells undergoing apoptosis began decreasing (Fig. 3). Interestingly, DP thymocytes decreased in number by 70% at 24 h and remained decreased by 67% on day 7, whereas SP cells decreased by 30% on day 1 and by 52% by day 7, when the percentage of cells undergoing apoptosis was close to the baseline level.

**Functional changes in T cells**

We have previously shown that injection of OVA into the thymus of normal mice almost completely inhibits peripheral Th1 cell responses as determined by Ag proliferation and cytokine production.

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**Figure 2.** Down-regulation of CD4 expression on apoptotic thymocytes. Thymocytes were stained with CD4-PE, CD8-FITC, and 7-AAD. CD4+CD8− (DP) and CD4−CD8+ (SP) thymocytes were gated, and each of the DP and SP populations was then gated according to its’ 7-AAD staining. Apoptotic cells (7-AAD+) had decreased expression of CD4 (filled histogram) compared with nonapoptotic (7-AAD−) cells (bold outline histogram). The background staining is shown in the stippled line histogram.

**Figure 3.** Kinetics of apoptosis in the thymus. Staining for 7-AAD is shown in the left panel for DP thymocytes and in the right panel for SP thymocytes. Staining was performed at different time points after intrathymic injection of HEL (open squares) or OVA (filled circles). Triangles represent naive animals. The results represent the mean of two to four experiments per time point.

**Figure 4.** IL-2 production by thymocytes. Thymocytes were collected on day 14 after intrathymic injection from animals injected with OVA (filled circles) or HEL (open squares). Thymocytes were cultured at 8 × 10^6 cells/well in serum-free media with various concentrations of OVA as shown on the x-axis. Cell-free supernatants were collected 48 h later, and IL-2 production was measured by ELISA. The results were adjusted to the number of CD4+ cells and reported as pg/ml per 10^6 cells.
In this report, we found that a significant number of thymocytes in intrathymically injected animals do not undergo apoptosis. This would suggest that surviving thymocytes migrating to the periphery may be anergic. Thus, we measured IL-2 production by thymocytes after in vitro stimulation with OVA and found that IL-2 production in mice injected intrathymically with OVA was 10-fold lower than in HEL-injected mice (Fig. 4). IL-2 production was normalized by adjusting to the number of SP thymocytes in the culture because the cultures from OVA- or HEL-injected mice have different numbers of mature thymocytes. The results in Figure 4 are reported as picograms per milliliter per million SP cells.

We investigated the functional characteristics of peripheral T cells of OVA TCR transgenics on day 7 after intrathymic injection of OVA. As seen in Figure 5, splenocyte proliferation and IL-2 production after OVA stimulation were suppressed, whereas IFN-γ production was unchanged when compared with control HEL-injected mice. There was no evidence of deletion in the periphery; the total number of splenocytes, the number of CD4+ cells, and the percentage of CD4+ cells were all unchanged compared with HEL controls. Furthermore, there was no detectable staining with 7-AAD (data not shown). A time course study of splenocyte proliferation to OVA is shown in Figure 6; proliferation of splenocytes from animals injected intrathymically with OVA remains suppressed compared with that from animals injected intrathymically with HEL up to day 14 after intrathymic injection. Furthermore, the defects in proliferation and IL-2 production were not corrected by adding rIL-2 (5 U/ml) in the cultures (proliferation was 60–70% suppressed and IL-2 production was 50–70% suppressed compared with HEL over a range of Ag concentrations), as we have previously demonstrated for nontransgenic T cells (10).

Discussion

The thymus plays a major role in the development of self tolerance, but recently there has been a renewed interest in studying the role of the thymus in acquired tolerance. Over the past few years, several investigators published data indicating that injection of Ag into the thymus of adult animals leads to specific systemic tolerance in clinically relevant transplantation and autoimmune models (1–8). More recently, Rooke et al. published data on intrathymic injection of adenoviral vectors indicating the potential for such an approach for gene therapy (19). However, the exact mechanisms responsible for the induction and maintenance of tolerance after intrathymic injection of Ag remain unclear. Understanding these mechanisms is essential for the clinical development of this strategy in humans as a treatment for autoimmune disease or to prevent rejection in organ transplantation (20). Our report is the first demonstration that intrathymic injection of Ag leads to Ag-specific thymocyte apoptosis in TCR-transgenic mice. Systemic injection of the same dose of Ag does not induce thymocyte apoptosis. This confirms the uniqueness of the intrathymic approach demonstrated in transplant and autoimmune models (1–8). Our data are consistent with previous reports demonstrating that circulating whole protein Ag lead to deletion of CD4+ SP but not DP thymocytes (21), because only very high concentrations of “whole protein” can reach the thymic cortex through the blood-thymic barrier (22) or through a transcapsular route (23). The induction of CD4+ SP apoptosis by circulating protein occurs when the protein concentration in the serum reaches a threshold (21), while apoptosis of DP thymocytes occurs with systemic administration of “peptide” Ag (14). In the case of intrathymic injection of whole protein, we observed apoptosis in both populations of thymocytes, again indicating the uniqueness of the intrathymic approach. Oehen et al. used a pigeon cytochrome c TCR-transgenic mouse crossed with a transgenic mouse expressing pigeon cytochrome c in the thymus to study thymic selection, and showed apoptosis of DP and SP thymocytes (24). Additionally, unlike our model, their study showed decreased numbers of peripheral CD4+ cells associated with peripheral unresponsiveness (24). This different outcome (decrease in peripheral CD4+ T cells) is perhaps due to the presence of Ag in the thymus throughout development, while in our model the Ag is injected into a mature adult animal.

Our results also demonstrate that DP and SP thymocytes have different sensitivities to undergoing apoptosis after encounter with Ag presented by thymic APCs. DP cells start to become apoptotic earlier and a majority are ultimately deleted (14), whereas only a subset of SP thymocytes become apoptotic and do so later than DP.
thymocytes (21, 25). Furthermore, we show down-regulation of CD4R during apoptosis in SP cells. This was previously reported for DP (18) but not SP cells undergoing apoptosis.

Interestingly, peripheral T cells from intrathymically injected animals are anergic to stimulation with OVA, similar to our findings in nontransgenic BALB/c mice (10). Anergy of peripheral T cells is manifested by suppressed proliferation and decreased IL-2 production, but no effect on IFN-γ production was evident. This is consistent with the described hierarchy of suppression for different lymphokines when T cells are anergized; IL-2 production being most affected, IL-3 less affected, and IFN-γ the least affected (26, 27). In BALB/c mice, we showed the failure of peripheral Ag-specific T cell expansion after intrathymic injection of Ag (10). We also showed an increase in clonotype-positive T cells in the thymus after immunization, confirming that activated T cells circulate through the thymus. Furthermore, thymectomy after intrathymic injection abrogates the effect of acquired thymic tolerance and restores Ag-dependent clonal expansion in vivo (10). In the current study, we show that, similar to peripheral T cells, thymocytes from intrathymically tolerized transgenic animals are also anergic to stimulation with OVA. Interestingly, a small decrease in peripheral T cell proliferation (by 26%) can be seen as early as day 2 after intrathymic injection of OVA. This may be related to the exit of thymic dendritic cells or mature thymocytes that have been anergized in the thymus by exposure to Ag. The suppression of proliferation becomes maximal by day 7 (83%) after intrathymic injection of Ag, presumably as more peripheral T cells become anergic. These various lines of evidence demonstrate the pivotal role of the thymus in initiating and maintaining peripheral tolerance in this model.

In summary, intrathymic injection of Ag leads to tolerance of Ag-specific T cells. In TCR-transgenic mice, this tolerance is mediated by apoptosis of CD4+CD8+ and some CD4+ thymocytes within hours of intrathymic injection and by prolonged anergy of peripheral Ag-specific T cells in vivo. Peripheral T cell anergy may be mediated in part either by migration of anergic mature thymocytes that have in turn anergized other T cells by a process of infectious tolerance (28) or by circulation of peripheral T cells to the thymus, where they are anergized (10). Alternatively, thymic dendritic cells may migrate out of the thymus and anergize peripheral T cells (9).

These data provide new information on the mechanisms of acquired thymic tolerance and may help in development of novel strategies to induce tolerance in autoimmunity and transplantation.

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