Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3031–3036, April 1996
Immunology

Intravenous injection of soluble antigen induces thymic and peripheral T-cell apoptosis

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Contributed by Hugh O. McDevitt, August 31, 1995

ABSTRACT **The mechanism by which tolerance is induced via systemic administration of high doses of aqueous antigen has been analyzed by using mice transgenic for a T-cell receptor specific for the influenza virus hemagglutinin (HA) peptide comprising amino acids 126–138. After intravenous injection of 750 (but not 75) μg of HA peptide, a state of hyporesponsiveness was rapidly induced. In the thymus, *in situ* apoptosis in the cortex and at the corticomedullary junction was responsible for a synchronous and massive deletion of $CD4^+$ $CD8^+$ thymocytes. In secondary lymphoid organs, HA-reactive T cells were initially activated but were hyporesponsive at the single cell level. After 3 days, however, those cells were rapidly deleted, at least partially, through an apoptotic process. Therefore, both thymic and peripheral apoptosis, in addition to T-cell receptor desensitization, contribute to high-dose tolerance.**

T-cell tolerance to self antigens is acquired through both thymic and peripheral events. In the thymus, developing T cells are clonally deleted or, in some instances, anergized upon encountering their specific major histocompatibility complex (MHC)–self peptide ligands (1, 2). Nevertheless, some autoreactive T cells do enter the periphery and several mechanisms normally operate to prevent these autoreactive T cells from initiating an autoimmune disease. These mechanisms have been studied extensively in recent years, particularly in systems using mice transgenic for neo-self antigens expressed in a tissue-specific manner and/or using mice transgenic for the T-cell receptor (TCR). By using this technology, evidence for peripheral self tolerance through anergy (3), modulation of TCR and coreceptor molecule expression (2), clonal deletion (2), and clonal diversion (4) has emerged. Other studies have suggested that autoreactive T cells are ignorant to the self antigens expressed in the periphery unless a second stimulus is provided (5).

Tolerance to foreign antigens has also been documented. The mechanisms of T-cell tolerance to foreign antigens largely overlap those proposed for tolerance to self antigens. For example, if a virus is present in the neonatal thymus, developing T cells reactive to this microorganism are deleted leading to a tolerant state (6). *In vivo* studies have shown that mature T cells can also be rendered tolerant to foreign antigens through anergy, deletion, or both (7, 8). The route of administration, the dose of the antigen, the presence of adjuvant, as well as the type of antigen-presenting cells (APCs) targeted, are important factors determining whether tolerance or an immune response will be induced. Thus, systemic injection of a high dose of antigen induces a state of long-lasting, antigen-specific unresponsiveness (9). The mechanisms by which this unresponsiveness is established are still unclear, mainly because of the low frequency of a given antigen-specific T-cell population and the absence of appropriate markers to follow the fate of these cells.

TCR-transgenic mice have been used to circumvent these obstacles since a large fraction of their T cells share the same specificity, thereby allowing the consequences of such treatments to be experimentally determined (10–12). We have analyzed the effects of intravenous (i.v.) injection of antigen in mice transgenic for an $I\text{-}A^d$-restricted, influenza virus hemagglutinin (HA)-specific TCR. We observed thymic apoptosis following injection of the appropriate peptide. Furthermore, peripheral T-cell unresponsiveness to the HA peptide was the result of a combination of TCR desensitization and peripheral deletion.

MATERIALS AND METHODS

Mice. The HNT-TCR transgenic mice were generated as described (4). For this study, one line, in which ≈50 copies of the TCR α transgene and 40 copies of the TCR β transgene had integrated in the same chromosomal location, was backcrossed at least five times on the B10.D2 ($H\text{-}2^d$) background. This line expresses the Vβ8.3 transgene on more than 99% of TCR^{hi} thymocytes and peripheral T cells. Mice were bred at Stanford in the Research Animal Facility and kept under barrier conditions. Where indicated, mice were thymectomized at 4–6 weeks of age and used for experiments 3 weeks later.

Peptides. The HA peptide comprising amino acids 126–138 (HNTNGVTAACSHE) and the control $I\text{-}A^d$-binding ovalbumin peptide OVA 323–339 (ISQAVHAAHAEINEAGR) were synthesized by standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptides were purified on HPLC before use, and their structures were confirmed by amino acid composition analysis and mass spectrometry, which was provided by the University of California, San Francisco Mass Spectrometry Facility. The peptides were solubilized in phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/81 mM Na_2HPO_4/1.7 mM KH_2PO_4) and administered i.v.

Flow Cytometry. Single-cell suspensions from lymph nodes, spleen, and thymus were stained at 4°C in PBS containing 1% fetal calf serum and 0.02% NaN_3. Biotin-, fluorescein-, and phycoerythrin-conjugated monoclonal antibodies (mAbs) specific for mouse TCR Vβ8 (F23.1), CD4, CD8α, CD69, B220, interleukin 2 receptor α chain (IL-2Rα) (PharMingen), and CD3ε (Caltag, South San Francisco) were used for double or triple staining. Viable cells ($30–50 \times 10^3$ cells per sample), gated by exclusion of propidium iodide-positive cells, were analyzed on a Flasher flow cytometer. The intracellular calcium level of indo-1-labeled $CD4^+$ lymph-node T cells from

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor; HA, influenza virus hemagglutinin; OVA, ovalbumin; APCs, antigen-presenting cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling reaction.

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HNT-TCR mice stimulated with peptide-pulsed APCs from sex-matched B10.D2 mice was determined as described (4).

T-Cell Proliferation Experiments. Spleen-cell suspensions were treated with Tris-buffered ammonium chloride to eliminate the red blood cells. Cells (8×10^5 per well) were cultured in triplicate in 96-well flat-bottomed microtiter plates with RPMI medium 1640 supplemented with 5% (vol/vol) fetal calf serum, 10 mM Hepes buffer, 2 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. After 24–48 h, cultures were pulsed with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) and harvested 16 h later. SEMs were <15% of the mean.

***In Situ* Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick-End-Labeling (TUNEL) Reaction.** The TUNEL reaction was carried out on formalin-fixed tissue sections as described (13). After the final washes, a brief (20–30 sec) counterstaining with lissamine green (Sigma) was performed to stain cytoplasm.

RESULTS

i.v. Injection of HA Peptide Induces Thymocyte Apoptosis. To study aspects of antigen-specific, induced immune tolerance *in vivo*, we assessed the functional and phenotypic consequences of i.v. administration of a single dose of HA 126–138 or control OVA peptide on T cells from HNT-TCR mice at different time points following injection.

In contrast to mice receiving OVA peptide, a dramatic reduction (a factor of 5-10) in the number of thymocytes, primarily CD4$^+$ CD8$^+$ double-positive thymocytes, was observed in HNT-TCR mice as early as 24 h after injection of HA peptide (Fig. 1). Importantly, injection of 750 μg of HA peptide in nontransgenic animals or injection of 750 μg of OVA peptide in HNT-TCR transgenic mice had no effect on either thymocyte number or subset distribution (data not shown). One day after HA peptide injection, the TCR^{lo} thymocytes were deleted to a greater extent than the TCR^{hi} cells (data not shown). However, a progressive decrease in the number of TCR^{hi} thymocytes was then observed such that, by day 5, as little as 1.5% (0.4×10^6) TCR^{hi} cells could be detected in the thymus. At days 15 and 30, the size of the thymus, the distribution within the four major thymocyte subsets, and the level of TCR expression were similar to that of control HNT-TCR littermate mice (Fig. 1*D* and data not shown). The CD4$^-$ CD8$^-$ double-negative thymocytes were affected only minimally (Fig. 1). Only the 750-μg dose of HA peptide induced massive thymic deletion. A dose of 75 μg of HA peptide only minimally affected thymocyte numbers and CD4, CD8, and Vβ8 expression, whereas a dose of 7.5 μg had no effect (data not shown).

To determine whether intrathymic apoptotic cell death was responsible for the deletion of the thymocytes, we utilized the TUNEL reaction to assess cellular apoptosis. As shown in Fig. 2 *B* and *C*, thymic apoptosis was detected as early as 6 h after i.v. injection of HA peptide, peaked at 12 h, and was essentially complete by 24 h. The majority of cells in the cortical areas of the thymus showed evidence of apoptosis, with only a few cells in the medulla appearing to stain positively, resulting in severe thymic cortex atrophy by 24 h. The apoptotic cells were frequently seen to be grouped as clusters found throughout the thymic cortex, although a preference for the corticomedullary junction was noted (Fig. 2*B*). These results demonstrate that deletion of immature cortical thymocytes induced by i.v. injection of peptide occurs through apoptosis. Interestingly, control HNT-TCR transgenic mice, as well as unmanipulated nontransgenic mice, also harbor some apoptotic cells in their thymus (Fig. 2*A*).

i.v. Injection of HA Peptide Induces Transient Peripheral T-Cell Activation and Long-Lasting Unresponsiveness. HNT-TCR mice receiving an injection of 750 μg of HA exhibited, on

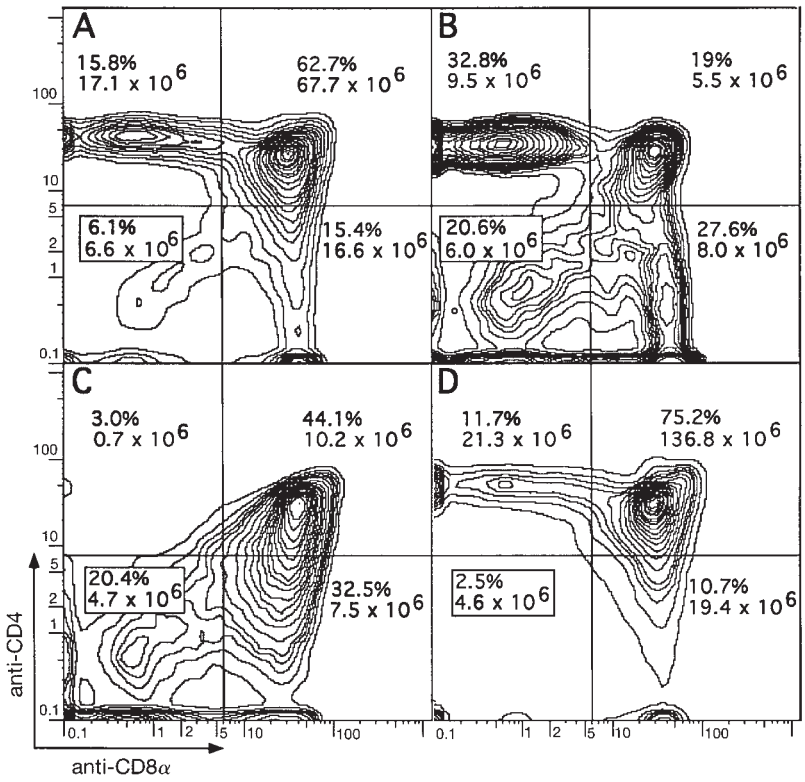

FIG. 1. The effects of i.v. injection of HA peptide on thymocyte numbers and distribution. Thymocytes isolated from HNT-TCR mice receiving a single i.v. injection of either 750 μg of OVA 323–339 (*A*) or 750 μg of HA 126–138 (*B, C*, and *D*) were analyzed by flow cytometry for CD4 and CD8 expression on day 1 (*A* and *B*), day 5 (*C*), or day 15 (*D*) following injection. The percentages and cell numbers represent the proportion and absolute number of thymocytes found in each of the four quadrants, respectively. Similar results were obtained in three other experiments.

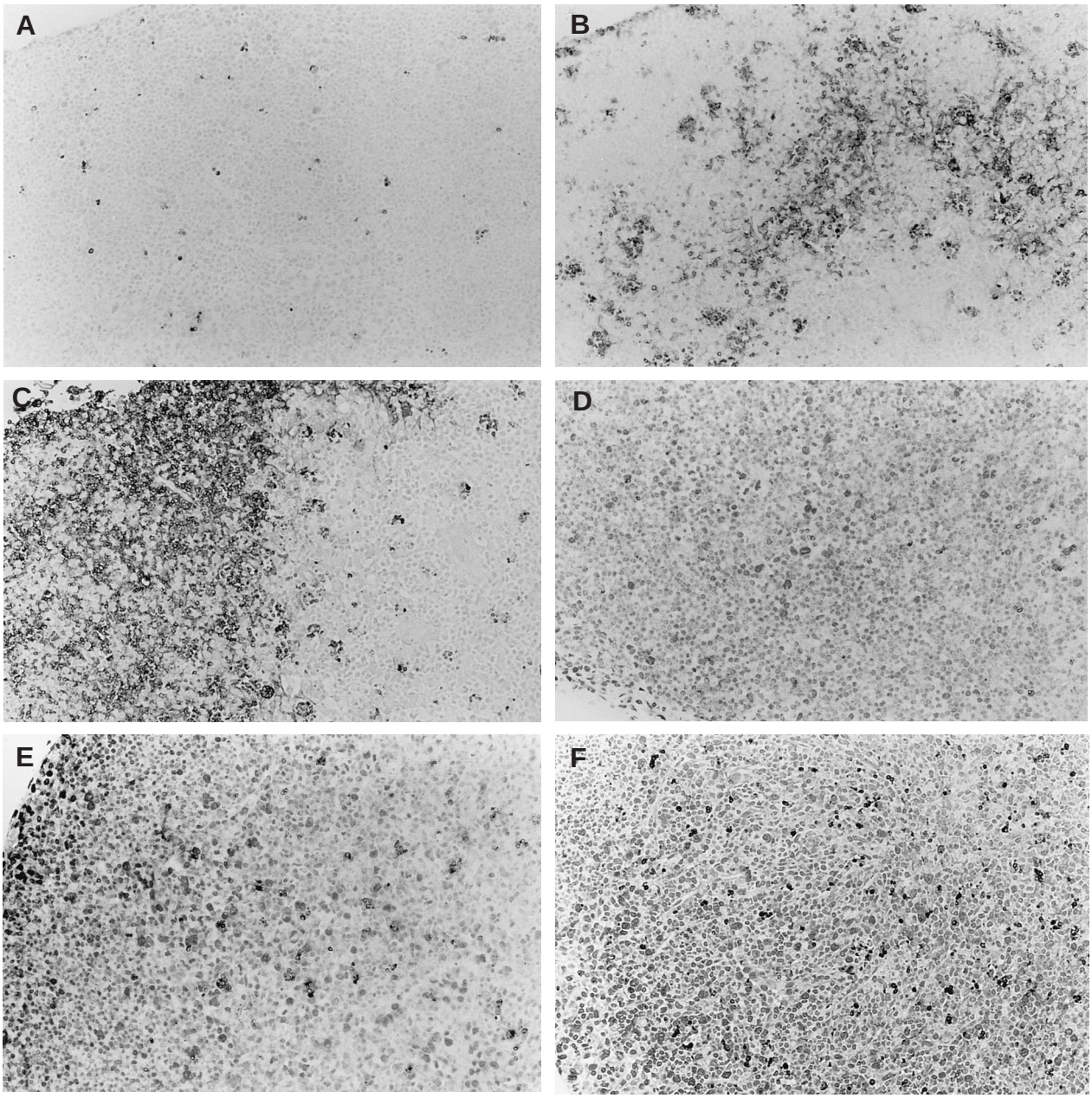

FIG. 2. Thymic and peripheral T-cell apoptosis in HNT-TCR transgenic mice following i.v. injection of HA peptide. Thymi (*A, B*, and *C*) or lymph nodes (*D, E*, and *F*) from HNT-TCR transgenic mice injected i.v. with 750 μg of OVA 323–339 (*A* and *D*) or injected i.v. once (*B, C*, and *E*) or twice (*F*) with 750 μg of HA 126–138 were harvested 6 h (*B*), 12 h (*A* and *C*), or 36 h (*D, E*, and *F*) after injection. The TUNEL staining reaction was performed as described (13). Staining of the nicked DNA in apoptotic cells appears dark. (×35.)

day 1, activation of $CD4^+$ peripheral T cells on the basis of three criteria. First, peripheral $CD4^+$ T cells had an increased cell size, as determined by forward light scatter (Fig. 3*B*). Second, as a consequence of the entry of $CD4^+$ T cells into cell cycle, the absolute number of $CD4^+$ T cells in the spleen of HA-injected mice was significantly increased at day 1 (see Fig. 5). Third, on day 1, surface expression of CD4, IL-2R (data not shown), and CD69 (Fig. 3*D*) was increased on $CD4^+$ T cells. The level of TCR expression remained unchanged (data not shown). HNT-TCR mice injected i.v. with OVA peptide showed no such T cell activation (Fig. 3 *A* and *C*). By day 3, the size of the cells and expression of these surface molecules were similar to that of controls indicating that the observed T-cell activation is transient.

To assess whether peripheral T cells were functionally impaired by i.v. injection of HA peptide, T-cell proliferation experiments were performed using splenocytes from peptide-injected animals. Mice injected with 750 μg of HA peptide exhibited up to a 95% decrease in T-cell proliferative responses to HA peptide *in vitro* compared with OVA-injected control mice (Fig. 4). Furthermore, in time course experiments, responses to HA peptide were decreased for at least 30 days, although by day 30 the effect was only marginal (Fig. 4 and Table 1). In addition, in spite of the increased number of $CD4^+$ $V\beta8^+$ T cells observed on day 1, peripheral T cells exhibited significantly reduced proliferative responses to both HA peptide and anti-CD3. At later time points, the responsiveness to anti-CD3 mAb was progressively restored, while the response to HA remained significantly reduced (Fig. 4 and data not shown). Finally, responses to HA peptide were enhanced by addition of exogenous IL-2 to cultures from HA-injected mice at day 1 (Fig. 4), suggesting that anergy was the major cause of the state of unresponsiveness at this time point. Injection of lower doses of HA peptide had minimal (75 μg) or no (7.5 μg) effect (data not shown).

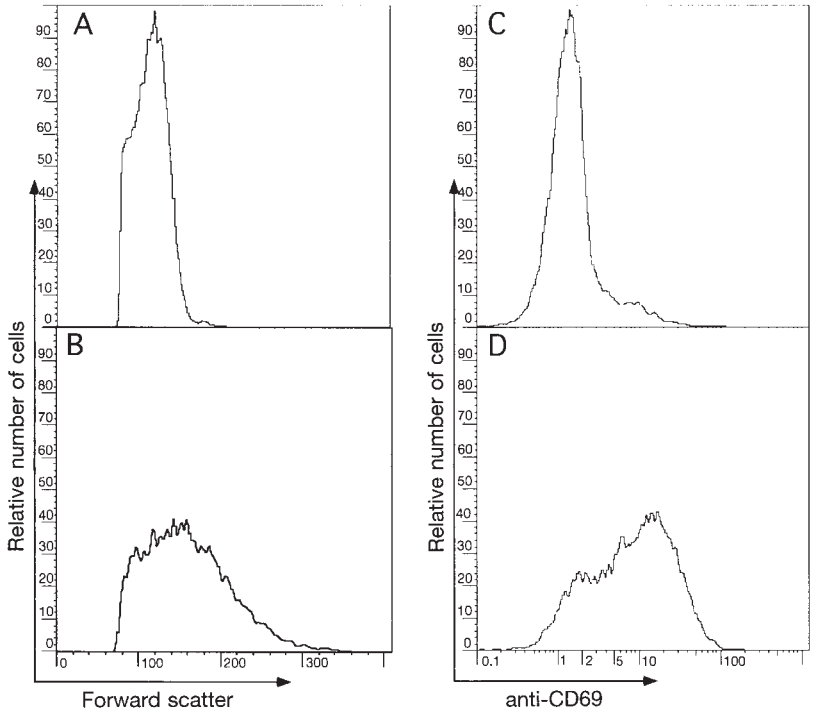

FIG. 3. Transient activation of peripheral CD4$^+$ T cells in HA peptide-injected HNT-TCR mice. Data were obtained after gating for CD4$^+$ splenic T cells. Cell size, as measured by forward light scatter (*A* and *B*), and CD69 (*C* and *D*) expression from mice injected 1 day earlier with 750 μg of either OVA 323–339 (*A* and *C*) or HA 126–138 (*B* and *D*) are displayed. Similar results were obtained in two to five other experiments.

To address whether the hyporesponsiveness was the result of TCR uncoupling, we assessed calcium flux *ex vivo* in lymph node cells from HA- and OVA-injected mice. The frequency of T cells mobilizing calcium in the presence of APCs and HA peptide was dramatically reduced in mice receiving 750 μg of HA peptide (Table 1). The mean intracellular calcium in the remaining responding T cells was also markedly reduced (Table 1).

Deletion and Apoptosis in Peripheral T Cells Induced by i.v. Injection of HA Peptide. Following the initial increase in the number of CD4$^+$ Vβ8$^+$ splenic T cells after injection of HA peptide, this number sharply decreased, and by day 15 it was lower by a factor of 3 than on day 1 and by a factor of 2 than in the OVA-injected mice (Fig. 5). A similar decline of CD4$^+$ Vβ8$^+$ T cells was observed in the lymph nodes (data not shown). The CD4$^+$ Vβ8$^+$ T cells may either die or home to tissues other than the spleen or lymph node after recognition of the HA peptide. Therefore, we evaluated tissue sections of lymph nodes from peptide-injected mice by the TUNEL reaction. A clear increase in the frequency of apoptotic cells in sections from HA-injected mice relative to OVA-injected mice was observed 36 h after injection (Fig. 2 *D–F*). Their number further increased following multiple HA peptide injections (Fig. 2*F*). Apoptotic cells were localized in the T-cell-rich paracortical areas and were also detected in the spleen, albeit at a lower frequency than seen in lymph nodes. No increase in the number of apoptotic cells was observed in the liver of HA-injected mice (data not shown). To assess whether peripheral T-cell tolerance and apoptosis was thymus dependent, thymectomized mice were injected with HA peptide. A similar pattern of unresponsiveness and temporal deletion of mature T cells was observed in thymectomized animals (data not shown). Importantly, lymph nodes from thymectomized HA-injected mice also exhibited increased apoptosis (data not shown). This indicates that the observed peripheral T cell apoptosis is not the result of leakage from the thymus or due to recent immature thymic emigrants.

DISCUSSION

The studies presented here were designed to define the mechanisms of unresponsiveness following i.v. administration of antigenic peptide. A single injection of peptide resulted in a synchronous and massive deletion involving ≈90% of thymocytes, suggesting that the vast majority of thymocytes expressed the TCR α and β transgenes. The double-positive TCR^{lo} thymocytes were the most sensitive to negative selection, with absolute numbers declining greater than a factor of 50, as previously reported (1). Coincident with the marked deletion of thymocytes was a dramatic increase in apoptotic cells in the thymus occuring within 6 h of antigenic peptide

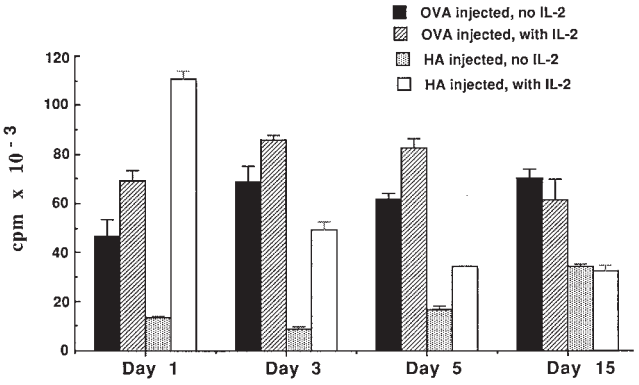

FIG. 4. Proliferation of splenic T cells from HA peptide-injected HNT-TCR mice is initially restored by addition of exogenous IL-2. Proliferation of spleen cells from OVA 323–339- or HA 126–138-injected HNT-TCR mice (750 μg of peptide per mouse) in response to 2 μg of HA 126–138 per ml was assessed in the presence or absence of 10 units of recombinant murine IL-2 per ml. Mean cpm without antigen varied from 1562 to 9204 cpm in the presence of recombinant IL-2, except at day 1 for cultures established from the HA 126–138-injected mouse, where it was 48,736. This is one representative experiment of a total of three.

Table 1. T-cell hyporesponsiveness induced by i.v. injection of HA peptide in HNT-TCR mice

Day after injection	$CD4^+$ T cells with intracellular Ca^{2+} >300 nM, %			
	T cells from OVA-injected mice stimulated with		T cells from HA-injected mice stimulated with	
	APCs* + OVA	APCs + HA	APCs + OVA	APCs + HA
1	1.8	55.4 (1175 nM)†	3.8	10.7 (660 nM)
3	3.9	54.2 (1650 nM)	5.7	23.7 (840 nM)
5	3.9	60.6 (1270 nM)	4.7	25.5 (540 nM)
15	4.9	66.3 (1080 nM)	5.3	29.9 (660 nM)
30	5.1	62.9 (1850 nM)	5.0	43.2 (1120 nM)

HNT-TCR mice were injected with 750 μg of either HA or OVA peptide and sacrificed at the indicated time point. Lymph node cells were used as responding cells after gating on $CD8^-$ and $B220^-$ cells. Each result is from one representative mouse from a minimum of four.
*APCs are spleen cells from normal B10.D2 mice pulsed with the indicated peptide.
†Mean intracellular calcium level in the cells that responded with $[Ca^{2+}]_i$ >300 nM.

administration and lasting ≈24 h. Similar antigen-mediated, *in situ* thymic apoptosis has been observed in another TCR transgenic system (14). The TUNEL reaction used here appears sensitive and provides anatomical information regarding the site of apoptosis. In agreement with the preferential deletion of double-positive thymocytes, apoptosis occurred primarily in the thymic cortex and at the corticomedullary junction in HA-injected mice. Interestingly, thymi from unmanipulated nontransgenic mice exhibited levels of apoptosis that, albeit low, did exceed those present in secondary lymphoid tissues. Similar results were published by Surh and Sprent (15). This apparent low degree of apoptosis, as compared with the high percentage of dying thymocytes, could be explained by rapid clearance of dying thymocytes by local $F4/80^+$ macrophages (15). Indeed, 24 h after injection of HA peptide in HNT-TCR mice, very few apoptotic cells remained in the thymus.

Following antigenic peptide injection, thymic deletion was consistently accompanied by peripheral T-cell activation. This suggests that in our *in vivo* model system, the thresholds for thymic deletion and for activation of peripheral T cells appear not to differ greatly (16). Similar observations were made in mice transgenic for an influenza nucleoprotein-specific class I-restricted TCR (17).

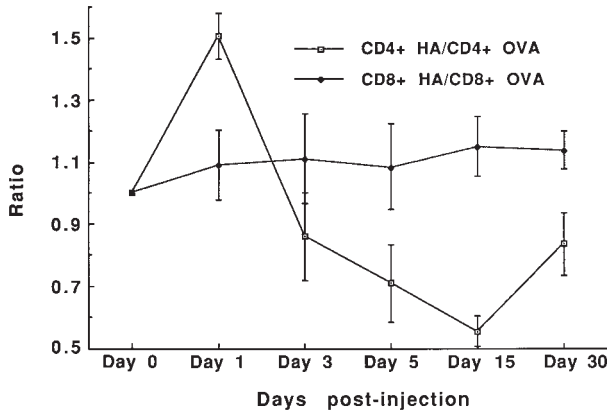

FIG. 5. Progressive depletion of peripheral $CD4^+$ T cells in HA peptide-injected HNT-TCR mice. The ratio of the number of $CD4^+$ and $CD8^+$ splenic T cells in HA- vs. OVA-injected HNT-TCR mice is displayed at various time points after the i.v. injection. Each value represents the mean ± SEM of four to eight mice for each time point in each treatment group.

Within 24 h after i.v. HA peptide injection, the mature $CD4^+$ T cells from HNT-TCR mice entered a refractory state to *in vitro* stimulation through the TCR, which was not due to downregulation of TCR or coreceptor molecules. It is likely that *in vivo* T-cell activation led to TCR uncoupling, resulting in a reduced capacity to mobilize Ca^{2+} and to proliferate (18). However, these T cells did respond vigorously upon addition of IL-2. Several factors may be associated with this unresponsive state. A high number of MHC–peptide complexes at the surface of APCs throughout the body after a high-dose i.v. injection may lead to intense, prolonged or repetitive stimulation of specific T cells culminating in anergy and deletion. In addition, the type of APCs could also be critical in determining the fate of the responding T cells. For example, resting B cells, which are unable to provide necessary costimulatory signals for efficient T-cell activation (19), far outnumber other MHC class II-expressing cells and would be targeted preferentially by a high dose of antigen, leading to tolerance.

In our system, high-dose antigen injection induced a transient expansion in the number of peripheral T cells, limited both in cell number and duration, followed by a rapid decline of $CD4^+$ $V\beta8^+$ T cells. This confirms that peripheral T-cell deletion is not necessarily preceded by massive expansion (20). A similar expansion/contraction of antigen-specific peripheral T cells has been noted following stimulation with superantigens (7, 21), alloantigens (22), male antigen (8), and conventional antigens (10–12, 23). $CD4^+$, as well as $CD8^+$ and $\gamma\delta$ T-cell subsets, follows this sequence of events (8, 11, 22). Following injection of large doses of antigen, specific T cells can be completely eliminated, leaving no detectable memory T cells (23). In several systems, including our own, the deletion is not complete, and some antigen-specific T cells do survive (8, 12, 22). Although these T cells are initially functionally impaired, their reactivity can be recovered in the absence of antigen (24). This expansion/contraction scheme is believed to represent a homeostatic mechanism regulating the intensity of an immune response and preventing the unrestricted growth of a particular clonotype under potent or chronic antigenic stimulation (25).

It has been suggested for some time that most activated T cells die in peripheral lymphoid tissues. Indeed, apoptosis of peripheral T cells has been observed *in vitro* after administration of super- and alloantigens (21, 22). We now provide *in situ* evidence for peripheral T-cell apoptosis after injection of a conventional antigen. However, a precise quantitative determination of the contribution made by apoptosis to peripheral deletion cannot be made by using the TUNEL reaction. Recent evidence suggests that the Fas/FasL system plays an important role in activation-induced cell death (AICD) of mature T cells (26). Fas-mediated signals could, alone or in association with other death-inducing signals, contribute to the peripheral $CD4^+$ T-cell apoptosis obtained in our system.

Following peptide treatment, some peripheral $CD4^+$ T cells continued to exhibit low intracellular calcium levels upon HA peptide stimulation, which may reflect an anergic state or low level of transgenic TCR α chain expression. This raises the question of whether the dying T cells belong to a population that is distinct from that which is anergized. We favor the idea that anergy and deletion are distinct stages in a continuing process. Thus, following antigen stimulation, T cells may either proliferate and secrete cytokines or become anergic depending on the intensity of the stimulation and the presence of costimulatory signals. If activated T cells are stimulated repeatedly through the TCR, a program leading to cell death is induced (27). Indeed, entry into the cell cycle is required for AICD (28). The fact that antigen doses which promote AICD

in vitro are higher than those inducing proliferation (25) and that *in vivo* T-cell deletion/apoptosis is more significant after high-dose or multiple antigen injections (refs. 11 and 23 and this report) would support such a model.

The mechanism of high-dose immune tolerance has remained unclear. Some investigators have suggested that high-dose administration of aqueous protein preferentially induces tolerance in T helper (Th)1-like cells while activating the Th2-like cells to secrete regulatory cytokines such as IL-4 and IL-10 (29). In our system, no evidence for the induction of active suppression was obtained (unpublished data). The observed hyporesponsive state was due to a combination of T-cell anergy and deletion.

Antigen-specific immunotherapy has great potential for the prevention or treatment of allograft rejection and allergic and autoimmune diseases. Indeed, such an approach has been used to prevent or treat a number of induced as well as spontaneous experimental autoimmune diseases (ref. 11; R.T., R.S.L., X.-d.Y., and H.O.M., unpublished results). The dose of antigen required for tolerance induction may depend in part on its stability, its affinity for the MHC molecule, and the TCR affinity for the MHC–peptide complex. To render such an approach clinically applicable to human autoimmune diseases, such as type 1 diabetes and multiple sclerosis, understanding the nature of the interaction between self peptide, MHC, and autoreactive TCR will be essential.

We would like to thank Drs. Andrew Cope, Mark Davis, and Cecelia Pearson for advice, and Peggy Sullivan, Sharon Phillips, and Mary Vadeboncoeur for expert technical assistance. R.S.L., R.T., and X.-d.Y. were supported by Institut National de la Santé et de la Recherche Médicale and Juvenile Diabetes Foundation International, National Institutes of Health, and American Diabetes Association fellowships, respectively. This work was supported by grants from the National Institutes of Health.

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