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Distinct Requirements for Deletion versus Anergy during CD8 T Cell Peripheral Tolerance In Vivo

William L. Redmond,2 Boris C. Marincek, and Linda A. Sherman3

Activation of naive T cells by quiescent APCs results in tolerance through deletion and anergy. The underlying basis for these distinct fates is unclear. Using clone 4 TCR transgenic animals as a source of naive CD8 T cells, we examined the requirements for peripheral deletion in vivo. Our results demonstrate that independent of the amount of Ag used for stimulation, a single dose was insufficient to achieve complete clonal deletion. Instead, further antigenic exposure was required to completely eliminate all of the activated T cells. Additionally, consecutive stimulations with low doses of Ag were highly effective in promoting deletion. In contrast, although stimulation with high doses of Ag initially led to the apoptosis of many of the activated T cells, it induced hyporesponsiveness in a portion of the responding cells, thereby sparing them from further activation and deletion. These data explain why some conditions promote tolerance through clonal deletion whereas others promote anergy. Furthermore, these data provide a framework to devise protocols for effective deletion of potentially autoreactive T cells. The Journal of Immunology, 2005, 174: 2046–2053.

During maturation in the thymus, most T cells with high avidity for self-peptide-MHC complexes are eliminated (1, 2). However, because not all self-Ags are expressed in the thymus under conditions required to induce such deletion, additional mechanisms exist to ensure T cell tolerance in the periphery, including clonal deletion, anergy, and immunoregulation (3, 4). Several groups have demonstrated that tolerance of CD8 T cells occurs in the periphery following TCR-mediated recognition of cognate peptide-MHC class I complexes on quiescent APCs that do not express high levels of costimulatory molecules. Activation under such conditions leads to an abortive proliferative response and tolerance (5–8). However, it remains unresolved as to whether such tolerance is due to clonal deletion or anergy.

We previously addressed the question of whether a single exposure to Ag presented by quiescent APCs was sufficient to effect the clonal elimination of naive CD8 T cells. Surprisingly, we found that a significant number of cells could survive following their initial exposure to Ag and that activation with quiescent APCs did not result in complete deletion (9). These data suggested that during tolerance induction, deletion of naive CD8 T cells was not programmed during the initial stimulatory event and that additional exposure to Ag was required to achieve complete clonal deletion. However, several groups have demonstrated that chronic Ag exposure leads to the induction of hyporesponsiveness or anergy in vivo (10–18). Maintenance of the anergic state typically requires the continuous presence of Ag, as T cells can regain their ability to respond to Ag once they are removed from the chronic antigenic milieu (10, 12, 19, 20).

The underlying mechanism that determines whether tolerogenic exposure to Ag results in either deletion or anergy is not understood. Nor is it known how the dynamics of Ag presentation affect subsequent CD8 T cell susceptibility to deletion. In the current study, we sought to investigate the requirements for CD8 T cell clonal deletion in vivo and to contrast these with the requirements for anergy induction. Our results indicate that, beyond the minimal requirement for T cell activation, the concentration of Ag present during the initial stimulatory event did not significantly affect the number of cells that ultimately survived. However, Ag concentration was determinative of the fate of the remaining cells. High dose Ag rendered the remaining cells anergic to further stimulation, thereby sparing them from further activation and deletion. In contrast, treatment with lower doses of Ag did not induce anergy and the residual cells remained susceptible to further activation, which ultimately led to their complete clonal deletion. This disparity in susceptibility to deletion was associated with changes in the regulation of TCR-mediated signaling molecules. Taken together, these data demonstrate that providing repeated or continuous exposure to relatively low doses of Ag may be a more effective means of completely purging the repertoire of CD8 T cells specific for a particular Ag.

Materials and Methods

Mice

BALB/c mice were purchased from the breeding colony of The Scripps Research Institute (La Jolla, CA). Insulin promoter expressing hemagglutinin (HA)3 transgenic mice (InsHA), homozygous for the HA gene, clone 4 TCR transgenic mice, and influenza HA-specific T cells (HNT) TCR transgenic mice were generated and characterized as previously described (6, 21, 22). Clone 4 mice were backcrossed with BALB/c mice for at least 10 generations and were then crossed with BALB/c Thy1.1 mice for two generations to obtain clone 4 TCR mice homozygous for Thy1.1. All mice were bred and maintained under specific pathogen-free conditions in The Scripps Research Institute animal facility. Experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3 Abbreviation used in this paper: HA, influenza virus hemagglutinin.
Purification of naive TCR transgenic T cells

Single cell suspensions were prepared from the lymph nodes of clone 4 Thy1.1 TCR transgenic mice. Cell suspensions were depleted of CD4+, CD11b+, CD45R, DX5, and Ter-119+ cells using the MACS CD8+ T cell isolation kit (Miltenyi Biotec). Clone 4 Thy1.1 T cells were purified by negative selection per the manufacturer’s instructions and had a naive phenotype (CD44+/low, CD62L+ high, and CD69-negative) as indicated by flow cytometry using FITC-conjugated Abs (BD Pharmingen) (unpublished observation). Purified clone 4 CD8 Thy1.1 T cells were washed once in cold HBSS and then resuspended at 5 x 10^6 cells/ml in HBSS. HNT CD4+ T cells were prepared as described earlier and then purified using the MACS CD4+ T cell isolation kit (Miltenyi Biotec). Purified HNT CD4+ T cells were washed in HBSS and then 4 x 10^6 cells were injected i.v. in 200 μl of HBSS into recipient mice.

CFSE labeling of naive clone 4 T cells

A total of 2 μl of a 5-mM solution of CFSE (Molecular Probes) in DMSO (Sigma-Aldrich) was added per 5 x 10^6 cells/ml in HBSS and incubated for 10 min at 37°C. Cells were then washed once with ice-cold HBSS. A total of 3 x 10^6 CFSE-labeled clone 4 T cells were injected i.v. in 200 μl of HBSS.

Activation of clone 4 T cells

After adoptive transfer, clone 4 Thy1.1 T cells were activated under the following conditions. For viral priming, 500 HA units of influenza virus strain A/FPR/34/ H1N1 that was grown in the allantoic cavity of 10- to 11-day-old hen eggs was injected i.p. into mice. HA peptide (H5HYS-TVASSL) was synthesized by the core facility of The Scripps Research Institute using an Applied Biosystems model 430A synthesizer. Purity was >85%, as determined by mass spectrometry and reversed-phase HPLC analysis on a Vydac C18 column. Peptide was given by i.v. injection of 100, 10, 1, or 0.1 μg of K^a HA peptide in 100 μl of HBSS.

Exogenous IL-2 treatment

A total of 5000 U of recombinant human IL-2 (obtained from the National Cancer Institute, Frederick, MD) was injected i.p. in 100 μl of HBSS into recipient mice on days 3 and 6 following viral priming.

Flow cytometry

Spleens were harvested and processed to obtain single cell suspensions. Ammonium chloride lysis buffer was added for 5 min at room temperature to lyse RBC. Cells were then rinsed with RPMI 1640 medium (Invitrogen Life Technologies) containing 10% heat-inactivated FCS (Gemini Bio-Products). Cells were incubated for 30 min on ice with the following Abs: anti-Thy1.2 FITC, anti-Thy1.1 PE, anti-CD8 PerCP, biotinylated anti-CD25, biotinylated anti-CD69, anti-Vα2+ biotin, anti-Vβ8.1 PE, or streptavidin-allophycocyanin. All mAbs and secondary reagents used for FACS analysis were purchased from BD Pharmingen. After washing three times with HBSS containing 0.1% w/v BSA (Sigma-Aldrich) and 0.02% w/v sodium azide, cells were analyzed with a FACS Calibur and CellQuest software (Becton Dickinson).

For detection of Erk phosphorylation, splenocytes were harvested and processed as described, then restimulated in vitro with no peptide, 1 μg/ml K^a HA peptide, or PMA (200 ng/ml) and ionomycin (12.5 μg/ml) in RPMI 1640 medium containing 10% heat-inactivated FCS for 5 min at 37°C. Immediately following Ag restimulation, cells were fixed in fresh 2% paraformaldehyde for 20 min at 4°C, then permeabilized with ice-cold 90% methanol for 1 h at 4°C. After washing, cells were stained with Thy1.1-allophycocyanin and CD8-TriColor (Caltag Laboratories) for 30 min at 4°C. After washing, phospho-Erk1/2-PE was added (BD Pharmingen) and cells stained for 1 h at room temperature. Cells were washed twice with HBSS containing 0.1% w/v BSA (Sigma-Aldrich) and 0.02% w/v sodium azide, then immediately analyzed with a FACS Calibur and CellQuest software (Becton Dickinson).

Results

Kinetics of Ag presentation in vivo

Our previous studies indicated that a single exposure to Ag was not sufficient to achieve complete clonal elimination of naive CD8 T cells activated by quiescent APCs. Therefore, we initiated the current studies to determine the antigenic requirements to achieve complete clonal deletion in vivo using naive CD8 T cells derived from clone 4 TCR Tg mice (clone 4 T cells) that are specific for a K^a-restricted peptide from the influenza HA (21). Because stimulation is a prerequisite for deletion, it was first necessary to assess the pharmacokinetics of peptide stimulation of the clone 4 T cells.

To this end, 100, 10, 1, or 0.1 μg of soluble HA peptide was injected i.v. into BALB/c mice at various time points (18, 9, 3, and 0 days) before the addition of 3 x 10^6 naive CFSE-labeled clone 4 T cells. Four days later, spleens were harvested and the percentage of activated clone 4 T cells, based on CFSE dilution, was measured by flow cytometry. As shown in Fig. 1A, 100 μg of HA peptide induced a potent proliferative response, whereas the lower doses stimulated fewer naive clone 4 T cells with the lowest dose, 0.1 μg of HA peptide, inducing only a limited proliferative response. Lower doses of HA peptide (<0.1 μg) were unable to induce the proliferation of a significant proportion of the clone 4 T cells (data not shown). To quantitate the kinetics of Ag elimination in vivo, we examined the percentage of naive clone 4 T cells activated by the remaining HA peptide available at each time point. After 18 days, the amount of Ag remaining in mice that initially received 100 or 10 μg of HA peptide was sufficient to activate ~85 or 50%, respectively, of the 3 x 10^6 CFSE-labeled naive clone 4 T cells (Fig. 1B). In contrast, the amount of Ag remaining in mice that initially received 0.1 and 1 μg of HA peptide was insufficient to stimulate a significant portion of newly transferred naive clone 4 T cells after ~6 and 9 days, respectively (Fig. 1B).

Phenotype of peptide-tolerized clone 4 T cells in vivo

Next, we compared the expression of several activation markers on the clone 4 T cells activated in vivo with the different doses of
soluble peptide. As before, $3 \times 10^6$ naive CFSE-labeled clone 4 T cells were adoptively transferred into BALB/c mice along with 100, 10, 1, or 0.1 $\mu$g of HA peptide. Four days later, mice were sacrificed and spleen cells were harvested to assess the expression of CD25 and CD69 on the activated clone 4 T cells by flow cytometry.

None of the cells exhibited up-regulation of CD25 (Fig. 2A), which is in agreement with previous results demonstrating a lack of CD25 up-regulation at this time point during peptide-mediated tolerance induction (8, 23). Activation with 100 $\mu$g of HA peptide led to increased expression of CD69 (Fig. 2A), reflecting their continuous exposure to HA peptide, which was consistent with the presence of persistent Ag observed in vivo (Fig. 1, A and B). Lower doses (10, 1, or 0.1 $\mu$g) of HA peptide induced less CD69 expression, which correlated with the reduced half-life of these peptides in vivo (Figs. 2A and 1B).

Another measure of T cell activation is down-regulation of TCR expression, which occurs as a consequence of internalization of TCR molecules (24). We observed a slight decrease in TCR expression on clone 4 T cells following activation with 100 or 10 $\mu$g, but not with 1 or 0.1 $\mu$g of HA peptide (Fig. 2B). Multiple treatments with high doses of HA peptide did not lead to additional TCR down-regulation (data not shown).

**Deletion vs anergy of peptide tolerized clone 4 T cells in vivo**

Before we could determine whether exposure to HA peptide could lead to CD8 T cell deletion, we first needed to ensure that all of the transferred clone 4 T cells were successfully activated by the tolerogenic peptide treatment in vivo. Small numbers ($1 \times 10^5$) of CFSE-labeled clone 4 T cells were transferred into BALB/c mice with varying concentrations of HA peptide. At this reduced cell number, nearly all clone 4 T cells became activated, even at the lowest Ag dose (Fig. 2C). To further assure complete activation of all clone 4 T cells, we decided to further reduce the number of transferred cells in subsequent experiments by an additional 10-fold.

To test the susceptibility of clone 4 T cells to deletion, $1 \times 10^4$ naive clone 4 T cells were transferred into BALB/c mice with 100, 10, 1, 0.1, or 0 $\mu$g of HA peptide. Three weeks later, recipient mice were immunized with influenza virus to induce expansion of the residual clone 4 T cells. Viral priming was necessary to promote sufficient expansion of the donor cells for detection by flow cytometry. Eight days after viral priming, spleens were harvested and the number of donor clone 4 T cells was quantitated by flow cytometry.

Transfer of naive clone 4 T cells into BALB/c mice followed by viral priming led to the accumulation of a large number of activated clone 4 T cells (Fig. 3A). In contrast, treatment with 100 $\mu$g of HA peptide 3 wk before viral priming led to the elimination of a significant proportion of the clone 4 T cells (Fig. 3A). These results are consistent with previous reports demonstrating that in the absence of proinflammatory signals, activation of naive CD8 T cells with soluble peptide can lead to significant levels of deletion (9, 13, 17, 18, 25, 26). Tolerance induction with 10, 1, and 0.1 $\mu$g doses of HA peptide also led to comparable deletion of the naive clone 4 T cells (Fig. 3A). Importantly, regardless of the dose of peptide Ag used to induce tolerance, some responsive cells remained indicating that complete clonal deletion could not be achieved with a single exposure to Ag.

To determine whether complete deletion could be achieved by providing multiple exposures to the soluble HA peptide, the experiment was performed again, except this time each group of mice received the indicated dose of peptide on three consecutive days. Three weeks later, recipient mice were immunized with influenza virus to induce expansion of the remaining clone 4 T cells. Eight days later, spleens were harvested and the number of donor clone 4 T cells was quantitated by flow cytometry. Surprisingly, treatment with multiple vs a single dose of 100 $\mu$g of HA peptide did not lead to increased deletion (Fig. 3, A and B), suggesting that the cells were refractory to further antigenic stimulation. A similar lack of responsiveness to antigenic signaling following the initial stimulatory period was shown recently for effector CD8 T cells, as they remained hyporesponsive for 3–4 days following activation (27, 28). In contrast to the incomplete deletion observed using a high dose of HA peptide, multiple treatments with lower doses (1 or 0.1 $\mu$g) of HA peptide led to substantially more efficient clone

**FIGURE 2.** Phenotype of peptide tolerized clone 4 T cells. A, A total of $3 \times 10^6$ CFSE-labeled clone 4 T cells were injected into BALB/c mice along with 100, 10, 1, or 0.1 $\mu$g of HA peptide on day 0. Four days later, spleens were harvested and the expression of CD25, CD69, or TCR (V$\beta$8.1) by the donor clone 4 T cells was determined by flow cytometry. For CD25 and CD69, data indicate the percentage of activated clone 4 T cells expressing the indicated marker. B, Clone 4 TCR V$\beta$8.1 expression is shown as naive clone 4 T cells (N, shaded histogram), activated clone 4 T cells (A, dark line histogram). Data indicate the mean fluorescence intensity of each population. C, A total of $1 \times 10^6$ CFSE-labeled clone 4 T cells were injected into BALB/c mice along with the indicated amounts of HA peptide on day 0. Four days later, spleens were harvested and the percentage of activated clone 4 T cells (based on CFSE dilution) was measured by flow cytometry. Data are representative of one of two independent experiments with similar results.
4 T cell deletion (Fig. 3B). These data suggested the possibility that activation of naive CD8 T cells by high doses of persistent Ag leads to partial deletion and induces hyporesponsiveness in the remaining cells, such that they resist further activation and deletion. In contrast, persistent activation with low doses of peptide allows the cells to remain responsive to Ag and exclusively promotes deletion.

Requirements for inducing complete clonal deletion of naive clone 4 T cells in vivo

It may be argued that the reason a single dose of peptide was insufficient to affect complete deletion was because at all Ag doses, there remained a few clone 4 T cells that were unresponsive to peptide Ag. Such cells would represent a population that could later respond to virus. To exclude this possibility, we used an adoptive transfer system that has been previously described (9). Naive CFSE-labeled clone 4 T cells were activated with HA peptide in vivo and after 4 days, the mice were sacrificed, spleen cells were sorted by flow cytometry, and the activated (dividing) cells that had diluted their CFSE-label were collected (Fig. 1A). A total of $1 \times 10^4$ of these previously activated clone 4 T cells were then adoptively transferred into nonirradiated BALB/c recipients. Recipient mice were treated at the indicated time points with either no additional Ag or given the same dose of HA peptide used for their primary stimulation (Fig. 4). Three weeks after the final peptide treatment, recipient mice were immunized with influenza virus and 8 days later, spleens were harvested and the number of donor clone 4 Thy1.1 T cells was quantitated by flow cytometry. The mean ± SD of two to three mice per group is indicated. Data are representative of one of three independent experiments with similar results.

![Figure 3](https://www.jimmunol.org/)

**FIGURE 3.** Deletion vs hyporesponsiveness following soluble peptide tolerance of naive clone 4 T cells in vivo. **A**, total of $1 \times 10^4$ naive clone 4 Thy1.1 T cells were injected into Thy1.2 BALB/c mice on day 0 along with no peptide, 100, 10, 1, or 0.1 μg of HA peptide. Three weeks later, mice were immunized with influenza virus and 8 days after viral priming, spleens were harvested and the total number of donor clone 4 Thy1.1 T cells was quantitated by flow cytometry. The mean ± SD of two to three mice per group is indicated. Data are representative of one of three independent experiments with similar results. **B**, total of $1 \times 10^4$ naive clone 4 Thy1.1 T cells were transferred into Thy1.2 BALB/c recipients along with no peptide, 100, 10, 1, or 0.1 μg of HA peptide on days 0, 1, and 2. Three weeks later, recipient mice were immunized with influenza virus and 8 days after viral priming, spleens were harvested and donor clone 4 Thy1.1 T cells were analyzed by flow cytometry. The mean ± SD of three mice per group is indicated. Data are representative of one of two independent experiments with similar results.

![Figure 4](https://www.jimmunol.org/)

**FIGURE 4.** Requirements for deletion of peptide tolerized clone 4 T cells. **A**, total of $3 \times 10^4$ naive clone 4 T cells were transferred into BALB/c mice along with 100 μg of HA peptide. Four days later, spleens were harvested and CFSE-diluted (dividing) clone 4 T cells were sorted by flow cytometry (as shown in Fig. 1A). A total of $1 \times 10^5$ activated clone 4 T cells were then adoptively transferred into BALB/c hosts and recipient mice were treated with 100 μg HA peptide at the indicated time points; D, Day. Three weeks after the last peptide treatment, groups of mice received either no additional treatment or $4 \times 10^6$ naive HA-specific CD4 T cells (HNT CD4). All groups of mice were immunized with influenza virus to induce expansion of the donor cells. On days 3 and 6, the indicated mice also received 5000 U of rIL-2 (rIL-2). Eight days after viral priming, spleens were harvested and donor clone 4 T cells were detected and quantitated by flow cytometry. **B** and **C**, total of $3 \times 10^4$ naive clone 4 T cells were activated with 10 μg (B) or 1 μg (C) of HA peptide and then, 4 days later, spleens were harvested and activated (dividing) clone 4 T cells were sorted by flow cytometry and $1 \times 10^4$ clone 4 T cells were adoptively transferred into BALB/c recipients. Recipient mice were treated with 10 μg (B) or 1 μg (C) HA peptide at the indicated time points; D, Day. Three weeks after the last peptide treatment mice were immunized with influenza virus to induce expansion of the donor cells. The mean ± SD of two to three mice per group is indicated. Data are representative of one of two independent experiments with similar results.
remained present, but were still anergic as late as 3 wk following exposure to peptide and therefore unable to respond to the viral immunization. The presence of IL-2 has been shown to restore the capacity of anergic CD8 T cells to proliferate in response to antigenic stimulation (27, 28). In addition, recent studies have demonstrated the importance of CD4 T cell help in promoting the optimal clonal expansion of CD8 T cells in vivo (29–31). Therefore, we tested the ability of either exogenous IL-2 or the addition of HA-specific helper CD4 T cells (from HNT TCR Tg mice) to rescue clone 4 T cell responsiveness to virus.

A total of 1 × 10^6 clone 4 T cells previously activated with 100 µg of HA peptide were injected into Ag-free recipient mice. Then, on days 7 and 14 after adoptive transfer, donor cells were treated with an additional 100 µg of HA peptide. Three weeks after the final peptide treatment, recipient mice were immunized with influenza virus to induce expansion of the remaining clone 4 T cells. One group of recipient mice also received exogenous rIL-2 on days 3 and 6 following viral priming. Another group of recipient mice received 4 × 10^6 purified HA-specific HNT CD4 T cells 1 day before viral priming. Eight days after viral immunization, spleens were harvested and the number of donor clone 4 T cells was quantitated by flow cytometry. Our results indicate that multiple peptide treatments were sufficient to cause deletion of the clone 4 T cells as no additional cells were recovered following treatment with exogenous IL-2 or by providing CD4 T cell help during viral priming (Fig. 4A).

**Reduced Erk phosphorylation of soluble HA peptide tolerized clone 4 T cells**

Our results suggested that for at least 4 days after treatment with 100 µg of HA peptide, clone 4 T cells were hyporesponsive to additional antigenic stimulation (Figs. 3B and 4A). However, clone 4 T cells regained responsiveness by day 7 after the initial treatment with 100 µg of HA peptide, indicating that the hyporesponsive state was only temporary (Fig. 4A). Several groups have demonstrated that T cell hyporesponsiveness following tolerogenic stimulation correlates with reduced phosphorylation of TCR-mediated signaling molecules, such as Erk (32, 33). Therefore, to determine the mechanism of hyporesponsiveness induced by HA peptide, we examined the effect of various doses of HA peptide on Erk phosphorylation in clone 4 T cells.

A total of 3 × 10^6 naive CFSE-labeled clone 4 T cells were adoptively transferred into BALB/c mice and at the time of cell transfer, mice received 100, 10, 1, or 0.1 µg of HA peptide. Four days later, spleens were harvested and donor cells were restimulated in vitro with either no Ag, HA peptide, or PMA and ionomycin. The baseline level of Erk phosphorylation under these stimulatory conditions was measured using naive clone 4 T cells freshly harvested from clone 4 TCR Tg mice. As expected, naive clone 4 T cells were able to up-regulate Erk phosphorylation following stimulation with HA peptide or PMA and ionomycin in vitro (Fig. 5, A and B). However, clone 4 T cells tolerized with 100 µg of HA peptide in vivo were unable to induce Erk phosphorylation in response to HA peptide in vitro (Fig. 5, A and B). These results were consistent with the inability of clone 4 T cells tolerized with 100 µg of HA peptide to respond to antigenic signaling in vivo (Figs. 3B and 4A). In contrast, clone 4 T cell tolerance induced in vitro with lower doses of HA peptide had less (10 µg) or no (1 or 0.1 µg) effect on Erk phosphorylation following in vitro restimulation with HA peptide (Fig. 5, A and B). This response correlated with the increased susceptibility of clone 4 T cells to deletion in vivo 4 days after their initial encounter with Ag (Fig. 4, B and C).

**FIGURE 5.** Erk phosphorylation in clone 4 T cells undergoing deletion vs the induction of hyporesponsiveness. A. A total of 3 × 10^6 naive clone 4 T cells were injected into BALB/c mice and given 100, 10, 1, or 0.1 µg of HA peptide on day 0. Four days later, spleens were harvested and donors splenocytes were stimulated in vitro for 5 min with no Ag, HA peptide, or PMA/ionomycin. Intracellular expression of phospho-Erk by donor clone 4 T cells was measured by flow cytometry. Bold line histogram represents donor clone 4 T cells that were restimulated in vitro, whereas dashed histogram represents clone 4 T cells that did not receive in vitro restimulation. B. The percentage increase in mean fluorescence intensity of phospho-Erk following in vitro restimulation was measured as compared with unstimulated controls (bold line histogram vs shaded histogram in A). C. A total of 3 × 10^6 naive clone 4 T cells were injected into BALB/c mice and given 100 µg of HA peptide on day 0. Four or seven days later, spleens were harvested and donor cells restimulated in vitro as described. The mean ± SD of two to three mice per group is indicated. Data are representative of one of three independent experiments with similar results.

In each case, Erk phosphorylation could be induced following stimulation with PMA and in the presence of the calcium ionophore, ionomycin (Fig. 5, A and B). Stimulation with PMA and ionomycin has been shown to signal through the protein kinase C pathway and can promote TCR-independent activation of Erk (34, 35), thus bypassing the potential TCR-mediated signaling defect induced during peptide-mediated tolerance (Fig. 5, A and B).

Although we observed a significant defect in Erk phosphorylation 4 days after clone 4 T cells were activated with 100 µg of HA peptide (Fig. 5), by day 7 the cells regained their ability to up-regulate phospho-Erk following HA restimulation in vitro (Fig. 5C). This increased responsiveness in vitro correlated with the ability of clone 4 T cells to undergo HA peptide-mediated deletion 7 days after their adoptive transfer into an Ag-free recipient (Fig. 4A).

**Discussion**

In a previous report, we showed that the initial stimulatory event experienced by naive CD8 T cells activated under tolerogenic
conditions was not sufficient to result in complete clonal deletion (9). This conclusion was based on the observation that after “parking” the cells for several weeks in an Ag-free host, we were able to detect residual cells that could respond to antigenic stimulation with influenza virus. In light of these results, we concluded that a single, brief exposure to Ag was insufficient to promote complete CD8 T cell clonal deletion. In contrast, no residual donor cells could be recovered following transfer into an Ag-bearing host, demonstrating that chronic exposure to Ag assured tolerance. However, in the latter case we could not be sure of the tolerance mechanism as chronic exposure to Ag can also result in T cell anergy, a condition in which cells persist in a nonresponsive state (36). Regardless of whether chronic Ag stimulation leads to CD8 T cell deletion or anergy, the mechanisms guiding these divergent responses are unclear. Therefore, in the current study, we sought to examine the requirements for CD8 T cell deletion vs the induction of anergy in vivo.

To this end we first compared the kinetics of high (100, 10 μg) vs low (1 or 0.1 μg) doses of HA peptide Ag presentation in vivo. Our results demonstrate that high doses of soluble HA peptide can persist for several weeks in vivo, whereas low doses are cleared more rapidly (Fig. 1). The kinetics of Ag presentation likely depend upon the characteristics of the individual peptide being examined (37–40), as well as the avidity of the T cell clone. A 200 μg dose of soluble OVA peptide was shown to persist for 10–14 days in vivo (14, 41). In contrast, some lymphocytic choriomeningitis virus -derived peptides needed to be presented with adjuvants, such as IFA, to stimulate CD8 T cell responses (12), most likely because of their short half-life in vivo (40).

Initially, we wished to determine whether the extent of clone 4 T cell deletion varied as a function of the amount of peptide tolerogen. Therefore, we compared the numbers of clone 4 T cells that survived a single in vivo treatment with different doses of peptide. It is important to note that even the lowest concentration of peptide used was sufficient to activate all of the clone 4 T cells (Fig. 2). We found that complete clone 4 T cell deletion could not be achieved with a single dose of Ag and furthermore, remarkably similar numbers of cells survived at all doses of Ag (Fig. 3A). We next asked whether additional exposure to peptide could induce complete elimination of the clone 4 T cells. Surprisingly, whereas three exposures to 100 or 10 μg of HA peptide was not sufficient to delete all of the clone 4 T cells (Fig. 3B), we could achieve complete deletion using similar treatments with lower doses of Ag (1 or 0.1 μg) (Fig. 3B). These results suggested that the clone 4 T cells remaining after the initial stimulation with high doses of HA peptide were rendered hyporesponsive to additional peptide treatments (Fig. 3B).

We then used an adoptive transfer system wherein only previously activated (based on Ag-specific proliferation) clone 4 T cells were collected and transferred into Ag-free BALB/c recipients. This system was used for two reasons: first, to ensure that only those clone 4 T cells successfully activated by the initial treatment with Ag were examined for their susceptibility to deletion. Second, we could control the timing of secondary Ag exposure and thus interrogate the antigenic responsiveness of the clone 4 T cells at specific time points. Together, these parameters allowed us to test the kinetic requirements for inducing complete clone 4 T cell deletion in vivo. Our results indicated that clone 4 T cells tolerized with 100 μg of HA peptide were hyporesponsive to subsequent tolerogenic treatments for at least 4 days after their initial Ag exposure (Fig. 4A). However, such hyporesponsiveness was transient as the cells again became susceptible to deletion by day 7 after transfer (Fig. 4A). In contrast, cells exposed to lower doses of Ag remained susceptible to deletion even at the earliest time points (Fig. 4, B and C).

It has been shown that anergic T cells exhibit reduced phosphorylation of signaling molecules, such as Erk or JNK (32–34) and that this leads to a defect in T cell-dependent activation-induced cell death (42, 43). Therefore, we examined the expression of phospho-Erk during clone 4 T cell deletion vs anergy induction in vivo. Activation of clone 4 T cells with high doses of HA peptide led to a temporary reduction in Erk phosphorylation, which correlated with their reduced susceptibility to deletion (Figs. 5A and 3B). This nonresponsiveness was transient as the clone 4 T cells regained responsiveness to Ag 7 days after the initial treatment, which correlated with increased TCR signaling and subsequent Erk phosphorylation (Figs. 4A and 5). Therefore, it is likely that following peptide-mediated tolerance in vivo, incomplete elimination of HA peptide-tolerized clone 4 T cells occurs due to a block in signal transduction molecules, such as Erk and JNK1/2, which are necessary for CD8 T cell activation and deletion. In contrast, when given repeated treatments of low doses of HA peptide, clone 4 T cells retain their ability to both up-regulate phospho-Erk as well as undergo deletion in vivo (Figs. 3B and 5). These data indicate that low doses of chronic Ag, as may occur for certain self-Ags cross-presented in vivo (4), promote the complete clonal deletion of CD8 T cells. However, in agreement with other studies, chronic activation with high doses of Ag can result in the survival of anergic T cells (44). The induction of anergy may include T cell tuning, a mechanism of anergy that has been found to occur for both CD8 and CD4 T cells chronically activated in vivo (45–50). A hallmark of tuning is the ability of a T cell to respond to higher doses of Ag than those used to induce anergy. Importantly, tuning did not appear to contribute to clone 4 T cell peripheral tolerance in vivo as high-dose anergized clone 4 T cells remained nonresponsive to stimulation with 10-fold higher doses of HA peptide (data not shown), although this also may be a parameter that varies with the avidity of a particular TCR.

Several groups have also demonstrated that the induction and maintenance of anergy during peptide-mediated tolerance can be due to TCR down-regulation or increased expression of CD5, a negative regulatory molecule (11, 16, 51, 52). Although we did not observe any change in CD5 expression following single or multiple exposures to HA peptide (data not shown), we did observe a slight decrease in clone 4 TCR expression following tolerance induction with 100 or 10 μg of HA peptide (Fig. 2B). This slight TCR down-regulation was independent of the extent of cell proliferation and was not enhanced following multiple exposures to HA peptide (data not shown). Thus, it is unlikely that modulation of TCR expression is a major contributing factor to the reduced responsiveness of clone 4 T cells exposed to high-doses of HA peptide; however, this does not rule out an important role for such down-modulation during tolerance induction in other situations. The lack of TCR down-regulation may be due to the relatively moderate affinity of the clone 4 TCR for HA, as it has been shown that in naive CD8 T cells, the efficiency of TCR down-regulation induced by a particular peptide correlates directly with TCR affinity (53). Thus, the strength of TCR signaling may play an important role in determining which mechanism of tolerance is induced.

An important question that remains unanswered by our results is why a single exposure to Ag is unable to affect complete clonal deletion. It is interesting to note that at all doses of tolerogen, the cells initially undergo some degree of clonal expansion that is followed by the death of the majority of clonal progeny. This is reminiscent of the process of memory T cell development that occurs following a productive immune response. There too, the
majority of activated T cells undergo apoptosis and a small number are able to survive and differentiate into memory cells (54, 55). It has been proposed that for memory T cell generation, the pro-survival signal initially involves expression of CD8α and is followed by up-regulation of the IL-7 receptor (56, 57). It is of interest to determine whether a similar mechanism may be responsible for preserving some of the clonal progeny after a single exposure to Ag under tolerogenic conditions.

The requirement for multiple exposures to Ag to promote complete clonal deletion has several important implications. First, this may provide a mechanism to prevent complete clonal elimination in response to an Ag that may appear only transiently. This would allow the repertoire to maintain TCR diversity, as cells that experience a single exposure to Ag are unlikely to be completely deleted from the repertoire. Second, these data suggest that for therapeutic tolerance protocols, in which the goal may be complete elimination of potentially harmful cells, one may wish to use multiple treatments with lower doses of short-lived Ag to optimize clonal deletion. This would avoid the induction of anergy and would facilitate complete clonal elimination. It should be noted that when dealing with a diverse TCR repertoire, the dose of Ag required to promote deletion might vary considerably among cells specific for the same Ag. Presumably, the highest avidity T cells would be most susceptible to anergy, and therefore, they could initially be targeted for elimination by using very low doses of Ag (46, 48). Progressively higher doses of Ag may later be used to target lower avidity T cells for deletion.

Acknowledgments

We thank Judith Biggs, Kristi Marquardt, and Rebecca Trenney for excellent technical assistance, The Scripps Research Institute Flow Cytometry core facility for assistance with cell sorting, and members of the Sherman laboratory for helpful discussions.

References


