Tolerant CD8 T Cells Induced by Multiple Injections of Peptide Antigen Show Impaired TCR Signaling and Altered Proliferative Responses In Vitro and In Vivo

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*J Immunol* 1998; 161:5260-5267; ;
http://www.jimmunol.org/content/161/10/5260
Tolerant CD8 T Cells Induced by Multiple Injections of Peptide Antigen Show Impaired TCR Signaling and Altered Proliferative Responses In Vitro and In Vivo

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The mechanisms responsible for peripheral CD8 T cell tolerance to foreign Ags remain poorly understood. In this study we have characterized the state of CD8 T cell tolerance induced in F5 TCR transgenic mice by multiple peptide injections in vivo. The tolerant state of CD8 T cells is characterized by impaired proliferative responses, increased sensitivity to cell death, and failure to acquire cytotoxic effector function after in vitro antigenic challenge. In vivo monitoring of CD8 T cell proliferation using 5-carboxyfluorescein diacetate succinimidyl ester showed that a large subset of the tolerant T cell population failed to divide in response to peptide. TCR down-regulation could not account for this loss of responsiveness to Ag since recombination-activating gene-1 (RAG-1)/F5 CD8 T cell responses were similar to those of RAG-1/F5 X RAG-1/F5 F1 lymphocytes, which express lower levels of the transgenic TCR. Analysis of early signal transduction in tolerant CD8 T cells revealed high basal levels of cytoplasmic calcium as well as impaired calcium mobilization and tyrosine phosphorylation after cross-linking of CD3ε and CD8α. Together these data indicate that repeated exposure to soluble antigenic peptide in vivo can induce a state of functional tolerance characterized by defective TCR signaling, impaired proliferation, and increased sensitivity to cell death. The Journal of Immunology, 1998, 161: 5260–5267.

Received for publication March 5, 1998. Accepted for publication July 13, 1998.

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1 This work was supported by institutional grants from the Centre National de la Recherche Scientifique and the Ministère de l’Éducation et de la Recherche and by additional support from the Association pour la Recherche sur le Cancer. P.M.D. is supported by a Training and Mobility of Researchers Fellowship from the European Union. M.P. was supported by a fellowship from the Association pour la Recherche sur le Cancer.

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We have previously shown that a single injection of soluble antigenic peptide induces a population of long-lived CD8 T cells that is hyper-reactive to Ag in vitro (48). In the present study we have used the F5 TCR transgenic system (49) to characterize the CD8 T cells that survive a tolerance induction protocol consisting of multiple exposures to soluble antigenic peptide. Our data show that 7 days after the last of three peptide injections administered at 4-day intervals, surviving CD8 T cells proliferate poorly in vitro and in vivo and have lost the ability to differentiate into cytotoxic effectors after in vitro stimulation in the absence of exogenously added cytokines. In addition, the tolerant F5 CD8 T cells are highly sensitive to cell death in vitro. Analysis of early signal transduction pathways revealed that these cells also had constitutively elevated cytoplasmic calcium levels and impaired early signal transduction in response to co-cross-linking of CD3 with CD8.

Materials and Methods

Mice and immunizations

C57BL/10, F5, RAG-1−/−, F51 TCR transgenic and RAG-1−/− mice were gifts from D. Kiossis (49). All animals were bred in the institute’s animal facility. Immunizations were performed by injecting 50 μl of the A/NT/60/68 influenza virus nucleoprotein peptide Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met-Ala-Ser-Asn-Glu-Asn-Met (Neosystems Laboratories, Strasbourg, France) in PBS into the peritoneal cavity. Control mice either were not treated or were injected with PBS alone.

Cell culture and proliferation assays

Spleen and lymph node T cells were cultured in RPMI 1629 (Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine (Life Technologies), 10 μg/ml gentamicin (Life Technologies), 6% FCS (Boehringer Mannheim, Mannheim, Germany), and 5% 10 M β-ME. F5 transgenic T cells (5 × 10^5 cells/well) were activated in 96-well plates with graded doses of peptide and 2 × 10^3 irradiated (3000 rad) C57BL/10 spleen cells and in the presence or the absence of 5% cell culture supernatant containing IL-2. The final IL-2 content ranged from 100 – 150 U/ml. After 3 days in culture, live spleen cells and in the presence or the absence of 5% cell culture supernatant containing IL-2. The final IL-2 content ranged from 100 – 150 U/ml. After 3 days in 24-well plates with 10 nM NP peptide and 2 × 10^3 irradiated C57BL/10 spleen cells in the presence of the absence of 5% cell culture supernatant containing IL-2 (100–150 U/ml). After 3 days in culture, live T cells were isolated by Ficoll-Hypaque (Lympholyte M, Cedarlane Laboratories) gradient centrifugation. Target P815 cells expressing a transfected H-2Db molecule (P815-Db) were pulsed with 1 mM Asp-Ala-Met (NP366–374; Neosystems Laboratories, Strasbourg, France) in 50 μl of ice-cold 1% Nonidet P-40, 0.1% NaN3, and 10^6 cells were incubated with Ab for 45 min at 4°C. Cells were then washed three times and incubated with 51 Cr-labeled targets with 2% PBS into the peritoneal cavity. Control mice either were not treated or were injected with PBS alone.

Cytotoxicity assays

F5 TCR transgenic CD8 T cells (1.5 × 10^5 cells/well) were activated for 3 days in 24-well plates with 10 nM NP peptide and 2 × 10^3 irradiated C57BL/10 spleen cells in the presence or the absence of 5% cell culture supernatant containing IL-2 (100–150 U/ml). After 3 days in culture, live T cells were isolated by Ficoll-Hypaque (Lympholyte M, Cedarlane Laboratories, Hornby, Canada) gradient centrifugation. Target P815 cells expressing a transfected H-2Db molecule (P815-Db) were pulsed with 1 mM Asp-Ala-Met (NP366–374) for 5 min and were harvested on day 3.

Analysis of intracellular calcium mobilization

This method has been described in detail previously (51). Total lymph node cells were loaded at a final concentration of 10^7/ml in fully supplemented RPMI 1629 containing the acetyl methyl ester of indo-1 at a concentration of 2 mM and 0.2% F127 (Molecular Probes, Junction City, OR). Loading was conducted at 37°C for 40 min in the dark. Cells were then washed, resuspended in fully supplemented medium, and stained at 4°C with PE-coupled Abs directed against CD4 (YTS 191.1, Caltag) and B220 (Sigma, St. Louis, MO). Analysis was performed on a Becton Dickinson FACScan-Star Plus. The UV excitation (351–364 nm) was provided by an argon laser; blue emission was detected at 480–520 nm, and violet emission was detected at 383–407 nm. Cells that were negative for PE staining were set as 100% CD8+.

Tyrosine phosphorylation assays

CD8 lymph node T cells were purified with magnetic beads using a negative selection strategy. Briefly, total lymph node cells were incubated with anti-CD4 (GK1.5) and anti-CD220 (RA3-6B2) hybridoma culture supernatants for 45 min at 4°C. Cells were then washed three times and incubated for 30 min at 4°C with magnetic beads coupled to goat anti-rat IgG (H & L) (Biomag, Perseptive Diagnostics, U.K.) at a ratio of 15 beads/cell. As determined by flow cytometric analysis, CD8 T lymphocytes represented 90–95% of the cells that remained after magnetic depletion. Samples of 1 × 10^7 CD8 T cells were incubated at room temperature with biotinylated mAbs to CD3ε (mAb 145 2C11) and CD8α (YTS169.4). Avidin was added 2 min after the beginning of the assay. Results are expressed as the mean indo ratio over time in the CD4+B220 population.

Labeling of cells with CFSE

The method used for labeling cells with CFSE is a modification of the protocol described by Westen and Parish (50). Briefly, T cells were resuspended at a concentration of 1–5 × 10^7/ml in fully supplemented growth medium before the addition of CFSE at a final concentration of 10 mM. Cells were then incubated for 10 min at 37°C, and the reaction was stopped by adding 5 vol of ice-cold medium. After two washes, CFSE-labeled cells were resuspended in growth medium for in vitro culture or in PBS for i.p. injection into C57BL/10 recipients. To achieve higher levels of CFSE fluorescence, RAG-1−/−F5 and F1 (RAG-1−/− F5 × RAG-1−/−) T cells were labeled with CFSE for 13 min instead of 10 min for some in vivo assays.

Results

Multiple systemic exposures to a soluble viral antigenic peptide has been shown to induce tolerance of CD8 peripheral T cells (44–46, 52). We first sought to determine how this tolerance induction protocol affected the peripheral CD8 T cell pool in F5 TCR transgenic mice. F5 mice were given three injections of the cognate influenza virus NP366–374 peptide at 4-day intervals. Seven days after the last peptide injection, the number of F5 CD8 T cells that could be recovered in the lymph nodes represented at least 30% of that in control animals. A subtle decrease in the expression levels of the transgenic TCR Vβ11 chain, CD3ε, and CD8α could be detected on the surface of CD8 T cells stimulated in vivo (Fig. 1A). A survey of surface molecules known to be up-regulated after in vivo T cell stimulation revealed that CD44 and Ly-6C were the only markers that were up-regulated on the surface of CD8 T cells stimulated 7 days before analysis with either single or multiple
peptide injections (data not shown). When cells stimulated multiple times in vivo were cultured in vitro in the presence of irradiated syngenic spleen cells and graded doses of Ag, they proliferated poorly compared with control populations (Fig. 1B) or CD8 T cells primed by a single peptide injection (data not shown). Similar results were obtained when proliferation was measured at different times after in vitro stimulation (data not shown). Proliferative responses were partially restored if tolerant F5 CD8 T cells were stimulated in the presence of exogenously added IL-2 (Fig. 1B).

These results indicated that multiple peptide injections in vivo rendered F5 CD8 T cells hyporesponsive to peptide stimulation in vitro.

Loss of ex vivo cytotoxic effector function has also been reported in tolerant virus-specific CD8 T cells (45). To determine whether F5 tolerant T cells were capable of becoming CTL effectors, control and tolerant F5 CD8 T lymphocytes were activated in vitro for 3 days and tested for the ability to lyse peptide-loaded P815-Db targets. The data presented in Fig. 2 show that tolerant T cells failed to become efficient CTL. However, in the presence of IL-2, tolerant T cells were able to differentiate into cytotoxic effector cells. Based on the hyporesponsive phenotype described above, F5 CD8 T cells stimulated by multiple peptide injections in vivo will be called tolerant.

Altered proliferation and increased cell death among tolerant F5 CD8 T cells in vitro

A number of mechanisms could account for the poor peptide-specific proliferative responses of tolerant T cells observed in vitro. These include a lower frequency of peptide-responsive cells, an increased rate of cell death, and slow or lack of proliferation in response to Ag in vitro. To follow the fate of tolerant CD8 T cells in vitro, we have used the fluorescent dye CFSE, which allows tracking of cell divisions using flow cytometry. Divisions could be detected as early as 40 h of in vitro stimulation in both control and tolerant CD8 T cell populations and at peptide doses ranging from 0.15–5 nM (data not shown). After 3 days in culture in the presence of 5 and 1.5 nM peptide, several divisions could be detected...
in both control and tolerant F5 CD8 T cell populations (Fig. 3A).
However, the number of cell divisions accomplished by activated
tolerant CD8 T cell populations after 3 days in culture was lower than
that observed in control F5 CD8 T cells. Moreover, at this time
point, the number of viable tolerant CD8 T cells was significantly
lower than that in control samples. At low Ag doses (0.5 and 0.15
nM) and after 3 days in culture, these numbers were consistently
10–20 times lower in tolerant CD8 T cell populations than in control
samples. By comparison, F5 CD8 T cells stimulated by a single
peptide injection 7 days before analysis performed the same num-
ber of divisions as naive cells (Fig. 3B). Together, these data show
that when tolerant F5 CD8 T cells were cultured in vitro they
performed fewer divisions in response to peptide and were more
sensitive to cell death compared with naive populations.

Ag-specific proliferative responses of tolerant F5 CD8 T cells
are impaired in vivo
To determine whether hyporesponsiveness of tolerant CD8 T cells
was also detectable in vivo, control and tolerant F5 T lympho-
cytes were labeled with the fluorescent dye CFSE and injected into
cS7BL/10 recipients. Three days later, mice were challenged with PBS (thin lines) or with
50 nmol of peptide (thick lines). Lymph node T cells were harvested on day
6 post-transplant and were stained with anti-CD8 coupled to PE. Results
show CFSE fluorescence in a viable cell population gated for CD8 expres-
These results are representative of three independent experiments.

FIGURE 4. Tolerant CD8 T cells are hyporesponsive to peptide in vivo.
Naive (A) and tolerant (B) RAG-1–/– F5 CD8 T cells were labeled with
CFSE and adoptively transferred by i.p. injection into C57BL/10 recipi-

ents. Three days later, mice were challenged with PBS (thin lines) or with
50 nmol of peptide (thick lines). Lymph node T cells were harvested on day
6 post-transplant and were stained with anti-CD8 coupled to PE. Results
show CFSE fluorescence in a viable cell population gated for CD8 expres-
These results are representative of three independent experiments.

majority of naive RAG-1–/– F5 CD8 T cells divided in response
to peptide challenge in vivo.

Role of cell surface transgenic TCR expression levels in tolerant
CD8 T cell response
The data presented above indicate that multiple injections of an
agonist peptide in vivo lead to hyporesponsiveness in vitro and in
vivo. Expression of a second TCR a-chain has been described in
mouse T cells (53, 54). Since a clonotypic Ab is not available in
the F5 system, we could not rule out the possibility that the F5
CD8 T cells that survived this tolerance induction protocol were,
in fact, cells that did not fully respond to the antigenic peptide. It
was thus conceivable that multiple peptide injections selected cells
that expressed lower levels of the transgenic F5 TCR a-chain. F5
to cells from naive RAG-1–/– F5, peptide-treated RAG-1–/– F5, or naive F1, mice were tested for surface expression of CD8 and Vβ11 7 days after
the last of three peptide injections. Results from one representative experiment of three are presented. B, In vitro proliferative responses of naive RAG-
1–/– F5, peptide-treated RAG-1–/– F5, and naive F1 CD8 T cells. CD8 T
cells (5 × 10⁴) were stimulated for 3 days with graded doses of peptide in
the presence or the absence of IL-2 as described in Materials and Methods.
C, Proliferation of naive RAG-1–/– F5 and RAG-1–/– F5 × RAG-1–/– F1
CFSE-labeled CD8 T cells in vivo was measured as described in Fig. 4.

FIGURE 5. Surface phenotype and proliferative responses of RAG-
1–/– F5 and F1 (RAG-1–/– F5 × RAG-1–/–) CD8 T cells. A, Spleen CD8
T cells from naive RAG-1–/– F5, peptide-treated RAG-1–/– F5, or naive F1,
were tested for surface expression of CD8 and Vβ11. B, In vitro proliferative responses of naive RAG-1–/– F5, peptide-treated RAG-1–/– F5, and naive F1 CD8 T cells. CD8 T
cells (5 × 10⁴) were stimulated for 3 days with graded doses of peptide in
the presence or the absence of IL-2 as described in Materials and Methods.
C, Proliferation of naive RAG-1–/– F5 and RAG-1–/– F5 × RAG-1–/– F1
CFSE-labeled CD8 T cells in vivo was measured as described in Fig. 4.

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were similar to those of control RAG-1 mice and also comparable to those seen in tolerant RAG-1 early TCR signaling defects. CD8+ vitro, we investigated whether hyporesponsiveness correlated with peptide in vivo. Multiple injections of F5 CD8 T cells are defective for signaling through the TCR complex. T cell activation using partial agonist peptides and superantigens, and anti-CD3 Abs in vitro and in vivo induces a state of T cell unresponsiveness that has been linked to alterations in TCR signal transduction. They include increased baseline activity of Fyn, inhibition of Ag receptor-mediated early tyrosine phosphorylation events, and diminished intracellular calcium mobilization. As multiple injections of peptide in vivo lead to inhibition of F5 CD8 T cell proliferation in vitro, we investigated whether hyporesponsiveness correlated with early TCR signaling defects. CD8+ T cells from F5 mice injected three times with antigenic peptide were tested for their ability to respond to co-cross-linking of CD3 with CD8. In these experiments, F5 CD8 T cells were used because down-regulation of CD3e was less pronounced than that seen in the RAG-1−/− background. In tolerant T cells, basal levels of intracellular free calcium were higher than those in control populations. In addition, intracellular free calcium responses (Fig. 6B) and tyrosine phosphorylation of several cellular substrates (Fig. 7) were both significantly reduced in tolerant CD8 T cells.

These results indicate that following multiple peptide injections, F5 CD8 T cells are defective for signaling through the TCR complex.

Discussion

The results presented in this study show that multiple systemic administrations of antigenic peptide into F5 TCR transgenic mice lead to functional defects in peptide-specific CD8 T cells. These cells displayed diminished proliferative responses to peptide both in vitro and in vivo. They also failed to differentiate into cytotoxic effectors when restimulated in vitro with peptide in the absence of exogenously added IL-2. Ag receptor down-regulation did not account for tolerant responses in vitro and in vivo. Instead, analysis of early signal transduction events revealed constitutively elevated resting levels of intracellular free calcium as well as impaired signaling after TCR/CD3 cross-linking with CD8.

The hyporesponsive phenotype described in this study does not appear to be linked to activation-induced T cell short term desensitization. Indeed, the proliferative responses of CD8 T cells activated in vivo by a single peptide injection 7 days before analysis were identical with those of naive populations. This is in agreement with the observation that a single exposure to peptide administered 10 days before exposure to virus may protect mice from infection with lymphocytic choriomeningitis virus (44). It is also consistent with induction of a memory-like F5 CD8 T cell population after a single exposure to antigenic peptide in vivo (48). In addition, we have found that 7 days after a single peptide injection, calcium mobilization triggered by co-cross-linking of CD3 and CD8. A

FIGURE 6. Regulation of cytoplasmic calcium levels in naive and peptide-tolerant F5 CD8 T cells. Lymph node cells from naive (open symbols) and peptide-injected (closed symbols) F5 mice were loaded with Indo-1, washed, and further incubated with PE-coupled Abs to CD4 and B220. A. The mean indo-1 violet/blue emission ratio of the baseline intracellular Ca2+ level in resting CD4+/B220+ control and tolerant F5 CD8 T cell populations. Data show results from three independent experiments. Each symbol represents a single measurement performed in individual mice. B. Calcium mobilization in response to co-cross-linking of CD3e with CD8α. In this assay, lymph node cells from naive or peptide-injected F5 mice were tested. Indo-1 and Ab-labeled cells were preincubated at 37°C with anti-CD3e-biotin (4 μg) and anti-CD8α-biotin (1.5 μg). Cells were then stimulated 2 min after the beginning of the assay by adding 25 μg of avidin. Results are representative of three independent experiments and are expressed as the mean indo-1 violet/blue emission ratio in the CD4+/B220− population over time.

FIGURE 7. TCR complex-mediated tyrosine phosphorylation in naive and tolerant F5 CD8 T cells. Cells were preincubated at 37°C with biotin-coupled Abs to CD3e and CD8α and stimulated with avidin as indicated in Materials and Methods. Western blotting was performed using a polyclonal anti-phosphotyrosine Ab on whole Nonidet P-40 cell lysates separated on 4–12% SDS-PAGE. Arrows indicate proteins for which the increase in phosphotyrosine content was lower in tolerant CD8 T cells compared with that in a naive population. Results are representative of three independent experiments.
CD8 was comparable to that seen in naive populations (data not shown).

A number of mechanisms have been proposed to account for hyporesponsiveness of tolerant peripheral T cells. One of these argues that T cell hyporesponsiveness after tolerance induction results from selection of a pre-existing population that cannot respond to peptide. Our results strongly argue against such a model by providing evidence indicating that hyporesponsiveness affected a population of cells that had responded to the first encounter with peptide. Indeed, using naive CFSE-labeled RAG-1\(^{-/-}\) F5 CD8 T cells transferred into C57BL/10 recipients, we have shown that the overall majority of RAG-1\(^{-/-}\)/F5 CD8 T cells had proliferated 3 days after peptide injection. These data are in agreement with our previous report showing that the bulk of F5 CD8 T cells incorporate bromodeoxyuridine in response to a single peptide injection (48) and make it unlikely that surviving CD8 T cells are derived from a RAG-1\(^{-/-}\)/F5 CD8 T cells subset that did not respond to Ag.

Another mechanism that could account for hyporesponsiveness in the surviving Ag-specific T cell population involves a decrease in the number of cell surface TCR molecules capable of recognizing the peptide. In the F5 system, this could be the consequence of a second TCR \(\alpha\)-chain rearrangement that has been reported in humans (58) as well as in normal and TCR transgenic mice (53, 54). One consequence of dual TCR \(\alpha\)-chain rearrangement is the expression of two Ag receptors. As a result, the number of cell surface TCR\(\alpha\beta\) receptors capable of recognizing a given Ag may be decreased. Since the ability to transduce activation signals correlates with TCR surface levels (59) in T cell clones, responses to limiting doses of Ag may be diminished in T cells expressing two Ag receptors. In vivo evidence for such a mechanism comes from studies in transgenic systems in which skin grafts are not rejected (62). In the F5 model, cells expressing a second TCR would be selected by CD8 T cells expressing lower levels of the Ag receptor (14, 60). In the F5 model, cells expressing a second TCR would be selected by CD8 T cells expressing lower levels of the Ag receptor (14, 60) in vitro and in vivo. These results show that mechanisms other than TCR down-regulation are responsible for the tolerant phenotype of F5 CD8 T cells. Indeed, modifications of TCR signaling have been demonstrated in anergic CD4 T cells in vitro (39, 40).

Following multiple peptide injections, CD8 T cells from RAG-1\(^{-/-}\)/F5 mice show some down-regulation of the TCR. In some systems, this is thought to be responsible for the lack of responsiveness of tolerant T cell populations to Ag (14, 60). However, using T cells that express cell surface levels of TCR V\(\beta\)11 comparable to those detected on the surface of tolerant RAG-1\(^{-/-}\)/F5 CD8 T lymphocytes (see Fig. 5), we found that this had no influence on the proliferative responses of naive cells to peptide both in vitro and in vivo. These results show that mechanisms other than TCR down-regulation are responsible for the tolerant phenotype of F5 CD8 T cells. Indeed, modifications of TCR signaling have been demonstrated in anergic CD4 T cells in vitro (39, 40).

In support of such a model, we found that F5 CD8-tolerant T cells exhibited fundamental alterations in early TCR signaling pathways. We first detected high levels of intracellular Ca\(^{2+}\), an observation that has been reported in anergic CD4 T cell clones (41). We further demonstrated that tolerant CD8 T cells were partially defective for intracellular signals such as cytoplasmic free calcium increases and tyrosine phosphorylation triggered by TCR/CD3 co-cross-linking with CD8. These results are consistent with previous reports showing that T cells that survive in vivo stimulation with superantigens display altered Ag receptor signaling (56, 57). They also suggest that alterations in intracellular calcium metabolism and TCR signaling contribute to block tolerant CD8 T cell responses in vitro and in vivo. Further analysis will be required to determine whether tolerant CD8 T cells share with anergic CD4 T cell clones increased Fyn activity (41), high Rap-1-GTP content (61), and impaired TCR-mediated activation of ERK and JNK (42, 43).

Using CFSE-labeled cells adoptively transferred into C57BL/10 recipients, we have shown that while the overall majority of naive F5 CD8 T cells did divide in response to peptide, a large fraction of the tolerant T cell population did not proliferate in the recipient’s lymph nodes. These results clearly indicate that 10 days after the last exposure to peptide and 3 days after transfer into an environment free of Ag, a significant proportion of the F5 TCR transgenic CD8 T cells remained unresponsive to peptide in vivo. To determine whether cytokines produced by naive F5 CD8 T cells could drive tolerant T cells to divide in vivo, we also injected tolerant CFSE-labeled F5 CD8 T cells into F5 recipients. In these mice, the large majority of peripheral CD8 T cells are activated and divide in response to the same dose of peptide (39). However, we found no evidence suggesting that tolerance could be reversed under these experimental conditions (data not shown).

Some tolerant CFSE-labeled CD8 T cells were capable of dividing in response to peptide stimulation in C57BL/10 recipients. These cells did not correspond to a subset of CD8 T cells that has escaped tolerance induction in the donor. Indeed, the division profiles that can be detected 3 days after peptide injection in the recipient mice were strikingly different when comparing naive and tolerant F5 CD8 T cells. Activation of naive cells leads to a normal distribution of division peaks, with a maximum at four divisions. By contrast, division peak distribution was not normal when tolerant T cells were analyzed, since the peak corresponding to one division contained the largest number of cells. This observation could mean that tolerant CD8 T cells that divided also migrated rapidly out of the lymph nodes. However, based on our in vitro results showing that CFSE-labeled tolerant T cells were highly sensitive to cell death, it seems more likely that those F5 tolerant T cells that responded to peptide stimulation in vivo died.

By comparing the percentages of control and tolerant CFSE-labeled F5 CD8 T cells detected in the lymph nodes of C57BL/10 recipients challenged with PBS, we also found that they were consistently lower in mice injected with tolerant F5 CD8 T cells (data not shown). One possibility is that the homing of naive and that of tolerant CD8 T cells are different. However, there is also evidence suggesting that tolerant T cells may have a shorter life span in vivo. First, T cells rendered anergic in vivo gradually disappear when parked in a recipient that does not express the Ag (62). Second, recent reports have shown that survival of naive TCR\(\alpha\beta\) T cells in the periphery is dependent on an environment expressing the restricting MHC (63–65). These findings show that a survival signal relayed by TCR is required for maintenance of mature T cells in peripheral lymphoid organs. This is compatible with a model proposed by Raff and co-workers (66) in which mammalian cells constitutively express proteins involved in triggering apoptosis, such that survival of these cells would require signals to inhibit this default death program. Since tolerant F5 T cells are partially defective for TCR and coreceptor signaling in our system, it is conceivable that the amount of signal transduced by the Ag receptor complex and/or CD8 upon interaction with H-2\(D^\alpha\) is not sufficient to ensure survival of these cells in vitro and in vivo.

Acknowledgments

We thank Dimitris Kionissis for the F5 and RAG-1\(^{-/-}\)/F5 TCR transgenic mice, Muriel Moser, Oberdan Leo, Patrick Bertolino, and Janet Maryanski for helpful comments and advice, and Annie Thomas for assistance with flow cytometry.
References


