

# High-Dose Soluble Antigen: Peripheral T-Cell Proliferation or Apoptosis

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## INTRODUCTION

A functional immune system reacts against foreign antigens while at the same time it remains unresponsiveness to self. Tolerance to self is acquired and maintained by both thymic and peripheral events. During thymic development, the T-cell receptor (TCR) repertoire is generated by gene rearrangement and pairing of TCR  $\alpha$  and  $\beta$  chains. This process is thought to occur in a random manner and, as a result, autoreactive T cells as well as T cells reactive to foreign antigens are generated. In the thymus, the developing T cells with specificity for self MHC are positively selected, but those specific for self MHC plus antigens having access to the thymus are deleted by apoptosis (Kisielow et al. 1988, Murphy et al. 1990). However, some autoreactive T cells do enter the periphery as a result of incomplete thymic deletion or absence of particular self antigens in the thymus. Several mechanisms collectively referred to as "peripheral tolerance" normally operate to prevent these autoreactive T cells initiating an autoimmune disease. These mechanisms include the induction of anergy, down-regulation of cell surface expression of TCR and co-receptor molecules, immune deviation, and peripheral deletion (Webb et al. 1990, Rocha & von Boehmer 1991, Fields & Loh 1992, Hammerling et al. 1993, Scott et al. 1994).

A major goal of clinical immunologists is to therapeutically manipulate the immune system in an antigen-specific manner, either to enhance an effective immune response against unwanted antigens (vaccine, anti-tumor immunotherapies) or to specifically down-regulate potentially harmful immune responses (autoimmune reactions, graft rejection). Studies assessing the events associated with the

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induction of self tolerance have raised the possibility of establishing and/or dissecting antigen-specific immunotherapeutic strategies. High-dose administration of antigen is one approach that has been shown to induce antigen-specific hyporesponsiveness (Dixon & Maurer 1955, Mitchison 1964). The mechanisms by which this unresponsiveness is established are still unknown. This is mainly due to the small size of a given antigen-specific T-cell population and the absence of appropriate markers to follow the fate of those cells. To circumvent these problems, we have analyzed the effects of high-dose intravenous (i.v.) injection of antigen in TCR-transgenic mice, in which a large fraction of T cells shares the same specificity. We found that, following the injection of the appropriate peptide, thymic deletion was obtained in two different transgenic systems. Histological analyses clearly showed that apoptosis mediated this thymic deletion. Peripheral unresponsiveness was seen in only one of the two systems and this was due to a combination of anergy and peripheral deletion via apoptosis. In the other system, peripheral T-cell activation and increased responsiveness were obtained.

#### THE TCR TRANSGENIC MOUSE SYSTEMS

We have generated several lines of transgenic mice (HNT-TCR mice) expressing a rearranged TCR which is hemagglutinin (HA) peptide residues 126–138-specific and I-A<sup>d</sup>-restricted (Scott et al. 1994). The  $\beta$  chain of this TCR is encoded by V $\beta$ 8.3-D $\beta$ 2-J $\beta$ 2.6, and the  $\alpha$  chain by V $\alpha$ 15-J $\alpha$ 20. Two of the HNT-TCR transgenic lines express the V $\beta$ 8.3 transgene in more than 99% of TCR bright thymocytes and peripheral T cells. There is no antibody to identify the TCR  $\alpha$  chain, but our studies indicate that 50–70% of peripheral CD4<sup>+</sup> T cells react *ex vivo* to antigen-presenting cells (APCs) pulsed with HA peptide 126–138 (Scott et al. 1994).

In addition to the HNT-TCR transgenic mice, we have established two lines of transgenic mice that express a TCR specific for a peptide of the self protein myelin basic protein (MBP). This self antigen induces experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (Zamvil et al. 1986). Encephalitogenic peptides and analogs of these peptides have been used successfully to inhibit or treat EAE (Wraith et al. 1989, Smilek et al. 1991, Metzler & Wraith 1993). Furthermore, there is evidence that the affinity of MBP peptide analogs correlates with the efficacy of treatment (Metzler & Wraith 1993). It is not clear how these peptide treatments affect the encephalitogenic T cells such that they no longer cause disease.

The two lines of transgenic mice (MBP-TCR mice) express a TCR from PJR-25, an encephalitogenic T-cell clone that is specific for the MBP peptide Ac1-11 bound to I-A<sup>u</sup> (Zamvil et al. 1986). The  $\beta$  chain is encoded by a V $\beta$ 8.2-D $\beta$ 1-J $\beta$ 2.3 rearranged genomic clone and the  $\alpha$  chain is encoded by a V $\alpha$ 4-J $\alpha$ 40 rearranged genomic clone (Acha Orbea et al. 1988). In one line, the transgenes have been

integrated into the X chromosome. Thus, in males, over 80% of CD4<sup>+</sup> single-positive thymocytes and peripheral T cells express V $\beta$ 8.2, while in females, about 50-60% of a similar population express V $\beta$ 8.2. The lower number of CD4<sup>+</sup>V $\beta$ 8.2<sup>+</sup> T cells in females, which are heterozygous for the transgenes, is probably due to random inactivation of the X chromosome carrying the transgenes on a cell-by-cell basis.

These two transgenic mouse systems allow us to compare the *in vivo* consequences of high-dose antigen administration on T cells bearing a receptor specific for HA, a classically defined foreign antigen, or for MBP Acl-11, a self peptide. HNT-TCR transgenic mice were injected once with HA peptide 126-138 or a peptide from ovalbumin that is nonstimulatory but binds to I-A<sup>d</sup>, OVA peptide 323-339. MBP-TCR transgenic mice were injected once or twice with Acl-11, or analogs Acl-11[4A] or Acl-11[4Y], which bind to I-A<sup>u</sup> with 1000 and 10000 times higher affinity, respectively, than does Acl-11, which is a relatively poor binder (Fugger et al. 1994). At various times post-injection, mice were sacrificed and cells from their lymphoid organs were analyzed for phenotype and function.

#### EFFECTS OF *i.v.* PEPTIDE INJECTION ON DEVELOPING THYMOCYTES

The effects of *i.v.* injection of HA peptide on the thymus of HNT-TCR transgenic mice were striking. At days 1 and 3 post-injection, the thymus size was severely reduced: the number of thymocytes was 10-20% of that of OVA 323-339 control peptide-injected mice. The CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) compartment was mostly affected with an almost complete deletion of CD4<sup>+</sup>CD8<sup>+</sup> TCR<sup>low</sup> thymo-

TABLE I  
*i.v. injection of peptide induces thymic deletion in HNT-TCR transgenic mice*

	Thymic Subset			
	CD4 <sup>-</sup> CD8 <sup>-</sup> (DN)	CD4 <sup>+</sup> CD8 <sup>+</sup> (DP)	CD4 <sup>+</sup> CD8 <sup>-</sup>	CD4 <sup>-</sup> CD8 <sup>+</sup>
Day 0	+	+++++	++	++
Day 1	++	+++	+++	++
Day 3	+++	+	++	++++
Day 5	++	++++	±	+++
Day 15	±	+++++	++	++
Day 30	+	+++++	++	+

Thymi were removed at various time points after *i.v.* injection of 750  $\mu$ g of HA 126-138 peptide. The total numbers of thymocytes were 100-150 $\times$ 10<sup>6</sup> at days 0, 15 and 30, and 25 $\times$ 10<sup>6</sup>, 12 $\times$ 10<sup>6</sup> and 40 $\times$ 10<sup>6</sup> at days 1, 3, and 5 respectively. This table shows the distribution of cells in the four major thymic populations as determined by staining with fluorescein-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4 monoclonal antibodies and analyzing by flow cytometry.  $\pm$ , <4%; +, 4-10%; ++, 10-25%; +++, 25-45%; +++++, 45-65%; ++++++, >65%.

cytes (Table I). The CD4<sup>+</sup> and CD8<sup>+</sup> single-positive compartments were consequently increased in relative numbers but were also reduced in absolute numbers. By day 5, increased numbers of CD4<sup>-</sup>CD8<sup>+</sup> and DP thymocytes could be detected. Furthermore, the size of the CD4<sup>+</sup>CD8<sup>-</sup> compartment was greatly reduced, probably due to the migration of these cells to the periphery coupled with a lack of production of additional mature thymocytes (Table I). By day 15, normal numbers of cells in the various thymocyte subsets were found.

To determine whether this dramatic thymic deletion resulted from accelerated thymocyte maturation and subsequent release into the periphery or from *in situ* apoptotic cell death, we analyzed thymic sections from HA peptide- or OVA peptide-injected mice using the Tdt-UTP-biotin-nick-end-labeling (TUNEL) reaction. This reaction is based on the fact that apoptotic cells contain a large number of free 3'ends of double-stranded DNA due to the endonuclease digestion of genomic DNA at nucleosomal intervals. Biotinylated UTP molecules are added to the 3'ends using terminal deoxynucleotidyl transferase. The sections are then covered with avidin-peroxidase and diaminobenzidine is used as a substrate. As a result, cells undergoing apoptosis can be microscopically visualized. In sections prepared from uninjected or OVA-injected mice, more labeled cells were seen in the thymus than in lymph nodes or spleen. This indicates that apoptosis is occurring in the thymus under physiological conditions. The relatively small percentage of thymocytes labeled by the TUNEL reaction compared to the large number of cells actually dying in the thymus might be explained by the ability of the thymus to rapidly phagocytose and degrade apoptotic cells. Therefore, at a given time point, few apoptotic cells are accessible to the TUNEL reaction. In contrast, 6 to 12 hours after i.v. injection of 750 μg of HA peptide, an intense apoptotic reaction could be detected in the thymic cortex of HNT-TCR transgenic mice (Table II), clearly demonstrating that *in situ* apoptosis has occurred in developing HA-specific thymocytes. Thirty-six hours post-injection, the thymic cortex had a considerably diminished size and was almost devoid of apoptotic thymocytes.

TABLE II  
*i.v. injection of HA peptide induces thymic apoptosis in HNT-TCR transgenic mice*

	Number of apoptotic cells in the thymus of peptide-injected HNT-TCR mice			
	Hour 6	Hour 12	Hour 24	Hour 36
OVA injected mice	+	+	+	+
HA injected mice	+++	++++	++	+

Thymi were removed at various time points after i.v. injection of 750 μg of HA 126-138 peptide. The TUNEL reaction was performed on sections of formalin-fixed tissues using biotinylated dUTP and avidin-peroxidase. Diaminobenzimide was used as a substrate. The staining of the nicked DNA in apoptotic cells is dark brown. Semi-quantitative results are shown since there were too many apoptotic cells in some samples to count.

Similarly, one or two doses of Ac1-11, Ac1-11[4A], and Ac1-11[4Y] were injected i.v. in MBP-TCR mice and thymocyte subsets were analyzed. Table III shows the results of two experiments. At day 1, in both experiments, none of the peptides induced thymic deletion. By day 4, however, 1.2 mg of both Ac1-11 and Ac1-11[4A] were able to induce modest (up to 40%) T-cell deletion of DP thymocytes. The same amount of Ac1-11[4Y] at day 4, in contrast, was able to induce up to a 75% decrease in the absolute number of thymocytes, as well as a 75% decrease in the number of DP thymocytes (Table III). By day 6, the thymi of Ac1-11- and Ac1-11[4A]-injected mice were indistinguishable from PBS-injected mice, whereas in Ac1-11[4Y]-injected mice T-cell deletion had reached a peak of almost 80% deletion of DP thymocytes and a 10-fold decrease in number of thymocytes. A higher dose (3.6 mg) of each of the three peptides yielded similar results on days 1 and 3. At day 6, however, 3.6 mg of both Ac1-11[4A] and Ac1-11[4Y] had induced profound deletion (>80%) of DP T cells and a concomitant 10-fold reduction in the number of thymocytes, and Ac1-11 had induced about a 60% reduction in DP T cells. By day 15, the thymi of mice injected with 3.6 mg of Ac1-11, Ac1-11[4A], or Ac1-11[4Y] were indistinguishable from thymi of PBS-injected mice. These experiments show that the affinity of the peptide for MHC correlates with the ability of the peptide to induce thymic deletion. The half life of Ac1-11[4A] bound to I-A<sup>u</sup> is approximately 10 minutes, while the half-life of Ac1-11[4Y] bound to I-A<sup>u</sup> is >4 hours (Fairchild et al. 1993), suggesting that longer exposure of a developing T cell to peptide/MHC complex favors thymic deletion.

TABLE III

*i.v. injection of peptides with increasing affinity correlated with induction of thymic deletion in CD4<sup>+</sup>CD8<sup>+</sup> (DP) cells of MBP-TCR mice*

Peptide	Dose	Day 1	Day 3	Day 4	Day 6	Day 15	Relative affinity for I-A
Ac1-11	1.2 mg	+++++	nd	++++	+++++	nd	1
	3.6 mg	+++++	++++	nd	+++	+++++	
Ac1-11[4A]	1.2 mg	+++++	nd	++++	+++++	nd	1000
	3.6 mg	+++++	+++	nd	++	+++++	
Ac1-11[4Y]	1.2 mg	++++	nd	+++	++	nd	10 000
	3.6 mg	+++++	++	nd	++	+++++	

Thymi were removed at various time points after i.v. injection of either one dose of 1.2 mg or two doses of 1.8 mg (3.6 mg total) of Ac1-11, Ac1-11[4A], or Ac1-11 [4Y]. The percent of CD4<sup>+</sup>CD8<sup>+</sup> (DP) cells in each thymus was determined as described in Table I. Data are presented as the percent of DP cells relative to the percent of DP cells in PBS-injected thymus as the following: +, 4-10%; ++, 10-25%; +++, 25-45%; +++++, 45-65%; ++++++, >65%. nd, not determined.

In both of our transgenic systems, injection of antigenic peptides resulted in a dramatic reduction of the total number of thymocytes, primarily due to the deletion of DP TCR<sup>low</sup> immature thymocytes. This is in accordance with data from several other groups also using TCR transgenic mice as a model system (Kisielow et al. 1988, Pircher et al. 1989, Murphy et al. 1990, Mamalaki et al. 1992). However, in other systems, most notably in MIs-mediated thymic deletion, negative selection appeared to occur after the DP stage. Thymic negative selection of DP thymocytes in TCR transgenic mice may represent an artifact due to early and high levels of expression of TCR/CD3 complexes on the thymocytes (Guidos et al. 1990). On the other hand, thymic deletion due to MIs superantigen could be due to the fact that MIs does not engage the TCR in the same manner as do conventional MHC/peptide complexes (Pircher et al. 1989).

Although it has previously been shown that antigen-mediated thymic negative selection occurs through *in situ* apoptosis (Murphy et al. 1990), our data provide additional insight. First, low but detectable levels of apoptosis occur in unmanipulated thymi. This apoptosis is not detectable using the DNA fragmentation assay. Second, in agreement with the preferential deletion of DP thymocytes, apoptosis occurs mostly in the thymic cortex and at the cortico-medullary junction following injection of antigen. Third, apoptotic cells are rapidly cleared from the thymus. Thus, 36 hours after injection of HA peptide very few dying cells can be detected. These cells are not seen in thymic vessels, suggesting that they are not exported to the periphery (Murphy et al. 1990). Furthermore, we observed no or limited apoptosis in liver, spleen and lymph nodes at the peak of thymic apoptosis. Thymic macrophages and dendritic cells are likely to be involved in the phagocytosis and elimination of apoptotic thymocytes.

Thymic deletion is believed to be a rapid process which is independent of cellular proliferation. In our experiments with the HNT-TCR mice, thymic deletion appears somewhat earlier than activation of mature peripheral T cells following injection of antigen. Interestingly, we could not, by varying the dose of peptide injected, induce thymic deletion without inducing concomitant activation in the periphery, suggesting that differences in antigenic thresholds for thymic deletion and activation of peripheral T cells (Pircher et al. 1991) do not exist in our systems. This has also been noted after intraperitoneal injection of peptide in another TCR transgenic mouse system (Mamalaki et al. 1992). This may reflect better diffusion of circulating antigens in the spleen and lymph nodes than in the thymus. However, this could have physiological importance as some self antigens may have access to the thymus only through the bloodstream and, therefore, thymic deletion of autoreactive T cells might not be complete even for circulating self antigens.

In both the HNT-TCR and MBP-TCR transgenic mice, deletion can be induced with a high i.v. dose of peptide, yet in the MBP-TCR system higher amounts of peptide are required and maximal deletion is seen between days 3 and

6, rather than at day 1. There are several possible reasons for the discrepancy between these two systems. First, the Ac1-11[4Y] peptide, although it has a high affinity for I-A<sup>u</sup>, may have a lower affinity for I-A<sup>u</sup> than does the HA peptide for I-A<sup>d</sup>. This would result in higher number of HA/I-A<sup>d</sup> complexes on APCs, such that the threshold for thymic deletion would be reached earlier in the HNT-TCR system. Second, if the HA peptide and Ac1-11[4Y] peptide have similar affinities for the MHC, then the delay in thymic deletion in the MBP-TCR mice may be due to a lower affinity of this TCR for Ac1-11[4Y] bound to I-A<sup>u</sup> than the HNT-TCR has for HA peptide bound to I-A<sup>d</sup>. Evidence suggests that the overall avidity of TCR/peptide/MHC interaction affects selection; a high-avidity interaction, which is influenced at least in part by peptide affinity for MHC and TCR affinity for peptide/MHC complexes, would favor thymic apoptosis, whereas a low-avidity interaction would permit expansion of developing T cells (Ashton-Richardt et al. 1994). Third, deletion in the thymus also is influenced by the density of TCR and co-receptor and accessory molecules on the surface of the T cells or APCs; the level of expression of these molecules may differ between the two systems. Finally, the susceptibility of these peptides to proteolysis is unknown. If Ac1-11 and its analogs are particularly susceptible, the doses of peptide given may be effectively much less.

#### EFFECTS OF *i.v.* PEPTIDE INJECTION ON MATURE T CELLS

One *i.v.* injection of 750  $\mu$ g of HA peptide 126-138 per mouse induced a marked hyporesponsiveness *in vitro* that lasted for about 15 days. A number of effects were observed in the mature T lymphocytes explaining the functional hyporesponsiveness. First, a transient activation phase was observed in CD4<sup>+</sup> mature T cells. At day 1 post-injection, but not at days 3 and 5, CD4<sup>+</sup> T cells had entered the cell cycle: the cells were larger in size and their numbers in the spleen and

TABLE IV

*i.v.* injection of HA peptide induces expansion and deletion of mature T cells in HNT-TCR transgenic mice

	CD4 <sup>+</sup> V $\beta$ 8 <sup>+</sup> /CD8 <sup>+</sup> V $\beta$ 8 <sup>+</sup> ratio in spleens of HNT-TCR animals at different days after injection of peptide					
	Day 0	Day 1	Day 3	Day 5	Day 15	Day 30
OVA injected mice	1.68	1.55	1.54	1.49	1.72	1.45
HA injected mice	1.68	2.85	1.41	0.81	0.73	1.13

Spleens were removed at various time points after *i.v.* injection of 750  $\mu$ g of HA 126-138 peptide. The CD4<sup>+</sup>V $\beta$ 8<sup>+</sup>/CD8<sup>+</sup>V $\beta$ 8<sup>+</sup> ratio was determined at each time points by staining with fluorescein-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4 monoclonal antibodies and analyzing by flow cytometry.

lymph node were increased. Indeed, the CD4<sup>+</sup>Vβ8<sup>+</sup>/CD8<sup>+</sup>Vβ8<sup>+</sup> T-cell ratio in the spleen of HA peptide-injected mice was approximately 60% higher than that seen in control mice treated with OVA peptide (Table IV). Cell surface expression of IL-2R was also increased on CD4<sup>+</sup> T cells. However, these CD4<sup>+</sup> T cells were functionally anergic; they proliferated weakly to HA peptide *in vitro*.

Interestingly, after the initial increase in the CD4<sup>+</sup>Vβ8<sup>+</sup>/CD8<sup>+</sup>Vβ8<sup>+</sup> ratio of splenic cells from HA peptide-injected mice at day 1, the ratio dropped to 49%, 28%, and 26% of this value on days 3, 5, and 15, respectively (Table IV). At days 5 and 15 this ratio was half of that seen in the spleen of OVA peptide-injected mice. At day 30 the CD4<sup>+</sup>Vβ8<sup>+</sup>/CD8<sup>+</sup>Vβ8<sup>+</sup> ratio had increased, possibly due to new mature T cells being generated by the repopulated thymus and to the clearance of HA peptide from APCs. This fluctuation in the CD4<sup>+</sup>Vβ8<sup>+</sup>/CD8<sup>+</sup>Vβ8<sup>+</sup> ratio was solely due to variations in CD4<sup>+</sup>Vβ8<sup>+</sup> T-cell numbers; the numbers of CD8<sup>+</sup>Vβ8<sup>+</sup> T cells remained constant. The deletion of CD4<sup>+</sup>Vβ8<sup>+</sup> T cells from peripheral lymphoid organs following HA peptide injection could either be due to the homing of the activated CD4<sup>+</sup>Vβ8<sup>+</sup> T cells to other tissues or to their death. To assess for their *in situ* death, we analyzed the lymph nodes of HA- or OVA peptide-injected mice using the TUNEL procedure. A substantial increase in the frequency of apoptotic cells in the tissues of HA peptide-injected mice was apparent 36 hours after injection (Table V). This indicates that apoptosis in the periphery contributes to the deletion of mature CD4<sup>+</sup>Vβ8<sup>+</sup> T cells observed after high-dose i.v. injection of HA peptide. However, it is not yet clear whether a linear relationship exists between the signal generated in the TUNEL reaction and the number of apoptotic cells in a tissue and, therefore, quantitative conclusions regarding the contribution of apoptosis to the peripheral deletion cannot be made.

While anergy or deletion was observed in the periphery of HNT-TCR mice following an injection of HA peptide, activation of peripheral T cells was observed in the MBP-TCR transgenic mice given 1.2 mg Ac1-11, Ac1-11[4A], or Ac1-11[4Y]. At day 1, the levels of expression of CD69, an early activation marker, and IL-2R positively correlated with increasing affinity of peptide for

TABLE V  
*i.v. injection of HA peptide induces peripheral apoptosis in HNT-TCR transgenic mice*

	Number of apoptotic cells in lymph nodes of peptide-injected HNT-TCR mice			
	Hour 6	Hour 12	Hour 24	Hour 36
OVA injected mice	9	11	5	8
HA injected mice	17	34	41	135

Lymph nodes were removed at various time points after i.v. injection of 750 μg of HA 126-138 peptide. The TUNEL reaction was performed as described in Table II. Numbers of apoptotic cells in one high power field are shown.

MHC (Table VI). Interestingly, the proliferative response in Acl-11- and Acl-11[4Y]-injected mice had increased in intensity over that of PBS-injected mice by about two times, while the proliferative response in Acl-11[4A]-injected mice had increased by three to four times. Furthermore, about 50% of CD4<sup>+</sup> cells fluxed calcium from PBS-, Acl-11-, and Acl-11[4A]-injected mice in response to Acl-11, while only 25–40% of CD4<sup>+</sup> cells from Acl-11[4Y]-injected mice fluxed calcium. These data suggest that some of the cells from Acl-11[4Y]-injected mice had become anergized. By day 4, both CD69 and IL-2R expression had been down regulated, while the proliferative responses of peripheral T cells to MBP peptide from Acl-11-, Acl-11[4A]-, and Acl-11[4Y]-injected mice were comparable to each other and were two to three times more intense than that of PBS-injected mice. By day 6, the proliferative responses from peptide-injected mice had subsided and were similar to that of PBS-injected mice. Despite evidence *in vitro* that these T cells are actively proliferating, there was no indication that MBP-specific T cells expanded *in vivo*, since the number of CD4<sup>+</sup>V $\beta$ 8.2<sup>+</sup> cells did not increase noticeably. These studies indicate that, at day 1 after injection of peptide, affinity of peptide for MHC correlates with ability to activate T cells in the periphery, but by day 4, this correlation has broken down, since T cells from all peptide-injected mice proliferate at an equally enhanced level.

Cellular expansion followed by rapid deletion of mature peripheral T cells after antigenic stimulation was initially described for superantigens (Jones et al. 1990, Webb et al. 1990, Kawabe & Ochi 1991, MacDonald et al. 1991). However, this type of expansion/deletion has also been seen for a number of other antigens (Table VII). In addition, CD8<sup>+</sup>, CD4<sup>+</sup> and  $\gamma\delta$  T cells all exhibit this sequence of

TABLE VI

*The effect of i.v. injection of peptides on the peripheral CD4<sup>+</sup> population in MBP-TCR mice*

Treatment	Day	CD69	IL-2R	Ca <sup>2+</sup> flux	Proliferative response
PBS	1	–	–	++	+
	4	–	–	++	+
Acl-11	1	+	±	++	++
	4	–	–	++	+++
Acl-11[4A]	1	++	+	++	+++
	4	–	–	++	+++
Acl-11[4Y]	1	+++	++	±	++
	4	–	–	++	+++

Lymph nodes were removed at days 1 and 4 after i.v. injection of PBS or 1.2 mg of Acl-11, Acl-11[4A], or Acl-11[4Y]. The expression of CD69, an early activation marker, and IL-2R in the CD4<sup>+</sup> population was determined by staining with a phycoerythrin-conjugated monoclonal antibody specific for CD4, and biotinylated monoclonal antibodies specific CD69 or the alpha chain of the IL-2 receptor (IL-2R) and analyzed by flow cytometry.

TABLE VII  
*Examples of tolerance through peripheral clonal deletion of mature T cells following antigen stimulation*

Type of antigen (route of injection)	T cells targeted	Expansion /Anergy	Peak of deletion	Evidence for apoptosis	Reference
<b>Superantigens</b>					
SEB (i.v.)	CD4 <sup>+</sup> >CD8 <sup>+</sup>	Yes/Yes	Day 7	Yes	Kawabe et al. 1991
SEB (i.v. or i.p.)	CD4 <sup>+</sup> >CD8 <sup>+</sup>	Yes/Yes	Day 4-7	No	MacDonald et al. 1991
SEA (i.v.)	CD4 <sup>+</sup> and CD8 <sup>+</sup>	Yes/nd	Day 4-9	No	McCormack et al. 1993
Mls (i.v.)	CD4 <sup>+</sup>	Yes/No	Day 4-7	No	Webb et al. 1990
Mls (NA)	CD4 <sup>+</sup> and CD8 <sup>+</sup>	Yes/nd	Day 7-9	No	Jones et al. 1990
<b>Membrane-bound</b>					
H-Y (NA)	CD8 <sup>+</sup>	Yes/Yes	Day 5-9	No	Rocha et al. 1991
H-Y (i.v.)	CD8 <sup>+</sup>	No/No	Day 2-7	No	Zhang et al. 1992
TL (i.v.)	$\gamma\delta$ CD4 <sup>-</sup> CD8 <sup>-</sup>	Yes/Yes	Day 4-8	Yes	Spaner et al. 1993
<b>Conventional</b>					
LCMV (i.v.)	CD8 <sup>+</sup>	Yes/Yes	Day 9-15	No	Moskophidis et al. 1993
LCMV peptide (i.p. +s.c.)	CD8 <sup>+</sup>	Yes/Yes	Day 5	<i>In vitro</i>	Kyburz et al. 1993
MBP peptide (i.v.)	CD4 <sup>+</sup>	nd/nd	Day 9	No	Critchfield et al. 1994
HA peptide (i.v.)	CD4 <sup>+</sup>	Yes/Yes	Day 3-5	Yes	Liblau et al. 1994

This table provides only some of the available examples of peripheral T cell clonal deletion and is not an exhaustive survey of the literature. SEA and SEB, *Staphylococcus aureus* enterotoxin A and B; LCMV, lymphocytic choriomeningitis virus; MBP, myelin basic protein; HA, hemagglutinin; NA, not applicable; nd, not determined.

events (Rocha & von Boehmer 1991, Spaner et al. 1993, Kyburz et al. 1993). Activation in the periphery, rather than deletion, has also been observed (Mamalaki et al. 1992), indicating that certain antigen/TCR combinations are more effective at peripheral deletion than others.

In the mid seventies, Sprent and Miller suggested that activated T cells follow one of three pathways (Sprent 1976, Sprent & Miller 1976). Some T cells would migrate to the intestine and be excreted into the lumen of the gut. Most T cells would home to the spleen and die *in situ*. A minority of T cells would survive and become recirculating memory cells. Although elimination through the gut has not been proven to be a major pathway, recent data show that activated mature T cells can die in peripheral lymphoid organs. Apoptosis has been observed in the periphery after administration of super- and alloantigens (Kawabe & Ochi 1991, Spaner et al. 1993). We provide evidence for peripheral apoptosis after injection of conventional antigen (Liblau et al. 1994). Whether memory cells are generated after the expansion/deletion of a specific T-cell pool remains controversial. After a typical immune response, in which expansion/deletion of antigen-specific T cells also occurs, the frequency of the remaining antigen-specific T cells is increased, providing a basis for immunological memory. However, following massive doses of antigen, in some experiments, specific T cells completely disappear and memory T cells are not detected (Moskophidis et al. 1993). In other experiments (Rocha & von Boehmer 1991, Spaner et al. 1993, Liblau et al. 1994), the deletion is not complete and some antigen-specific T cells survive. Although these cells are initially functionally hyporesponsive, they can revert in the absence of antigen and, in turn, may contain memory cells.

One interesting point is that, at the peak of cellular proliferation prior to deletion, the antigen-specific cells are anergic (Moskophidis et al. 1993, Liblau et al. 1994). This chronology favors the idea that anergy and deletion are distinct stages in a continuing process. Since entry into the cell cycle appears to be a requirement for activation-induced cell death (Russell et al. 1991, Boehme & Lenardo 1993), it is unlikely that, following a single antigenic stimulation, a T cell enters an activation state, becomes anergic and subsequently dies. We favor the notion of a multistep process. Following antigen stimulation, T cells are activated to proliferate and secrete cytokines or to become anergic depending on the type of APCs involved. If the activated T cells are triggered again through the TCR, a program leading to cell death is activated. This is reminiscent of data obtained in a transgenic system studying the induction of self tolerance in which mature thymocytes were rendered anergic by contact with antigen in the thymic medulla and were then deleted by additional stimulation with the same antigen in the periphery (Hammerling et al. 1993). The fact that antigen doses promoting *in vitro* activation-induced T-cell death are higher than those inducing proliferation (Russell et al. 1991, Lenar-

do 1991) and that *in vivo* T-cell deletion/apoptosis is more complete after high-dose or multiple antigen injections are consistent with such a model (Moskophidis et al. 1993, Critchfield et al. 1994). Apoptotic death may be promoted by repetitive stimulation of the TCR itself or in conjunction with local factors. These factors may result from the initial stimulation and could include cytokines such as interferon- $\gamma$  and IL-2 (Liu & Janeway 1990, Lenardo 1991), and cell surface molecules such as Fas antigen (Russell et al. 1993).

The type of APC also influences the decision between proliferation, anergy and death. Dendritic cells, owing to their expression of costimulator molecules such as members of the B7 family, promote proliferation and generation of effector T cells. Non-professional APCs, such as resting B cells, lacking the appropriate costimulator molecules, mediate anergy and possibly death (Eynon & Parker 1992). Massive antigenic challenge could induce preferential loading of antigen on non-professional APCs resulting in tolerance by anergy and deletion.

In this scenario, a situation similar to positive and negative thymic selection is mimicked in the periphery. If mature T cells receive optimal stimulation through the TCR then activation, proliferation and differentiation into effector cells occurs. However, if the stimulation is above this threshold (e.g. repetitive TCR stimulations), or perhaps also if it is below this threshold (e.g. lack of costimulation), then the activated T cells would undergo "peripheral negative selection". Thus, the HA system may reflect a situation where the TCR/peptide/MHC interaction is of high avidity and requires less antigen for repetitive stimulation and subsequent apoptosis. The MBP system, because of the low affinity of Ac1-11 for I-A<sup>u</sup>, and possibly a low affinity of the TCR for Ac1-11/I-A<sup>u</sup>, may require repeated doses of antigen to induce peripheral deletion. This has already been shown to be true for another Ac1-11-specific, I-A<sup>u</sup>-restricted TCR (Critchfield et al. 1994). Despite these findings, a single dose of Ac1-11, Ac1-11[4A], or Ac1-11[4Y] given either *i.v.* or through nasal inhalation can inhibit EAE at the time of disease onset (Metzler & Wraith 1993). Although the mechanisms of these peptide therapies are not yet understood, the difference may be that they treat a polyclonal T-cell population, rather than a monoclonal population of T cells, that expresses TCR with a wide range of affinities for peptide/MHC.

This expansion/deletion scheme can be viewed as a homeostatic mechanism regulating the strength of an immune response and preventing the unrestricted growth of a particular clonotype under potent or chronic antigenic stimulation (Lenardo 1991). This is necessary to allow naive and memory T cells with other specificities to persist. In *lpr* mice, the Fas mutation results in a defective activation-induced apoptotic program in peripheral T cells, which is likely to be the reason for the accumulation of mature T cells observed in the periphery of these mice (Russell et al. 1993). In addition to providing space for T cells with other specificities, peripheral deletion may prevent the potentially harmful effects of factors secreted by large numbers of activated T cells.

## CONCLUDING REMARKS AND SUMMARY

The ability to delete mature T cells has important consequences for tolerance induction. Deletion of mature T cells could play a role in the acquisition of self tolerance. This may apply specifically for those autoreactive T cells that have not been deleted in the thymus due to the restricted expression of the autoantigens in the periphery (Fields & Loh 1992), their low thymic concentration, their inappropriate cellular or tissue thymic location (Hammerling et al. 1993), or their low affinity for TCR and/or MHC. Peripheral deletion also offers the possibility to irreversibly eliminate T cells with given specificities. We have attempted to define the amount of antigen required to induce both thymic and peripheral tolerance in two different TCR transgenic mice. The antigenic dose of HA peptide that induced apoptosis in the thymus of HNT-TCR mice correlated with that required to induce hyporesponsiveness in the periphery. In the MBP-TCR mice, however, high-dose injection of peptide induced thymic deletion and, paradoxically, peripheral T-cell activation. Although the mechanisms underlying the differences in induction of peripheral and thymic tolerance remain to be defined, it is possible that the nature of the TCR/peptide/MHC interactions account at least in part for the discrepancies. Nonetheless, the results in the MBP-TCR mice with Acl-11, Acl-11[4A], and Acl-11[4Y] suggest that, for some TCR, peripheral deletion may be more difficult to achieve. (This finding needs to be tested with higher doses of peptide, and at later time points than have been studied to date). Despite these findings, Acl-11[4A] and Acl-11[4Y] have been shown to be highly effective in preventing and treating EAE.

High-dose tolerance has been extensively studied but the mechanisms remain elusive. Administration of large doses of aqueous protein antigens *i.v.* could favor processing by non-professional APCs resulting in tolerance by anergy or deletion (Eynon & Parker 1992). Other investigators have stressed that high-dose administration of aqueous protein may have different effects on different Th subsets (De Wit et al. 1992, Burstein & Abbas 1993). Tolerance is induced in the Th1 subset through anergy/deletion while Th2-like cells, more resistant to tolerization, are activated to secrete cytokines such as IL-4 and IL-10, thereby contributing to the unresponsiveness. Recent experiments have demonstrated that high-dose antigen activates and expands peripheral T cells, and sometimes induces anergy and/or deletion. The dose of antigen required for apoptosis, which may be induced through successive ligations of the TCR on activated T cell, depends in part on its affinity for MHC, as well as its stability.

A number of experimental autoimmune diseases can be prevented by *i.v.* injection of large doses of an autoantigen (Cremer et al. 1983, Kaufmann et al. 1993, Critchfield et al. 1994, Tisch et al. unpublished). In one such study, the mechanism was clearly peripheral deletion of autoreactive T cells (Critchfield et al. 1994). To

render such an approach clinically applicable to human autoimmune diseases such as insulin-dependent diabetes mellitus and multiple sclerosis, knowledge of the nature of the interaction between self peptide, MHC and autoreactive TCR is essential.

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