Developmental Regulation of Thymocyte Susceptibility to Deletion by “Self”-Peptide

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Summary

The specificity of the T cell receptor (TCR) repertoire for foreign peptide bound to self-major histocompatibility complex (MHC) molecules is determined in large part by positive and negative selection processes in the thymus, yet the mechanisms of these selection events remain unknown. Using in vitro organ culture of thymi isolated from mice transgenic for a TCR-α/β specific for cytochrome c peptide bound to I-E^k, we analyzed the developmental timing of negative selection (deletion). On the basis of the experiments described below, we conclude that all CD4+8+ thymocytes, and only CD4+8+ thymocytes, are susceptible to negative selection mediated by the cytochrome c peptide antigen. First, we found that deletion of thymocytes resulting from addition of the cytochrome c peptide to the thymic organ cultures can occur at the earliest stage of TCR, CD4, and CD8 coexpression. Second, we found that CD4+8+ thymocytes isolated from positively selecting or nonselecting MHC haplotypes were equally efficiently deleted in vitro, suggesting that positive selection is not a prerequisite for deletion. Third, we examined the effects of TCR/ligand avidity on the developmental timing of deletion by varying the concentration of cytochrome c peptide added to the organ cultures. We detected deletion only at the CD4+8+ stage: intermediate concentrations of peptide that resulted in partial deletion of CD4+8+ cells did not eliminate the appearance of mature CD4+8− cells. Finally, we found that CD4+8− thymocytes were resistant to deletion as well as activation by peptide antigen added to the intact organ cultures. Nevertheless, the CD4+8− thymocytes isolated from the peptide-treated organ cultures responded vigorously to peptide presented by spleen cells in vitro. Thus, the T cells were tolerant of (but not anergized by) self-antigen encountered in thymic organ culture. Together, these results indicate that thymocytes susceptible to negative selection are not developmentally distinct from those susceptible to positive selection, and further, that the thymic microenvironment plays a role in regulating the outcome of TCR/ligand interactions.
scribed above, studies analyzing negative selection of super-antigen-reactive T cells in normal mice have indicated that deletion occurs late in the CD4+8+ stage (13, 14) or at the transition from CD4+8+ to CD4+ or CD8+ (15).

The experiments of Pircher et al. (16) provide an attractive explanation for the observed differences in the timing of deletion mediated by superantigens as compared with conventional MHC/peptide antigens. Using a transgenic strain bearing a TCR reactive to both Mls superantigen and a conventional MHC-restricted viral antigen lymphocytic cho- riomeningitis virus, they examined the developmental timing of deletion mediated by these two antigens. They found that the Mls superantigen deleted thymocytes at the CD4+8+ to CD4+8+ transition and the viral antigen deleted thymocytes at the more immature CD4+8+ stage. To explain their results, the authors speculated that the timing of deletion could depend on the avidity of the TCR for the deleting ligand. For example, high-avidity interactions (in their system, viral antigen) would cause deletion early in the CD4+8+ stage (when cells have very low levels of TCR/CD3), and low-avidity interactions (such as Mls+) would occur later in the CD4+8+ stage, or at the CD4+8+ to CD4+ or CD8+ transition, when TCR/CD3 levels have been upregulated.

The hypothesis that avidity controls the timing of deletion has not been directly addressed in a system where the relative affinities of TCR/antigen interactions are known. Furthermore, the in vivo analyses of deletion described above have not taken into account the levels, developmental timing, or anatomical location of expression of antigens in the thymus. As a result, these in vivo studies have not been able to resolve the question of when and by what mechanism developing thymocytes gain and lose their susceptibility to deletion.

We have overcome these difficulties by using in vitro thymic organ culture of TCR transgenic mice to study the developmental timing of deletion. This system allows manipulation of the time and concentration at which a specific antigen is encountered by developing T cells, providing an advantage not present in in vivo systems. Specifically, we have analyzed T cell selection in thymic organ cultures derived from transgenic mice expressing the 2B4 TCR on >95% of thymocytes (1). In this transgenic strain, in contrast to others (10), the transgene-encoded TCR is expressed efficiently on developing T cells. Thus, thymocytes expressing endogenous receptors are rare, even under selection pressure (1; this study). The 2B4 TCR, originally isolated from the T helper hy- bridoma, 2B4 (17, 18), is specific for the carboxy-terminal fragment of the pigeon cytochrome c (PCC)1 protein bound to the MHC class II molecule, I-E\(^\text{a}\). 2B4+ T cells also respond ~10-50 times more strongly to the homologous peptide of moth cytochrome c (MCC) bound to I-E\(^\text{a}\) than to PCC bound to I-E\(^\text{a}\) (17, 19; our unpublished results).

Using this system we have been able to determine at which points in development thymocytes gain and lose their susceptibility to deletion. Furthermore, we have examined the effects of prior positive selection as well as differences in TCR/ligand avidity on the developmental timing and efficiency of deletion.

### Materials and Methods

**Mice.** 2B4 transgenic (9) and nontransgenic B10.BR/SgSnJ and C57 Bl/10J (obtained from The Jackson Laboratory, Bar Harbor, ME) mice were maintained and bred under standard conditions. Pregnancies were timed from the first day of plug observation (day 0).

**Thymic Organ Cultures.** Fetal thymi were cultured according to procedures previously described (20, 21). Briefly, thymic lobes were removed from fetuses on day 14 or 16 as indicated. A small piece of liver was removed from each fetus for genotype determination. Lobes were placed on a strip of nitrocellulose supported by a sterile gelatin sponge (Upjohn Co., Kalamazoo, MI) soaked in RPMI/10% FCS plus 5 x 10^-5 M \(\beta\)-mercaptoethanol, gentamycin, penicillin, and streptomycin. Cultures were fed on the third day by complete medium replacement. Newborn thymic cultures were established similarly, except that each thymus was cut into six pieces using small curved scissors. Each experiment was repeated at least three times (unless otherwise noted in the figure legend), and a representative example is given in the figures.

**Genotype Determinations.** The liver fragments were dissociated by rapid pipetting, and the cells pelleted, resuspended, lysed, and amplified by the PCR as has been previously described (22). 1 \(\mu\)l of each sample was analyzed by simultaneous PCR using primers specific for the two 2B4 TCR transgenes, and primers specific for the relevant H-2\(^\text{a}\) and H-2\(^\text{b}\) MHC alleles.

**FACS\(^\text{©}\) Analysis.** Directly conjugated rat anti-mouse CD4-PE-conjugated (clone GK1.5) (23) and directly conjugated rat anti-mouse CD8-red 613-conjugated (clone 53-6.7) (24) mAbs were purchased from Gibco-BRL (Gaithersburg, MD). Other antibodies used were hamster anti-mouse CD3 (clone 500A2) (25), biotinylated anti-V\(\beta\)3 (clone KJ25) (26), and FITC-conjugated anti-2B4 \(\alpha\) chain (clone A2B4-2) (18). FITC-conjugated goat anti-hamster IgG-conjugated affinity-purified antibody was purchased from Caltag. Cultured thymic organs were dissociated and the cells stained using above reagents for CD4, CD8, and CD3 simultaneously, and in some cases, parallel samples were stained for CD4, CD8, and CD3 simultaneously, and in some cases, parallel samples were stained for CD4, V\(\beta\)3, and clonotypic 2B4 \(\alpha\) chain. Flow cytometric analysis was performed on a FACSScan\(^\text{©}\) instrument (Becton Dickinson & Co., Mountain View, CA). Ungated data (20,000-30,000 events per sample) were collected in list mode using FACSScan\(^\text{©}\) Research software and analyzed using LYSIS software (Becton Dickinson & Co.). Before analysis of antibody staining, samples were gated on live cells based on forward and side scatter parameters. Sorting was done on a FACStar Plus\(^\text{©}\) (Becton Dickinson & Co.; MIT Flow Cytometry Lab).

**Magnetic Bead Sorting.** Cells were stained using biotinylated anti-CD4 or anti-CD8 (Gibco-BRL), avidin FITC (Cappel Laboratories, Cochranville, PA), and biotinylated magnetic microbeads (Miltenyi Biotec). Stained and unstained populations were separated using a magnetic column (Miltenyi Biotec, Bergish-Gladbach, Germany), and the purity was determined by flow cytometry. Purity of fractions was 80-90%.

**Antigenic Stimulation.** 10\(^6\) irradiated B10.BR (I-E\(^\text{a}\)) spleen cells were plated with 10\(^4\)-10\(^5\) sorted thymocytes in triplicate wells. After 48 h, each well was labeled for 16 h with \(^{3}H\)thymidine (5 \(\mu\)Ci/ml), harvested on filter discs (Skatron, Inc., Sterling, VA), and counted in Biofluor (DuPont Co., Wilmington, DE).

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1 **Abbreviations used in this paper:** MCC, moth cytochrome c; PCC, pigeon cytochrome c.
**Results**

**Peptide Antigen-mediated Deletion Occurs at the Earliest Stage of TCR and Coreceptor Expression.** To determine the earliest stage at which developing thymocytes become susceptible to antigen-mediated deletion, we cultured thymic lobes isolated from mice on the 14th day of gestation. The 14th day of gestation is 3 d before expression of CD4, CD8, or TCR in vivo (data not shown); CD4 + 8 + thymocytes are not observed in significant numbers until the 17th day of gestation. Organ-cultured thymic lobes from both transgenic and nontransgenic littermates were dissociated and analyzed each day beginning at 2 d in culture (gestational day 14 plus 2 d in culture; 14 + 2). As shown in Fig. 1 A, the majority of thymocytes were CD4 + and CD8 + at day 14 + 2. After 3 d in culture, day 14 + 3, the thymocytes began to coexpress CD4 and CD8. By day 14 + 5, the cultures contained significant numbers of CD4 + 8 + and CD4 - 8 + thymocytes. These results are consistent with previous reports describing normal development of thymocytes in organ culture (28). In addition, in all cases >90% of organ cultured thymocytes expressed both chains of the transgenic TCR as detected by specific antibodies (data not shown).

To study antigen-mediated deletion, MCC peptide was added to parallel cultures at day 14 + 1. As shown in Fig. 1 A, the addition of MCC peptide to cultures of nontransgenic thymi had no effect on T cell development. In contrast, H-2k 2B4 α/β transgenic organ cultures showed a marked change in the CD4 vs. CD8 profile in the presence of peptide antigen (Fig. 1 A). In particular, MCC peptide eliminated TCR transgenic thymocytes before or immediately after CD4 and CD8 expression (day 14 + 3). At no stage was CD4/CD8 coexpression observed on thymocytes developing in the presence of peptide. The absolute number of total thymocytes recovered from transgenic lobes on day 14 + 5 was reduced in the presence of peptide (Fig. 1 A). This result suggests that MCC peptide specifically deletes 2B4 transgenic CD4 + 8 + thymocytes as soon as they are formed. In similar experiments, we found that a cytochrome c peptide variant containing an amino acid change that does not affect MHC binding but eliminates the ability of the peptide to stimulate 2B4 T cells (K to Q at position 99 of MCC) (29) does not delete 2B4 + T cells even at 100 μM (data not shown). In addition, we find that essentially all CD4 + 8 + cells can be deleted by peptide, as <1% of the remaining cells express the CD4 molecule on their surface. These results are in contrast to those predicted by the in vitro studies of Finkel et al. (30, 31), and the in vivo studies of Guidos et al. (15).

The immediate susceptibility of TCR transgenic CD4 + 8 + thymocytes might have been due to the slightly higher average levels of TCR we and others have found in TCR transgenic strains relative to nontransgenic littersmates (9, 32). In the thymic organ cultures we found that although the levels of TCR/CD3 expression on transgenic thymocytes were on average slightly higher than on those of nontransgenic littersmates, most transgenic thymocytes expressed TCR/CD3 levels comparable to nontransgenic thymocytes (Fig. 1 B). Furthermore, we have not found any greater sensitivity of thymocytes expressing relatively higher levels of TCR to limiting amounts of peptide (data not shown). Therefore, we think it unlikely that premature TCR upregulation can account for the sensitivity to deletion of all the transgenic thymocytes.

**Positive Selection Is Not Required for Deletion.** It has been suggested that positive selection is required before deletion occurs (15). This hypothesis is compelling because it can explain how similar signals through the same TCR can induce either differentiation (positive selection) or deletion (negative selection). This model predicts that positive and negative selection act on distinct populations of immature thymocytes that differ by the downstream signals induced by TCR engagement. The molecular and cellular events of positive selection, or even the developmental stage at which they take place, are not well understood. However, most evidence suggests that positive selection occurs at the CD4 + 8 + stage. For example, the presence of a TCRhigh subset of CD4 + 8 + cells very strongly correlates with the presence of selecting MHC alleles in TCR transgenic mice (32–34; our unpublished results), suggesting that upregulation of TCR levels on CD4 + 8 + cells is the first observable consequence of positive selection. Moreover, kinetic studies of CD4 + 8 + TCRhigh thymocytes in normal mice suggest that these cells are postselection precursors to mature T cells (35). We have previously determined that differentiation of CD4 + 8 + thymocytes in 2B4 TCR transgenic mice is 20 times more efficient on the selecting...
as compared with the nonselecting MHC background (1). Our results described above demonstrate that CD4⁺8⁺ thymocytes are susceptible to deletion as soon as they are formed, before TCR^{high} CD4⁺8⁺ thymocytes are detected in fetal thymi (Fig. 1 B), suggesting that prior positive selection is not required for deletion. However, we tested this directly by determining whether CD4⁺8⁺ thymocytes that developed in the nonselecting MHC H-2\(^b\) (I-E\(^-\)) background, and thus were at least 20-fold less likely to have been positively selected, could be deleted by antigen. Since the nonpositively selecting MHC haplotype is I-E\(^-\), and therefore cannot bind or present cytochrome \(c\) peptide to 2B4 TCR transgenic thymocytes, peptide added to H-2\(^b\) thymic organ cultures has no effect (data not shown). Therefore, we used a disrupted culture system to which we added APC bearing the I-E\(^k\) MHC protein (36). In addition, newborn mice were used for these experiments since they contain greater numbers of thymocytes than fetal mice. We isolated TCR transgenic thymocytes from newborn mice bearing either the H-2\(^k\) (selecting) or H-2\(^b\) (nonselecting) MHC alleles and cocultured them with spleen adherent cells in the presence or absence of 1 μM MCC peptide. After 24 h, the cultures

Figure 1. Time course of deletion in fetal thymic organ culture. (A) Thymi from day 14 2B4 α/β transgenic or nontransgenic fetal mice were cultured in the absence or presence of 1 μM MCC peptide for the indicated number of days. Thymi were disrupted and the cells stained with anti-CD4, anti-CD8, and anti-CD3 mAbs as described in Materials and Methods. Results shown are log-log dot plots of CD4 vs. CD8 staining of cells previously gated on live cells based on forward- and side-scatter parameters. The number of total thymocytes ± SD × 10\(^{-5}\) recovered at the day 14+5 time point is shown in the upper right for each sample and condition. (B) CD3 expression levels on CD4⁺8⁺ thymocytes (indicated in the box) from transgenic and nontransgenic mice from gestational day 14 thymi after 4 and 5 d in culture are shown. The vertical lines are for reference in comparing the different histograms.
were harvested and analyzed by flow cytometry for expression of the transgenic TCR α chain, CD4, and CD8. Fig. 2 shows a representative CD4 vs. CD8 staining of 2B4+ H-2b, or H-2k thymocytes cultured in the presence or absence of peptide. We found that the percentage of CD4+8+ thymocytes from the nonselective as well as the selective MHC backgrounds were reduced five- to sevenfold in the presence of peptide. As shown in Fig. 3, regardless of MHC haplotype, the number of 2B4+ CD4+8+ thymocytes was significantly (p ≤ 0.001, student’s t test) reduced in the presence compared with the absence of MCC peptide. These data indicate that H-2b and H-2k thymocytes are equally susceptible to deletion despite the large difference in efficiency of positive selection in the two haplotypes.

It remained possible that some positive selection occurred during the coculture of H-2b thymocytes with H-2k splenocytes. To test this, FAC§-purified CD4+8+ thymocytes were cocultured with spleen adherent cells and analyzed for CD4, CD8, and TCR expression each day for 3 d. In no case were 2B4+ CD4+8+ thymocytes observed at any time in the cultures. These experiments strongly suggest that spleen adherent cells are not sufficient to positively select thymocytes in vitro. This result is consistent with experiments by one of us and others indicating that hematopoietically derived thymic stromal cells do not mediate positive selection in vivo (1, 37). Taken together, our results strongly suggest that CD4+8+ immature thymocytes are susceptible to antigen-mediated deletion before positive selection.

TCR/Ligand Avidity Does Not Influence the Developmental Stage of Deletion. Our results demonstrate that transgenic thymocytes are susceptible to deletion very early in development. Pircher et al. (16) have suggested that the avidity of the TCR Peptide/MHC interaction could determine the developmental stage at which thymocytes are deleted. We decided to test this model in our TCR transgenic thymic organ culture system, in which we can selectively alter the avidity of the TCR-peptide/MHC interaction by varying peptide concentration. For these experiments, gestational day 16 mice were used since day 16 thymocytes developed into mature CD4+8+ thymocytes more efficiently in organ culture than did thymocytes from day 14 mice (data not shown). Furthermore, after 1 d in culture (day 16+1 or day 0 of peptide addition), thymi were found to contain ≤50% CD4+8+, and no detectable CD4+8− (Fig. 4 A), allowing us to assess the effects of peptide on thymocytes undergoing the CD4+8+ to CD4+8− transition. The thymi used were obtained from heterozygous H-2k/b mice, which express twofold less I-E on their MHC class II-positive cells than homozygous H-2k mice. MCC peptide was added at the indicated concentration on day 16+1, and samples were stained with antibodies against CD4 and CD8 on day 16+6 (Fig. 4 B). It can be seen that at the different peptide concentrations at which deletion is occurring (>0.01 μM), the percentage of CD4+8− cells is reduced roughly in proportion to the reduction of the CD4+8+ population. We counted the number of cells present in cultures on day 16+6 and calculated the number of thymocytes in each subset. As shown in Fig. 5, addition of peptide reduces the total number of cells recovered due to the elimination of CD4+8+ thymocytes. These results in-

![Figure 2](image-url)

Figure 2. Peptide-mediated deletion of H-2b and H-2k CD4+8+ thymocytes. Thymocytes from newborn H-2b and H-2k mice as indicated were dissociated and plated in suspension with H-2k spleen cells in the presence or absence of 1 μM MCC peptide as described in Materials and Methods. After 24 h of cocultivation, cells were stained with antibodies against CD4, CD8, and the TCR transgenic α chain, 2B4. Results shown are log-log dot plots of CD4 vs. CD8 staining. Samples were gated based on scatter parameters for live cells and for transgene-expressing cells. The percent of cells present in each quadrant is shown.

![Figure 3](image-url)

Figure 3. The absolute number of 2B4+ CD4+8+ thymocytes recovered after 24 h in the presence or absence of peptide plus spleen adherent cells (see Fig. 2 and Materials and Methods) was determined for 5 H-2k and 6 H-2b individual animals. The average and standard deviation error bars are shown for each group, plus and minus peptide.
Figure 4. MCC peptide titration fails to detect a difference in the timing of deletion dependent on TCR avidity. Results shown are log-log dot plots of CD4 vs. CD8 staining of cells previously gated on live cells based on forward and side scatter parameters, as described in Materials and Methods. Day 16 fetal thymi from 2B4 α/β transgenic mice were cultured for 6 d in the presence of varying concentrations of MCC peptide. To demonstrate that the majority of CD4+8- thymocytes present on day 6 arose in organ cultures during this time period, CD4 and CD8 expression on thymocytes of a representative thymus analyzed on day 16+1 (day 0 of peptide addition) is shown in A. (B) CD4 vs. CD8 expression at day 6 for thymi incubated in concentrations of peptide varying from 0.003 to 1 μM are shown. At intermediate concentrations of peptide, e.g., 0.03 μM, only partial deletion of CD4+8+ thymocytes is observed with no visible effect on CD4+ cells; at high concentrations of peptide, e.g., 1 μM, both subsets of cells are gone. This experiment was repeated in its entirety once with identical results.

Figure 5. Culture of fetal thymi in the presence of MCC peptide prevents the accumulation of CD4+8+ and CD4+8- thymocytes. Fetal thymic lobes cultured for 6 d, as described in the legends to Figs. 1 and 4, were dissociated and the cells counted. The number of cells in each subset was calculated based on the quadrant percentages shown in Fig. 4.

dicate that CD4+8+ thymocytes are deleted at very low peptide concentrations, and that cells in transition to becoming CD4+8- thymocytes are not preferentially susceptible to deletion by levels of antigen that fail to delete CD4+8+ cells.

CD4+8- Thymocytes Are Resistant to Antigen-mediated Deletion. To determine the last point at which developing thymocytes are susceptible to antigen-mediated deletion, we added peptide to transgenic thymic organ cultures after allowing the thymocytes to develop for several days in vitro. Again, for these experiments gestational day 16 mice were chosen since day 16 thymocytes are initially CD4-8-, yet efficiently develop into mature CD4+8- thymocytes in organ culture. Day 16 fetal thymic organ cultures from 2B4 α/β transgenic mice were established, and 1 μM MCC peptide was added on day 0, at which point CD4+8+ thymocytes are undetectable, or on day 3, at which point CD4+8+ cells are present representing 2-4% of thymocytes (data not shown). All cultures were analyzed on day 16+6. As shown in Fig. 6 A, the addition of peptide on day 0 led to a loss of both CD4+8+ and CD4+8- thymocytes. In contrast, addition of peptide on day 3 resulted in the deletion of CD4+8+ cells but not CD4+8- cells. These data suggest that CD4+8- thymocytes are resistant to peptide-mediated deletion.

The last point at which thymocytes are susceptible to deletion, as well as the kinetics of deletion, were also examined in thymi cultured from newborn mice. The larger size of the newborn thymi allowed us to sample the same thymus at different time points and thus determine the kinetics of peptide-mediated deletion in vitro. Fragments of TCR transgenic thymi were incubated in the presence or absence of peptide and analyzed for expression of CD4, CD8, and TCR at the indicated times. 12 h after the addition of peptide (1 μM), we observed a twofold downregulation of CD4 and CD8 (Fig. 6 B). A similar downregulation was an early result of deletion observed in suspension cultures of TCR transgenic thymocytes exposed to antigen (36). By 36 h, virtually all CD4+8+ cells were eliminated (Fig. 6 B). In contrast, the CD4+8- thymocytes appeared to be relatively resistant to antigen-mediated deletion. We next counted the number of cells present in cultures at the endpoint, 48 h, and calculated the number of thymocytes in each subset. As shown in Fig. 7, slightly more CD4+8- thymocytes, and significantly fewer CD4+8+ thymocytes, were present in cultures incubated in the presence vs. absence of peptide. We also determined the average number of thymocytes present at the time of peptide addition, and found that most CD4+8+ thymocytes present at 48 h were already present in the cul-

Developmental Regulation of Thymocyte Susceptibility to Deletion
A

no peptide

pep. added day 0

pep. added day 3

CD4

CD8

CD4

CD8

CD4

CD8

Figure 6. CD4⁺8⁻ cells are resistant to deletion in thymic organ culture. Results shown are log-log dot plots of CD4 vs. CD8 staining of cells previously gated on live cells based on forward- and side-scatter parameters, as described in Materials and Methods. (A) Fetal thymi from day 16 2B4 α/β transgenic mice were placed in organ culture, and analyzed on day 16 + 6. 1 μM MCC peptide was added to parallel cultures on day 0 or 3 of culture, as indicated. (B) The kinetics of antigen-mediated deletion of neonatal thymocytes in vitro are shown. Thymi isolated from newborn transgenic mice were cut into fragments and then cultured in the absence or presence of 1 μM MCC peptide for 12, 24, or 36 h, as indicated. A decrease in CD4 and CD8 staining of CD4⁺8⁺ thymocytes is visible after only 12 h, and by 36 h the bulk of these cells are deleted. In contrast, CD4⁺8⁻ thymocytes are not deleted by peptide. Anti-CD3 staining profiles (log scale) of the CD4⁺8⁻ (as shown in A) and CD4⁺8⁺ cells (B) are given in the right-most panels for the 36-h time point, plus and minus peptide.

tures at time 0 (data not shown). Thus, the CD4⁺8⁻ thymocytes resistant to deletion had not differentiated in the organ cultures in the presence of peptide. In these experiments, as well as those described previously, >95% of the thymocytes (including CD4⁺8⁻ thymocytes in the presence of MCC peptide) expressed both chains of the transgenic TCR (data not shown). This result indicates that CD4⁺8⁺ but not CD4⁺8⁻ thymocytes were deleted by peptide. The levels of TCR on CD4⁺8⁻ and CD4⁺8⁺ thymocytes in the presence or absence of peptide were also examined. As shown in Fig. 6 B, peptide was found to cause a TCR/CD3 down-modulation on the CD4⁺8⁻ population. Therefore, the deletion resistance of CD4⁺8⁻ thymocytes is not due to their inaccessibility to peptide.

We tested whether the presence of peptide antigen might activate some of the CD4⁺8⁻ transgenic thymocytes in the organ cultures, and induce cell division. If some CD4⁺8⁻ thymocytes are deleted by peptide, and a resistant subset is induced to divide, it could appear that the number of CD4⁺8⁻ thymocytes is remaining constant. To assess the proliferative activity of thymocytes in organ culture, we determined their DNA content by propidium iodide staining, and DNA content analyzed by FACS® (27). In both the presence and absence of peptide, >90% of the thymocytes were in the G0/G1 stage of the cell cycle based on analysis of their DNA content (Fig. 8 A). By comparison, 75% of normal mouse bone marrow cells were in G0/G1 (Fig. 8 A). In other experiments, the cultured thymocytes were purified by magnetic bead sorting into CD4⁺ and CD4⁻ fractions before DNA content analysis. In these experiments >90% of the thymocytes were also in G0/G1, in the presence or absence of peptide (data not shown). We conclude that peptide did not induce the 2B4 transgenic thymocytes to divide in organ cultures.

Expression of the IL-2 receptor α chain is induced on mature T cells by antigenic stimulation (38). As an independent measure of the activation state of thymocytes in organ cultures, we analyzed the expression of the IL-2 receptor α chain on thymocyte subsets by three-color FACS®. CD4⁺8⁻ thymocytes did not express the IL-2 receptor α chain with or without exposure to peptide (data not shown). These data indicate that 2B4 TCR-α/β-expressing CD4⁺8⁻ thymocytes are not activated by antigen before their exit from the thymus.

We next determined whether thymocytes exposed to antigen in thymic organ culture could respond in conventional stimulation assays. This was of interest since the stimulation
of T cells in vitro by fixed APC (39), or by peptide presented by purified MHC in planar membranes (40), has been observed to induce a state of antigen-specific proliferative nonresponsiveness, termed anergy. Since peptide presented in organ culture was found not to activate CD4+8- thymocytes, we hypothesized that they might be anergized. To test this, we determined the response of organ culture-derived thymocytes to peptide in conventional in vitro stimulation assays. Transgenic thymi incubated for 48 h in the presence or absence of 10 μM MCC peptide were separated into CD4+8-, CD4-8-, and CD4-8+/CD4+8+ populations by magnetic bead sorting. The purified cells were incubated in the presence or absence of peptide with irradiated H-2 k spleen cells and pulse-labeled 48 h later with [3H]thymidine. All fractions from peptide-treated or untreated organ cultures were able to mount a vigorous proliferative response to antigen. As shown in Fig. 8 B, CD4+8- thymocytes pretreated with peptide were found to respond two times better than their untreated counterparts. Therefore, the CD4+8- thymocytes were not anergized by peptide in organ culture.

Discussion

We have described a system useful for the analysis of the molecular and cellular regulation of T cell selection. We have found that TCR transgenic thymi will develop faithfully in organ culture in vitro. This system has allowed us to manipulate the temporal and quantitative exposure of developing thymocytes to their peptide antigen. Using this system, we have been able to determine the developmental time course of deletion susceptibility, and to test the predictions of models that have been put forward to explain the developmental regulation of T cell selection.

These experiments have demonstrated that deletion of self-reactive thymocytes can occur at the earliest CD4+8+ stage. In fact, in fetal organ cultures incubated with peptide from the initiation of culture, no CD4+8- cells are ever observed. These results are consistent with a previous report indicating that the superantigen SEB deletes newly formed Vβ8-bearing CD4+8+ thymocytes (41). These results suggest that thymocytes do not pass through an immature CD4+8- developmental stage during which they are resistant to deletion, as has been proposed (15, 30, 31).
It has also been suggested that thymocytes may not be sensitive to deletion until after they have been positively selected (15). We find that thymocytes do not require prior exposure to positively selecting thymic epithelium to become susceptible to deletion. Thus, our results agree with those of others indicating that deletion can occur both before and after positive selection (16, 32, 42). These results argue against the developmental stages during which they are first susceptible to positive selection and then to deletion. Instead, our results suggest that thymocytes are susceptible to deletion at the same developmental stage at which they are susceptible to positive selection. Significantly, these data imply that the outcome of TCR engagement may not be determined by developmentally regulated coupling of TCR signaling to distinct signal transduction mechanisms. Instead, the outcome of TCR engagement may be determined by the strength of the interaction, by modifications of the primary TCR signal by secondary interactions determined by APC, or both.

We have extended this analysis further by testing the model proposed by Pircher et al. (16) to explain how, depending on the antigen, thymocytes bearing the same TCR can be deleted either before or after positive selection (16). This model predicts that TCR/ligand avidity determines the developmental stage at which thymocytes are deleted. By modulating the number of antigen receptor interactions, we have found no effect of TCR avidity on the developmental stage at which deletion takes place. Our result indicates that avidity alone cannot explain the stage-specific deletion observed for certain antigens.

Although clearly responsible for a large part of the tolerance of the immune system to self-proteins, intrathymic deletion cannot account for all instances of self-tolerance. There are many self-proteins that are not present in the thymus, and therefore are not able to induce deletion of reactive thymocytes. Nevertheless, the immune system makes no response to these peripheral self-proteins. This problem, termed peripheral tolerance, has been investigated using a variety of experimental protocols, which together reveal a variety of mechanisms of immune tolerance. For example, transgenic mouse strains that have been engineered to express foreign proteins outside the thymus are usually tolerant to these proteins. In one case, in vivo tolerance was correlated with the downregulation of reactive TCRs and CD8 coreceptors from the cell surface (43). In this experiment and others involving MHC antigens expressed on pancreas cells (44, 45), the tolerance observed in vivo was found to be reversible in vitro. Antigen-specific in vivo and in vitro nonresponsiveness or anergy was observed in another case (46). In a third case, peripheral tolerance to Mls superantigens occurred by a nonthymus-dependent clonal elimination mechanism (47, 48).

We have found that CD4+8- thymocytes exposed in organ culture to high concentrations of peptide do not proliferate. Our results are in contrast with those of Jenkinson et al. (41), who showed that the superantigen SEB induces mature thymocytes to proliferate when added to day 14 fetal thymic organ cultures on the 16th day of culture. There are at least two possible explanations for this difference. First, it may be that conventional peptide/MHC complexes and superantigens differ in their ability to induce proliferation of thymocytes in organ culture. Alternatively, differences in the developmental stage of the responding thymocytes or thymi may account for the different results obtained. In any case, our experiments do not rule out the possibility that some mature thymocytes may be capable of antigen-induced proliferation in situ. For example, the thymocytes may need to be exposed to peptide for longer than 48 h in order to proliferate.

CD4+8- thymocytes exposed in organ culture to high concentrations of peptide are not made anergic. Nevertheless, CD4+8- thymocytes contact their antigen in intact organ cultures, since peptide induced a twofold downregulation of surface levels of TCR/CD3. These results suggest that the thymic microenvironment plays a role in regulating the outcome of TCR/ligand interactions. The nonresponsiveness and TCR downregulation of CD4+8- cells in intact thymus is perhaps most similar to the cases of peripheral tolerance to MHC expressed in transgenic pancreas or neuroectodermal cells described above (43-45). Since CD4+8- thymocytes are found primarily in the thymic medulla (49), our results suggest that the medullary APC of the organ-cultured thymus are, like pancreas or neuroectodermal cells, insufficient to prime an immune response. This result was unexpected; although the thymic epithelium is readily tolerated by allogeneic graft recipients (50, 51), the hematopoietic stromal cells of nontreated thymic grafts, many of which reside in the thymic medulla, are targets for graft ejection across allogeneic barriers (52). To resolve this issue it will be necessary to determine which APC are presenting peptides to thymocytes in the medulla of cultured thymus.

Our experiments have addressed the developmental stage requirements for antigen-mediated deletion in the thymus. This TCR transgenic thymic organ culture system should allow us to address other specific questions pertaining to the mechanisms of positive and negative selection. For instance, it may be possible to directly address the role of peptides in positive selection. Our data in Fig. 4 appear to show a slight increase in the efficiency of positive selection in the presence of low levels of MCC peptide. More experiments are in progress to determine if added peptides, whether MCC, PCC, or substituted peptides with intermediate activities in stimulation assays (29), have effects on the efficiency of positive selection.
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