A Role for Fas in Negative Selection of Thymocytes In Vivo

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Summary

To seek information on the role of Fas in negative selection, we examined subsets of thymocytes from normal neonatal mice versus Fas-deficient lpr/lpr mice injected with graded doses of antigen. In normal mice, injection of 1–100 μg of staphylococcal enterotoxin B (SEB)-induced clonal elimination of SEB-reactive Vβ8+ cells at the level of the semi-mature population of HSA hi CD4+ 8− cells found in the thymic medulla; deletion of CD4+ 8+ cells was minimal. SEB injection also caused marked elimination of Vβ8+ HSA hi CD4+ 8− thymocytes in lpr/lpr mice. Paradoxically, however, elimination of these cells in lpr/lpr mice was induced by low-to-moderate doses of SEB (≤1 μg) but not by high doses (100 μg). Similar findings applied when T cell receptor transgenic mice were injected with specific peptide. These findings suggest that clonal elimination of semi-mature medullary T cells is Fas independent at low doses of antigen but Fas dependent at high doses. Previous reports documenting that negative selection is not obviously impaired in lpr/lpr mice could thus reflect that the antigens studied were expressed at only a low level.

Self-tolerance induction is largely a reflection of negative selection (clonal deletion) of immature T cells during maturation in the thymus (1–4). Despite the importance of central (thymic) tolerance, some self-antigens, e.g., tissue-specific antigens, are poorly represented in the thymus. Hence, unresponsiveness of T cells to these antigens is thought to involve peripheral mechanisms. Of the various mechanisms proposed to account for peripheral tolerance, considerable attention has been focused on the role of Fas (CD95) (5–8). This cell-surface molecule is upregulated after TCR stimulation and results in activation-induced cell death (AICD)1 through interaction with Fas ligand during the late stages of the primary response. In support of this view, the normal elimination of T cells after the primary response (9–11) is impaired in Fas-deficient lpr/lpr mice (11–16).

During later life lpr/lpr mice develop massive lymphadenopathy and auto-antibody production (17). This syndrome is considered to reflect a breakdown of peripheral tolerance as the result of defective AICD (8, 11–17). The possibility that lpr/lpr mice have a defect in central tolerance seems unlikely since most groups have failed to find evidence for impaired negative selection in the thymus of lpr/lpr mice (11, 12, 17–25). Nevertheless, a recent study found reduced apoptosis of cortical thymocytes in lpr/lpr mice after injection of specific peptides or anti-TCR mAb (26). However, this effect was only apparent within the first 24 h after injection.

Most studies on thymic tolerance have focused on negative selection occurring in the cortex. Recently, we obtained evidence that negative selection can operate at the level of the semi-mature subset of heat-stable antigen (HSA) hi CD4+ 8− cells found in the medulla (27). Thus, combined TCR/CD28 ligation in vitro induced rapid (<24 h) induction of apoptosis in HSA hi CD4+ 8− cells; by contrast, for fully mature HSA lo CD4+ 8− thymocytes TCR/CD28 ligation caused T cell activation rather than death. An unexpected finding in these experiments was that Fas played a decisive role in TCR/CD28-mediated apoptosis, but only when TCR ligation was induced with a high concentration of anti-TCR mAb. With a low-to-moderate concentration of this mAb, apoptosis induction was Fas-independent. Therefore, the implication is that Fas expression might play an important role in negative selection, but only for antigens expressed at a high level.

Since the above data were derived from a highly artificial in vitro model, the relevance of the data to normal negative selection in vivo is questionable. To seek direct evidence on the possible role of Fas in negative selection, we have now examined the effects of injecting normal versus lpr/lpr mice with various doses of Staphylococcus enterotoxin B (SEB), a soluble superantigen (SAg) recognized by Vβ8+

Abbreviations used in this paper: AICD, activation-induced cell death; BrdU, bromodeoxyuridine; DP, double positive; guinea pig C, guinea pig complement; HSA, heat-stable antigen; ova, ovalbumin; SEB, staphylococcal enterotoxin B; SP, single positive; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

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CD4+ T cells (28); previous studies have shown that injection of this antigen induces clonal elimination of T cells in the thymus (29). We also examined negative selection in D011 TCR transgenic mice (30) after injection of specific ovalbumin (ova) peptide. In each situation, injection of antigen caused elimination of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>+</sup> thymocytes. However, in lpr/lpr mice, negative selection failed to occur when the dose of antigen was raised to a high level.

**Materials and Methods**

Mice. C3H/H<sub>E</sub> (C3H), MRL/M or Fa<sup>sr</sup> (MRL/L<sup>pr</sup>/), C3H, MRL-Fa<sup>sr</sup> (C3H<sup>pr</sup>/), C57BL/6 (B6), and B6-Fa<sup>sr</sup> (B6<sup>pr</sup>/) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility. D011 TCR transgenic mice (30) were bred in our facility and backcrossed three times to MRL/L<sup>pr</sup>/, when used in experiments, neonatal mice were typed individually for Fa<sub>s</sub> and TCR clonotype expression using specific mAbs.

Antibodies. Antibodies specific for the following markers have been previously described (30, 31): CD4 (R172, rat IgM), CD8 (3.163.8, rat IgM), CD25 (7D4, rat IgM), HSA (J11D, rat IgM), Thy 1.2 (J1), rat IgM), D011 TCR (KJ1-26, mouse IgG), TCR V<sub>b</sub> (F23.1, mouse IgG), and class II (M5/114, rat IgG). Purified mAbs from ascites specific for TCR-8.1 and V<sub>b</sub> mAbs were purchased from The Jackson Laboratory (Bar Harbor, ME) and Harlan Sprague-Dawley (Indianapolis, IN), and biotinylated anti-V<sub>b</sub> (MR5-2) or anti-CD28 (37.51) mAbs or medium alone for 20 h (27). For flow cytometry analysis, purified CD8<sup>+</sup> thymocytes were stained with a mixture of biotinylated anti-V<sub>b</sub> (MR5-2) or anti-CD28 (37.51) mAbs, followed by TRI-COLOR conjugated streptavidin (Calbiochem, La Jolla, CA). For TUNEL staining, purified cells were stained with FITC-conjugated anti-HSA mAbs, followed by PE-streptavidin (GIBCO BRL). After staining for surface markers, cells were fixed with paraformaldehyde and then incubated with FITC-conjugated anti-HSA mAbs, followed by PE-streptavidin (GIBCO BRL). A Role for Fas in Negative Selection of Thymocytes In Vivo

**Results**

Effects of SEB Injection in Adult versus Neonatal C3H Mice. To examine the effects of SEB on negative selection, SEB was injected intraperitoneally into normal C3H versus Fa<sub>s</sub>-deficient MRL/L<sup>pr</sup>/ mice. In these immunogenic I-E<sup>k</sup> strains, SEB is recognized by V<sub>b</sub> T cells, especially CD4<sup>+</sup> cells (29). In a previous study, injection of SEB was reported to cause a twofold reduction in the proportion of V<sub>b</sub> T cells in unseparated thymocytes 1 d later both in normal and Ipr/lpr mice (16); whether this finding was V<sub>b</sub>-specific was unclear. The V<sub>b</sub>-specific effects of SEB injection on subsets of thymocytes in normal C3H mice is discussed below. In all experiments a single dose of SEB was injected.

At the level of CD4<sup>+</sup> 8<sup>+</sup> double-positive (DP) thymocytes, the effects of SEB injection in adult (10 wk) and neonatal (4 d) C3H mice were quite different (Fig. 1). In adult mice, injection of a large dose of SEB (200 μg) caused pronounced destruction of DP cells and reduced the total cellularity of the thymus by ~80% at 1 d after injection; the elimination of DP cells was associated with a reciprocal increase in single-positive (SP) CD4<sup>+</sup> 8<sup>+</sup> and
In neonatal mice, by contrast, a large dose of SEB (50 μg) caused little or no reduction in the proportion of DP cells and led to a smaller (20–50%) reduction in total cellularity. In contrast to DP cells, SEB injection had a clear VB-specific effect on CD4\(^{18}\) SP cells, in both adult and neonatal mice (Fig. 1). At 2 d after injection, SEB injection caused little or no alteration in the total proportion of SEB-reactive VB\(^{8}\) CD4\(^{18}\) cells. However, when these cells were typed for HSA expression, the VB\(^{8}\) CD4\(^{18}\) subset showed a marked and selective reduction of semi-mature HSA\(^{hi}\) cells and a reciprocal increase in fully mature HSA\(^{lo}\) cells. In control PBS-injected mice, by contrast, the majority of the VB\(^{8}\) CD4\(^{18}\) cells were HSA\(^{hi}\). The effect of SEB injection appeared to be specific for VB\(^{8}\) cells since there was no change in the HSA phenotype of VB\(^{6}\) cells.

SEB injection thus led to substantial elimination of HSA\(^{hi}\) VB\(^{8}\) CD4\(^{18}\) cells while causing apparent expansion of HSA\(^{lo}\) cells; these findings applied in both adult and neonatal mice. Unless stated otherwise, the experiments discussed below refer to neonatal (4-d-old) C3H mice.

**Figure 1.** SEB-mediated negative selection of VB\(^{8}\) CD4\(^{18}\) thymocytes in adult and neonatal C3H mice. Adult (10-wk-old) or newborn (4-d-old) mice were injected intraperitoneally with PBS or the indicated amount of SEB, and thymuses were harvested 2 d after injection; thymocyte suspensions were four-color stained for CD4, CD8, HSA, and VB8 or VB6 and were FACS\(^\text{®}\) analyzed as described in Materials and Methods. The following FACS\(^\text{®}\) plots are shown: CD4 versus CD8 on all thymocytes (left); VB8 and VB6 expression on gated CD4\(^{18}\) cells (middle); and HSA expression on gated VB8 CD4\(^{18}\) and VB6 CD4\(^{18}\) cells (right). The average number of total thymocytes recovered from each mouse is shown at the left. The FACS\(^\text{®}\) plots refer to thymocytes from individual mice and are representative of data from 2 to 3 mice per group.

**Figure 2.** The kinetics of negative selection of VB\(^{8}\) CD4\(^{18}\) cells induced by injection of SEB. Neonatal (4-d-old) C3H mice were injected intraperitoneally with SEB (25 μg) at 1, 2, or 3 d before mice were killed. Thymocytes from SEB-injected mice were then stained with mAbs specific for VB8 or VB6, CD4, CD8, and HSA. (A) Left: Total cell numbers (mean data from 5–8 mice per group) and CD4 versus CD8 expression (data from one representative mouse). MIDDLE AND RIGHT: (a) VB8 expression on gated CD4\(^{18}\) cells; (b) HSA expression on gated VB8 CD4\(^{18}\) cells; (c) VB8 expression on gated HSA\(^{hi}\) CD4\(^{18}\) cells; (d) VB8 expression on gated HSA\(^{lo}\) CD4\(^{18}\) cells. (B) Total numbers of CD4\(^{18}\) thymocyte subsets per mouse recovered from SEB-injected mice at the time points shown. Total numbers of VB8\(^{8}\) HSA\(^{hi}\) versus VB8\(^{6}\) HSA\(^{lo}\) cells (left) and VB6\(^{6}\) HSA\(^{hi}\) versus VB6\(^{6}\) HSA\(^{lo}\) cells (right) are shown. The data represent the mean values obtained from two different experiments involving 5–8 mice per group.
that the proportion of Vβ8+ cells was slightly reduced on day 1 (20 h), increased on day 2 (44 h), and greatly reduced on day 3 (68 h). Examining HSA expression on Vβ8+ CD4+ 8− cells (Fig. 2A, column b) showed that HSA hi cells were present on day 1, greatly reduced on day 2, but again visible on day 3. When the data were expressed as total numbers of cells/thymus (Fig. 2B, left), numbers of Vβ8+ HSA hi CD4+ 8− cells were slightly reduced on day 1 and substantially reduced on days 2 and 3; levels of Vβ6+ cells, by contrast, remained largely unchanged (Fig. 2B, right). Essentially similar findings applied when Vβ8 expression was examined on gated HSA hi CD4+ 8− cells (Fig. 2A, column c). Thus, Vβ8+ cells in the HSA hi subset were present at day 1 but markedly reduced on days 2 and 3. This applied to both the proportion of cells (Fig. 2A, column d) and total cell numbers (Fig. 2B).

The data on Vβ8+ HSA lo CD4+ 8− cells were more complex. Thus, total numbers of these fully mature cells were moderately reduced on day 1, elevated on day 2, and greatly reduced on day 3 (Fig. 2B, left). Similar findings applied to the proportion of Vβ8+ cells in gated HSA lo CD4+ 8− cells (Fig. 2A, column d).

Other Thymocyte Subsets. Since CD4+ 8− thymocytes arise from CD4+ 8+ precursors through progressive down-regulation of CD8, FACS-gated CD4+ 8− thymocytes include a proportion of CD4+ 8+ cells, which are presumably less mature than CD4+ 8− cells. The tolerance susceptibility of CD4+ 8+ (CD4+ 8 hi), CD4+ 8−, and CD4+ 8lo cells to SEB is shown in Fig. 3 (note the “sharper” FACS® gates for CD4+ 8− cells in Fig. 3 than in Figs. 1 and 2). At day 2 after SEB injection, it can be seen that the proportion of Vβ8+ cells (the data in parentheses refer to mean values) was considerably reduced in the CD4+ 8− subset but markedly elevated in the CD4+ 8+ subset; proportions of Vβ6+ cells remained relatively unchanged. For CD4+ 8 hi cells, Vβ8+ cells consisted mostly of TCRlo cells with very few TCR hi cells. Significantly, SEB injection failed to deplete either Vβ8 hi or Vβ6+ cells from the CD4+ 8+ subset.

The above data suggest that, for the FACS® gates used, SEB-induced elimination of thymocytes was restricted to CD4+ 8hi cells and did not include either CD4+ 8lo or CD4+ 8− cells. Here two points should be made. First, before injection, nearly all of the Vβ8 hi CD4+ 8− cells were HSA hi (90%), attesting to their relative immaturity. However, after SEB injection the depleted population of Vβ8+ cells found on day 2 was enriched for HSA lo cells (50%), implying that SEB selectively deleted the HSA hi Vβ8+ cells and caused expansion of the small subset of Vβ8+ HSA lo cells (data not shown). Second, HSA lo cells also accounted for a considerable proportion of CD4+ 8− cells (60%). However, at 2 d after SEB injection the expanded population of Vβ8+ cells consisted almost entirely of HSA hi cells (>90%) (data not shown).

Collectively, these data appear to indicate that the Vβ8+ cells eliminated by SEB injection were predominantly HSA hi CD4+ 8+ cells. However, whether deletion also affected the slightly more mature population of HSA lo cells found in the CD4+ 8+ subset could not be accurately established because of the high levels of fully mature HSA lo cells in the CD4+ 8+ subset. To seek direct information on this issue, we prepared purified populations of HSA lo CD4+ 8+ cells by positive panning (for HSA lo cells) and negative selection with anti-CD8 mAb + C. This treatment removed both CD4+ 8+ and CD4+ 8− cells. The HSA hi cells surviving this procedure were thus CD4+ 8− rather than CD4+ 8+. The susceptibility of these purified HSA hi CD4+ 8− cells to SEB-mediated deletion in vitro is discussed below. For the in vivo experiments that follow, it
should be pointed out that the FACS\textsuperscript{b} gate used to define CD4/CD8 expression was the same as in Figs. 1 and 2 (rather than in Fig. 3); with this gate the cells defined as being CD4\textsuperscript{+} CD8\textsuperscript{−} consisted predominantly of CD4\textsuperscript{+} CD8\textsuperscript{−} cells plus a small proportion of CD4\textsuperscript{−} CD8\textsuperscript{+} cells (∼30%). For convenience, the HSA\textsuperscript{hi} cells in this mixed population will be referred to hereafter as HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells.

Disappearance of HSA\textsuperscript{hi} Cells Reflects Elimination. In interpreting the above data, a key issue is whether the disappearance of HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells on day 2–3 after SEB injection (Fig. 2) simply reflected a switch to HSA\textsuperscript{lo} cells. This possibility is unlikely because previous studies showed that culturing purified HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells with a combination of cross-linked anti-TCR and anti-CD28 mAb in vitro caused many of the cells to undergo apoptosis (27) but failed to downregulate HSA expression on the surviving cells (our unpublished data). Similar findings applied when purified HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells (depleted of CD8\textsuperscript{+} cells; see above) were cultured overnight with SEB plus spleen APCs in vitro (Fig. 4). As manifested by TUNEL staining, this treatment induced substantial apoptosis of V\textsuperscript{b8+} HSA\textsuperscript{hi} cells (but not V\textsuperscript{b8−} cells) relative to cells cultured with APCs without SEB (Fig. 4, A and B). However, there was no reduction of HSA expression on the surviving (TUNEL−) cells (Fig. 4 C). With mature HSA\textsuperscript{lo} cells, exposure to SEB failed to cause apoptosis of V\textsuperscript{b8+} cells (Fig. 4 D); since the culture period was brief (20 h), expansion of V\textsuperscript{b8+} HSA\textsuperscript{lo} cells was not apparent. These findings applied to normal cells stimulated with SEB in vitro. If the disappearance of V\textsuperscript{b8+} HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells from the thymus between day 1 and 2 after SEB injection (Fig. 2) reflected death of these cells (rather than a switch to HSA\textsuperscript{lo} cells), onset of apoptosis would be expected if the cells were harvested on day 1 after in vivo SEB injection and then cultured in vitro without further stimulus. This was indeed the case. Thus, when HSA\textsuperscript{hi} versus HSA\textsuperscript{lo} CD4\textsuperscript{+} CD8\textsuperscript{−} thymocytes were purified from mice given SEB 1 d before and cultured in vitro overnight without APCs, there was significant apoptosis of V\textsuperscript{b8+} HSA\textsuperscript{hi} cells but no apoptosis of V\textsuperscript{b8+} HSA\textsuperscript{lo} cells (relative to the background apoptosis in total HSA\textsuperscript{hi} CD4\textsuperscript{+} CD8\textsuperscript{−} cells).

Figure 5. Susceptibility of HSA\textsuperscript{hi} versus HSA\textsuperscript{lo} subsets of CD4\textsuperscript{+} 8\textsuperscript{−} thymocytes to apoptosis in vitro after exposure to SEB in vivo. HSA\textsuperscript{hi} versus HSA\textsuperscript{lo} CD4\textsuperscript{+} CD8\textsuperscript{−} thymocytes from neonatal C3H mice were cultured in vitro for 20 h with PBS or SEB (50 μg/ml) 28 h earlier were purified by panning and mAb + C (see Materials and Methods). The purified cells were cultured in normal tissue culture medium in vitro for 20 h at 37°C without SEB or APCs and then stained for V\textsuperscript{b8} or V\textsuperscript{b6} followed by TUNEL staining after fixation. Levels of apoptosis found for comparable cultured populations of thymocytes prepared from un.injected control mice have been subtracted from the data shown. The data represent the mean values obtained from three separate experiments.

Figure 4. SEB-induced negative selection of CD4\textsuperscript{+} 8\textsuperscript{−} thymocytes in vitro. Purified HSA\textsuperscript{hi} or HSA\textsuperscript{lo} CD4\textsuperscript{+} 8\textsuperscript{−} thymocytes from neonatal C3H mice were cultured in vitro for 20 h with or without SEB (10 μg/ml) in the presence of splenic APCs. After culture, cells were surface stained for HSA, V\textsuperscript{b8}, and V\textsuperscript{b6}, expression; after washing, cells were fixed and stained by TUNEL for apoptotic cells. (A) Proportion of TUNEL− (apoptotic) cells in gated V\textsuperscript{b8+} cells (left) and V\textsuperscript{b6+} cells (right) in HSA\textsuperscript{hi} versus HSA\textsuperscript{lo} subsets. TUNEL staining is plotted against forward scatter (FSC). (B) Representation of the data shown in A after subtraction of the background values for apoptosis found for cells cultured with APCs without SEB. (C) HSA expression on viable (TUNEL−) V\textsuperscript{b8+} cells (left) and V\textsuperscript{b6+} cells (right) after culture with APCs ± SEB. (D) Proportion of V\textsuperscript{b8+} cells in the viable (TUNEL−) subsets of HSA\textsuperscript{hi} versus HSA\textsuperscript{lo} cells after culture with APCs ± SEB. The data shown are representative of three separate experiments.
and representative of three separate experiments. For the HSAlo cells, the transient re-injection reflected elimination of these cells rather than a cated proliferation of mature T cells to SEB.

BrdU (2 injections 12 h apart) was prepared 1, 2, or 3 d later; BrdU (2 injections 12 h apart) was killed 1, 2, or 3 d later (1 d later for PBS); starting at 20 h before death, the mice were injected with SEB and thymocytes were pre-

is consistent with this possibility. The expansion of V8 cells after SEB injection (Fig. 5) was suggested that a small subset of these cells was eliminated at day 1. The minor degree of apoptosis seen when the cells were cultured in vitro at 1 day after SEB injection (Fig. 2) suggested that a small subset of these cells was eliminated at day 1. The minor degree of apoptosis seen when the cells were cultured in vitro at 1 day after SEB injection (Fig. 2) was consistent with this possibility. The expansion of V8 cells after SEB injection (Fig. 5) was suggested that a small subset of these cells was eliminated at day 1.

The minor degree of apoptosis seen when the cells were cultured in vitro at 1 day after SEB injection (Fig. 5) is consistent with this possibility. The expansion of V8 cells after SEB injection presumably indicated proliferation of mature T cells to SEB.

To examine this possibility, groups of neonatal C3H mice were injected with SEB and thymocytes were prepared 1, 2, or 3 d later; BrdU (2 injections 12 h apart) was administered for the last 20 h before preparation of thy-
mocytes. The data in Fig. 6 make three points. First, in agreement with the known high turnover of CD4+ 8-cells in the neonatal thymus (34), 20 h of exposure to BrdU labeled a high proportion of the V8- and V8+ CD4+ 8-thymocytes in PBS-injected control mice; labeling was higher for HSAhi cells than for HSAlo cells and, for V8+ HSAhi cells of the SEB-injected groups, labeling declined progressively as the 3-d-old mice aged over the 3-d period studied. Second, relative to V8+ cells, SEB injection caused no obvious change in BrdU labeling of V8+ cells at 1 day after injection; this applied to both HSAhi and HSAlo V8+ cells. Third, labeling of V8+ HSAlo cells increased markedly at day 2 after SEB injection and then declined abruptly on day 3. Labeling of V8+ HSAlo cells, by con- trast, was below normal on both day 2 and 3. Collectively, these data indicate that SEB injection caused V8+ HSAlo cells to proliferate for a brief period (evident only on day 2) and then disappear. This transient proliferative response did not affect V8+ HSAhi CD4+ 8-cells.

Dose of SEB and Role of Fas. As discussed earlier (see Introduction), apoptosis of purified HSAhi CD4+ 8-thymocytes in response to combined TCR/CD28 ligation in vitro was found to be Fas independent with a low concentration of cross-linked anti-TCR mAb, but was completely Fas dependent with a high dose of this mAb (27). This finding is illustrated in Fig. 7, where it can be seen that TCR/CD28 ligation of Fas-deficient B6lpr/lpr HSAhi CD4+ 8-cells in culture caused significant apoptosis at a low concentration of anti-TCR mAb (0.1 μg/ml) but not at a high concentration (10 μg/ml). By contrast, apoptosis of HSAhi CD4+ 8-cells from normal B6 mice was higher with a high concentration of anti-TCR mAb than with a lower concentration (Fig. 7). Similar findings applied when Fas expression on normal B6 thymocytes was blocked with Fas-ligand fusion protein (27).

To examine whether this role of Fas in negative selection also applied in vivo, we tested the effects of injecting normal versus lpr/lpr neonatal mice with graded doses of SEB. Based on the results considered above, the expectation was that a moderate-to-low dose of SEB would induce negative selection of HSAhi V8+ CD4+ 8-cells via a

Figure 6. BrdU incorporation by CD4+ 8-thymocyte subsets after SEB injection. Neonatal C3H mice were injected with PBS or SEB and killed 1, 2, or 3 d later (1 d later for PBS); starting at 20 h before death, the mice received two intraperitoneal injections of BrdU 8 h apart (−20 h and −12 h). Purified CD8+ thymocytes from normal B6 mice were co-cultured with anti-CD4 mAb + C) were surface stained for V8 or V8, CD4, and CD8, and then fixed and stained for BrdU. The data show BrdU versus HSA expression on gated V8+ CD4+ 8- (left) and V8+ CD4+ 8- (right) cells. The data are representative of three separate experiments.

Figure 7. Relative sensitivity of normal B6 and B6lpr/lpr HSAhi CD4+ 8-thymocytes to SEB-induced negative selection in vitro. Purified HSAhi CD4+ 8-thymocytes prepared from adult (6-wk-old) mice were cultured in vitro for 20 h in wells coated with a fixed concentration of anti-CD28 mAb (10 μg/ml) plus a low or high concentration of anti-TCR mAb (0.1 μg/ml or 10 μg/ml). After culture, cells were stained for TUNEL to de-
tect apoptotic cells. The data show percentage of apoptotic (TUNEL+) cells after subtraction of the background levels for cells cultured alone. The data represent the mean of four separate experiments.
Fas-independent pathway and thus cause cell deletion in both normal and lpr/lpr mice. By contrast, with a high dose of antigen, negative selection would be Fas dependent and thus occur only in normal and not lpr/lpr mice.

In accordance with this prediction, injecting graded doses of SEB into normal neonatal C3H or MRL mice induced strong deletion of HSA\(^{hi}\) V\(\beta\)8\(^{+}\) CD4\(^{+}\)8\(^{-}\) cells at doses ranging from 1 to 100 \(\mu\)g SEB/mouse (Fig. 8 A and data not shown). When injected into either MRL\(^{lpr/lpr}\) or C3H \(^lpr/lpr\) hosts, by contrast, SEB induced strong deletion at up to 1 \(\mu\)g but caused little or no deletion at higher doses, e.g., 100 \(\mu\)g. This difference between normal and lpr/lpr mice was restricted to HSA\(^{hi}\) cells and did not apply to control V\(\beta\)6\(^{-}\) cells (Fig. 8). Similar findings applied when SEB was injected into adult mice (Fig. 8 B) and also when anti-TCR mAb was injected into neonatal mice (Fig. 8 C).
A moderate dose of antigen induced strong deletion of HSA\textsuperscript{hi} CD4\textsuperscript{+} 8\textsuperscript{+} cells in both normal and lpr/lpr mice, whereas a high dose of antigen induced deletion only in normal and not lpr/lpr mice.

Negative Selection in lpr/lpr TCR Transgenic Mice. To seek further information on the role of Fas in negative selection, we examined the effects of injecting specific peptide into neonatal (4-d-old) normal versus Fas-deficient D011 TCR transgenic mice; mature CD4\textsuperscript{+} 8\textsuperscript{+} cells from this line are strongly reactive to an ova peptide, ova 323–339, presented by IAd (30).

The features of thymocytes from uninjected neonatal D011 mice are shown in Fig. 9A. Gating on the subset of CD4\textsuperscript{+} 8\textsuperscript{+} thymocytes revealed that nearly all of these cells were clonotype-positive (Id\textsuperscript{+}). In contrast to the discrete subsets of HSA\textsuperscript{hi} and HSA\textsuperscript{lo} CD4\textsuperscript{+} 8\textsuperscript{+} thymocytes found in normal nontransgenic mice (Fig. 9A, bottom, thick line), the vast majority of CD4\textsuperscript{+} 8\textsuperscript{+} thymocytes from D011 mice were HSA\textsuperscript{hi} (~90%) and therefore are potentially tolerance susceptible. For this reason, detecting negative selection of D011 thymocytes after ova peptide injection was relatively easy and merely entailed examining CD4/CD8 expression (rather than enumerating HSA\textsuperscript{hi} CD4\textsuperscript{+} 8\textsuperscript{+} cells).

The effects of injecting various doses of ova peptide into neonatal D011 mice are shown in Fig. 9B; thymocytes were examined at day 2 after injection. For normal Fas\textsuperscript{+} D011 mice, injecting either a moderate dose (1 \mu g) or a high dose (100 \mu g) of ova peptide caused a marked deple-
tion of (total) CD4<sup>+</sup> 8<sup>-</sup> thymocytes. With Fas-deficient D011<sup>lpr/lpr</sup> mice, by contrast, depletion of CD4<sup>+</sup> 8<sup>-</sup> thymocytes was apparent only with the lower dose of peptide; with a high dose of peptide deletion of CD4<sup>+</sup> 8<sup>-</sup> thymocytes was minimal. Similar findings applied when the data were expressed as total numbers of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> thymocytes (Fig. 9 C, left). These data apply to thymocytes examined at day 2 after injection. As for SEB, elimination of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells at day 1 after ova peptide injection was quite limited (data not shown).

Injecting D011 mice with ova peptide caused little if any expansion of the residual population of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> thymocytes. Indeed, especially in Fas<sup>-</sup> D011 mice, peptide injection appeared to delete both HSA<sup>hi</sup> and HSA<sup>lo</sup> CD4<sup>+</sup> 8<sup>-</sup> cells (Fig. 9 C, middle). In interpreting this finding it should be noted that, in contrast to normal thymocytes, D011 thymocytes lacked a discrete subset of HSA<sup>lo</sup> CD4<sup>+</sup> 8<sup>-</sup> cells (Fig. 9 A, bottom): HSA expression was relatively homogeneous and the few HSA<sup>lo</sup> cells detected expressed intermediate levels of HSA and formed a continuum with the HSA<sup>hi</sup> cells. Hence it is not surprising that the HSA<sup>lo</sup> cells resembled HSA<sup>hi</sup> cells in being tolerance susceptible.

Unlike CD4<sup>+</sup> 8<sup>-</sup> thymocytes, D011 CD4<sup>+</sup> 8<sup>-</sup> thymocytes were comparatively resistant to negative selection (Fig. 9 B and C, right). Thus, even large doses of peptide caused only minimal (30%) depletion of CD4<sup>+</sup> 8<sup>-</sup> cells.

**Discussion**

The data in this paper make two main points. First, injecting mice with antigen (either SEB or ova peptide) caused marked antigen-specific elimination of the semi-mature population of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> thymocytes within 2 d. Second, based on studies with normal versus lpr/lpr mice, the disappearance of these cells was Fas independent at low doses of antigen but Fas dependent at high doses. Before discussing the role of Fas, several features of the elimination of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells require comment.

The finding that the disappearance of Vβ<sup>+</sup> HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells after SEB injection was associated with a reciprocal increase in HSA<sup>lo</sup> cells raised the possibility that the HSA<sup>hi</sup> cells were not deleted but simply switched to HSA<sup>lo</sup> cells. This possibility is unlikely because exposing HSA<sup>hi</sup> cells to SEB plus APCs in vitro caused rapid onset of apoptosis of Vβ<sup>+</sup> cells and failed to reduce HSA expression on the surviving cells. In addition, when purified HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells were prepared from SEB-injected mice at 1 d after injection, the Vβ<sup>+</sup>-subset of these cells underwent apoptosis in vitro without further contact with SEB, implying that the prior in vivo exposure to SEB had already signaled the cells to die. In light of these findings, the disappearance of Vβ<sup>+</sup> HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells after SEB injection appeared to reflect clonal deletion of these cells rather than differentiation into HSA<sup>lo</sup> cells. For D011 mice, the paucity of HSA<sup>lo</sup> CD4<sup>+</sup> 8<sup>-</sup> thymocytes in these mice makes it most unlikely that the deletion of HSA<sup>hi</sup> cells reflected a switch to HSA<sup>lo</sup> cells. Thus, in this situation, peptide injection caused extensive deletion of total CD4<sup>+</sup> 8<sup>-</sup> thymocytes.

In tissue culture, deletion of HSA<sup>lo</sup> CD4<sup>+</sup> 8<sup>-</sup> thymocytes occurred quite rapidly. Thus, when HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells were exposed to SEB plus APCs in vitro, apoptosis of Vβ<sup>+</sup> cells was clearly apparent after overnight culture; whether these rapid kinetics also apply to ova peptide was not tested. However, under in vivo conditions the elimination of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells was limited on day 1 but prominent at days 2 and 3 both for SEB and ova peptide. Why the disappearance of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells was delayed in vivo is unclear. One possibility is that the intra-peritoneal route of injection entry of antigen into the bloodstream, and thus led to relatively slow accumulation in the thymus. In support of this idea, injecting SEB intravenously rather than intra-peritoneally accelerated the onset of Vβ<sup>+</sup> HSA<sup>hi</sup> cell elimination by ~10 h (our unpublished data). For ova peptide, we have yet to examine the effects of peptide injection beyond day 2. For SEB, the maximal depletion of Vβ<sup>+</sup> HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells after intraperitoneal injection of SEB was at days 2 and 3. These cells began to reappear in the thymus at about day 5 after injection and reached normal levels by day 7 (our unpublished data). The implication is that SEB was cleared from the thymus within a few days, thus allowing rapid replacement of the deleted cells by a new wave of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells derived from DP precursors in the cortex.

With regard to the site of negative selection, many workers consider that the deletion of immature T cells occurs largely in the cortex (3). In favor of this idea, it is well documented that injecting adult mice with antigen causes massive apoptosis in the cortex (30). However, in this situation cortical apoptosis could be a reflection of stress induced by stimulation of mature T cells in the periphery (37). Such non-antigen-specific destruction of cortical thymocytes is less of a problem in neonatal mice, which have few peripheral T cells. Here, it is of interest that, for both SEB and ova peptide, injecting neonatal hosts with antigen caused marked elimination of HSA<sup>hi</sup> CD4<sup>+</sup> 8<sup>-</sup> cells but minimal deletion of CD4<sup>+</sup> 8<sup>-</sup> cells. We considered the possibility that injection of antigen caused deletion of a small subset of CD4<sup>+</sup> 8<sup>-</sup> cells, e.g., TCR<sup>+</sup> cells. However, gating on typical CD4<sup>+</sup> 8<sup>-</sup> cells after SEB injection showed no detectable deletion of either Vβ<sup>+</sup> or Vβ<sup>-</sup> cells (Fig. 3). Similarly, we failed to see deletion of either TCR<sup>+</sup> or TCR<sup>-</sup> CD4<sup>+</sup> 8<sup>-</sup> cells in D011 mice after ova peptide injection (our unpublished data). Hence, for neonatal hosts and the two antigens studied here, negative selection of typical cortical CD4<sup>+</sup> 8<sup>-</sup> cells appeared to be very limited. It should be emphasized that these data do not exclude the possibility that the cortex is an important site of negative selection in other systems. Nevertheless, it is notable that, even in adult mice, SEB-induced deletion of Vβ<sup>+</sup> thymocytes is reported to be undetectable in mice expressing MHC class II molecules only in the cortex (on cortical epithelium) but not in the medulla (38).

In contrast to typical cortical CD4<sup>+</sup> 8<sup>-</sup> cells, the imme-
mediate progeny of these cells, namely TCR hi HSA hi CD4+ 8 lo cells, were strongly deleted after antigen injection (Fig. 3). Since the anatomical localization of CD4+ 8 lo cells in the thymus is unclear, it is possible that some of these cells were deleted in the cortex before entering the medulla. However, it is important to emphasize that, for both SEB and ova peptide, the elimination of SP thymocytes applied not only to HSA hi CD4+ 8 lo cells but also to the larger population of HSA hi CD4+ 8 lo cells, which are presumably more mature than HSA hi CD4+ 8 lo cells. In fact, most of the experiments with SEB concerned CD4+ 8 lo cells rather than CD4+ 8 lo cells. Similarly, for D011 mice, injecting peptide deleted both HSA hi CD4+ 8 lo and HSA hi CD4+ 8 thymocytes (Fig. 9B).

Since the vast majority of lymphoid cells in the medulla are typical SP cells (39), most HSA hi CD4+ 8 lo thymocytes are probably situated primarily in the medulla rather than the cortex. In support of this idea, the medulla contains considerable numbers of HSA hi cells in tissue sections (our unpublished data), suggesting that many medullary T cells are HSA hi. In addition, TUNEL staining has revealed that SEB injection leads to increased numbers of apoptotic cells in the medulla at day 2 after injection (our unpublished data). For these reasons, it would seem highly likely that the deletion of HSA hi CD4+ 8 lo cells takes place mainly in the medulla (although the possibility that these cells initially receive a death signal in the cortex cannot be excluded[40]).

The elimination of HSA hi CD4+ 8 lo cells after antigen injection did not appear to involve cell division. Thus, cell cycle analysis of HSA hi CD4+ 8 lo thymocytes has shown that the rapid death of these cells after TCR ligation in vitro does not involve entry into cell cycle (our unpublished data). Likewise, the elimination of HSA hi CD4+ 8 lo cells in vivo was not preceded by an increase in BrdU incorporation (Fig. 6). This finding contrasts with fully-mature T cells where TCR-mediated apoptosis (AICD) generally occurs slowly and is preceded by an overt proliferative response (41). Hence, for the fully mature subset of HSA lo CD4+ 8 thymocytes, we expected initial contact with SEB to be directly immunogenic for these cells. In line with this prediction, SEB injection caused Vβ8+ HSA lo CD4+ 8 lo cells to proliferate (incorporate BrdU) at 2 days after injection and undergo considerable expansion. Surprisingly, however, the expansion of Vβ8+ HSA lo cells was preceded by a transient decrease in numbers of these cells at day 1 after injection, probably via deletion. Why a proportion of these mature T cells succumbed to early deletion is unclear, although a similar finding has been reported for peripheral T cells after SEB injection (10, 42). It is of interest that the expansion of Vβ8+ HSA lo thymocytes was evident only at day 2 after injection and was followed by a marked reduction in numbers of these cells on day 3; the disappearance of Vβ8+ HSA lo cells was also prominent on day 5 and levels of these cells did not return to normal until after day 7, i.e., ~2 d later than for HSA hi cells (our unpublished data). At face value the rapid elimination of proliferating Vβ8+ HSA lo thymocytes after day 2 could reflect AICD and be the counterpart of the rapid sequence of expansion followed by deletion reported for peripheral Vβ8+ cells after SEB injection (10). However, whether the Vβ8+ HSA lo cells in the thymus died in situ or migrated to the periphery is still unclear.

For SEB, it is worth noting that the near-complete elimination of Vβ8+ cells, including mature HSA lo cells, seen at days 3–5 after a single injection of SEB, is in line with the original report that multiple injections of SEB caused marked deletion of Vβ8+ SP thymocytes 8 d after the last injection (29). Since earlier time points were not examined, the authors may have missed the prior expansion of Vβ8+ cells reported here for HSA hi SP cells.

With regard to Fas, the main finding in this paper is that in lpr/lpr mice the strong elimination of HSA hi CD4+ 8 lo thymocytes induced by SEB, ova peptide, and anti-TCR mAb injection failed to occur when the dose of Ag/Ab was increased to a high level. The implication therefore is that negative selection is critically dependent upon Fas, but only for antigens expressed at a high level. This finding would seem to disagree with reports that injecting large doses of specific peptide into TCR transgenic mice reduced the cellularity of the thymus by >10-fold, in both normal and lpr/lpr mice (11, 25). But because adult mice were used in these experiments, the possibility remains that thymocytes were not eliminated via negative selection but destroyed nonspecifically by stress (37). In another study, giving multiple injections of SEB to neonatal mice over a 2-wk period caused equivalent deletion of Vβ8+ cells in both normal and lpr/lpr mice (43). Since the effects of varying the dose of injected SEB was not tested, the relevance of this finding to our data is unclear.

Much of the evidence that Fas is not involved in negative selection has come from the finding that lpr/lpr mice show relatively normal thymocyte deletion in response to endogenous SAgs and the male (HY) antigen (8, 12, 15, 17–20, 22–24). A corollary of the present data is that Fas is irrelevant for negative selection to endogenous self-antigens unless the concentration of these antigens in the thymus is unusually high. Hence if endogenous SAgs and the male antigen are expressed at only an “average” level (equivalent to a low-to-moderate dose of SEB or ova peptide), there is no discrepancy with the present data.

Although the range of self-antigens causing negative selection is unknown, one can envisage that some antigens, e.g., peptides derived from histones or other common intracellular proteins, are expressed at a relatively high level. Hence, based on our findings with SEB and ova peptide, the elimination of T cells specific for these common self-proteins might be Fas dependent and thus allow the reactive T cells sensitive to the self-antigens to escape central tolerance induction in the thymus. Upon maturation and exit from the thymus, these nontolerant T cells would continue to see these antigens at a high concentration, but now in immunogenic rather than tolerogenic form. Accordingly, the onset of lymphadenopathy and autoimmune disease in lpr/lpr
mice (8, 17) might be a reflection of an ongoing immune response of nontolerant T cells directed to ubiquitous self-antigens expressed at a high level.

This scenario rests on two assumptions. First, one has to argue that the peripheral T cells in lpr/lpr mice are indeed responding to self-antigens. Such a possibility has yet to be proved, although the finding that all subsets of T cells in lpr/lpr mice, including CD4+ 8- and CD4- 8+ cells, have an abnormally high turnover in the periphery is consistent with this idea (24, 44). The second assumption is that if T cells in lpr/lpr mice are indeed autoreactive, the antigens recognized by these cells are expressed at a high level relative to other antigens. Assessing this idea will hinge on defining the peptide specificity of autoreactive T cells from lpr/lpr mice.

As a final comment, it is important to emphasize that our data suggest that Fas regulates negative selection at a relatively high level relative to other antigens. Assessing this idea will hinge on defining the peptide specificity of autoreactive T cells from lpr/lpr mice.

In conclusion, the data in this paper indicate that clonal elimination of antigen-specific T cells in the thymus after antigen injection occurs at a relatively late stage of differentiation and does not involve Fas when the dose of antigen is kept to a low-to-moderate level. However, with a high dose of antigen the presence of Fas becomes crucial for negative selection. In light of these findings, the prior failure to find a clear role for Fas in central tolerance may reflect that the antigens studied were expressed at a relatively low level.

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References


16. Mogil, R.J., L. Radvanyi, R. Gonzalez Quintiliano, R. Miller, G. Mills, A.N. Theofilopoulos, and D.R. Green. 1995. Fas(CD95) participates in peripheral T cell deletion and asso-