Steroid Production in the Thymus: Implications for Thymocyte Selection

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Summary

The mouse thymus was assessed for its ability to produce steroids. Cultured thymic non-T cells produced soluble pregnenolone and deoxycorticosterone, and immunohistochemistry demonstrated steroidogenic enzymes in radioresistant thymic epithelial cells but not in thymocytes. Inhibition of thymic corticosterone production or blockade of the glucocorticoid receptor with RU-486 resulted in enhanced TCR-mediated, antigen-specific deletion of immature thymocytes. These data indicate that locally produced glucocorticoids, because of their antagonism of TCR-mediated signaling for death, may be a key element of antigen-specific thymocyte selection.

Developing thymocytes undergo selection based upon the ability of their clonally distributed TCRs to recognize self-antigens associated with MHC-encoded molecules (1). The mechanistic basis for selection is unknown, but it is generally thought to depend upon the avidity of the TCR for self-molecules (2, 3). Thymocytes whose TCRs have inadequate avidity for self-antigen/MHC die within the thymus, whereas those whose TCRs have high avidity, and are thus potentially harmful, are negatively selected and undergo programmed cell death (apoptosis). Thymocytes whose TCRs have moderate avidity for self-antigen/MHC survive (positive selection) and develop into the T cells that populate the periphery. How ligand recognition by one receptor can lead to two such different fates, death vs. survival and expansion, is an unresolved paradox.

In addition to occupancy of the TCR, glucocorticoids are a potent means of inducing thymocyte apoptosis (4, 5). The thymocytes most sensitive to the lethal effects of glucocorticoids are the same that undergo selection: $CD4^+CD8^+$ (double positive) immature cells located in the cortex; medullary $CD4^+CD8^-$ and $CD4^-CD8^+$ (single positive) cells, like peripheral T cells, are relatively resistant (6, 7). Although no clearly defined role has been elucidated for steroids in thymocyte development, there is reason to believe that such an influence might exist. Stress-induced increases in circulating glucocorticoids cause thymus involution (8, 9), and adrenalectomy in adult animals results in thymus hypertrophy (10). Surprisingly, although both stimuli are lethal by themselves, glucocorticoids and TCR-mediated signals prevent each other's induction of T cell hybridoma and thymocyte programmed cell death in vitro (mutual antagonism), prompting us and others to speculate that the balance of these two stimuli may in part regulate the process of thymocyte selection (11–13). One difficulty with such a model is that the level of circulating glucocorticoids during neonatal life, a time during which thymocyte selection is particularly active, is very low (14, 15). It is intriguing in this regard that there is accumulating evidence that the thymus has endocrine properties. The neuropeptides oxytocin and vasopressin (16), as well as insulin-like growth factors (17, 18), are produced by thymic epithelial cells. The thymus has also been found to express corticotropin-releasing factor (CRF)[1] (19) and adrenocorticotrophic hormone (ACTH) (20), a peptide that induces glucocorticoid production in adrenal cortical cells (21). Given these observations, we asked if the thymus can produce steroids, and if so, whether locally produced steroids influence TCR-directed thymocyte selection.

Materials and Methods

Mice. BALB/c mice were obtained from the Developmental Therapeutics Program, NCI (Frederick, MD). In some experiments, mice were sublethally irradiated (600 cGy, Gammacell 40; Atomic Energy of Canada, Kanata, Ontario, Canada) 4 d before killing and removal of the thymus.

Media and Reagents. In fetal thymic organ cultures and steroid synthesis assays, HL-1, a serum- and corticosteroid-free medium (Ventrex Hycor, Portland, ME) was supplemented with penicillin, 0.015% L-glutamine, 100 mM nonessential amino acids, 1 mM sodium pyruvate, 20 mM Hepes, 250 μg/ml gentamicin, and 50 μM

[1] *Abbreviations used in this paper:* ACTH, adenocorticotrophic hormone; CRF, corticotropin-releasing factor.

2-ME (HL-1 medium). In in vitro deletion experiments, Iscove's modified Dulbecco's medium plus 10% fetal calf serum was used. RU-486 was the generous gift of E. E. Baulieu (Université de Paris-Sud, Paris, France). Trilostane, an inhibitor of 3βHSD, was obtained from Sterling Drug, Inc. (Rensselaer, NY), and aminoglutethimide, an inhibitor of P450scc, was obtained from Ciba-Geigy Corp. (Summit, NJ). Antisera to pregnenolone and deoxycorticosterone, and [^{3}H]pregnenolone and [^{3}H]deoxycorticosterone, were purchased from ICN (Irvine, CA). 22R-hydroxycholesterol and ACTH fragment 1–24 was obtained from Sigma Chemical Co. (St. Louis, MO). Metyrapone was purchased from ICN. All reagents for immunohistochemistry were purchased from Zymed Laboratories Inc. (S. San Francisco, CA). Anti-P450scc, anti-P450c11, and anti-P450aromatase (Oxygene Dallas, Inc., Dallas, TX) and anticytokeratin (Boehringer-Mannheim Corp., Indianapolis, IN) were used for immunohistochemistry. GK1.5 (anti-CD4 [22]), 53-6.7 (anti-CD8 [23]) were used to stain thymocytes, and 30H12 (anti-Thy-1.2 [23]) plus complement was used to deplete thymocytes.

Radioimmunoassays. For secreted steroids, thymi were isolated from 3-wk-old BALB/c mice, minced finely, and treated with collagenase solution (0.5 mg/ml collagenase, 0.5 mg/ml dispase, 4 μg/ml DNase) at 37°C until no fragments remained. Cells were washed and titrated numbers were cultured in supplemented HL-1 medium. For some experiments the medium was supplemented with 6.25 μM 22R-hydroxycholesterol, 10 nM ACTH fragment 1–24, and 50 μM trilostane. Cultures were performed in triplicate and maintained at 37°C with 5% CO_2 for 4 d. Supernatants were collected at 4 d, stored at −20°C, and pregnenolone concentrations determined by RIA (ICN). For measurement of mitochondrial steroids, mitochondria were prepared and assayed for P450scc activity as described (24). Briefly, tissues were crushed and collagenase-treated to a single cell suspension, then homogenized in 250 mM sucrose/10 mM Tris, pH 7.4. Homogenates were centrifuged at 800 *g* for 10 min to pellet nuclei, then supernatants centrifuged at 10,000 *g* for 15 min. Mitochondrial pellets were resuspended in a solution containing 250 mM sucrose, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 15 mM triethanolamine-HCl, 20 mM KCl, and 5 μM trilostane. Equal amounts of mitochondrial protein were incubated with 15 mM malate and 0.5 mM NADP, with or without 25 μM 22R-hydroxycholesterol, for 12 h at 37°C in 100 μl. The reaction was stopped with 500 μl of ice-cold ethanol and extracted 3 times with 2.5 volumes of hexane. The ethanol fraction was collected and evaporated. The pellet was resuspended and assayed for pregnenolone. RIAs for pregnenolone and deoxycorticosterone were performed according to the manufacturer's instructions.

Immunohistochemistry. Tissue and slide preparation was performed as described (25). Briefly, thymi were placed in Bouin's solution for 24 h. Tissues were dehydrated with increasing concentrations of ethanol (70, 90, 95, and 99%) at 1-h intervals, then placed in 100% ethanol. Tissue embedding and sectioning was performed by American Histolabs (Gaithersburg, MD). 5-micron sections were placed on silanated slides. Before staining, the slides were dewaxed in xylene for 5 min, then rehydrated in decreasing concentrations of ethanol (95, 90, and 70%) for 10-min intervals. The final concentration of 70% ethanol had 1% $LiCO_3$ to remove the picric acid remaining from the Bouin's solution. Slides were incubated in 300 mM glycine in PBS, then 1% BSA in PBS for 5 min each. Slides were then incubated with periodic acid (Zymed Laboratories, Inc.) for 45 s to block endogenous peroxidase activity, followed by 10-min incubations each with avidin and biotin (Zymed Laboratories, Inc.). Antibody staining was performed with the Histostain-SP kit (Zymed Laboratories, Inc.) according to the manufacturer's instructions, with the primary antibody incubation lasting for 3 h. Primary antibodies were used at the following concentrations: rabbit Ig (Zymed Laboratories, Inc.) 1:100; anti-P450scc 1:15; and anti-P450c11 1:15 (both from Oxygene Dallas). Slides were counterstained with hematoxylin. The anti-P450scc and anti-P450c11 antibodies stained adrenal tissue intensely but did not stain fibroblasts (data not shown).

Enrichment of Thymic Cell Subsets. Thymic epithelial cells and thymic rosettes, containing macrophages and dendritic cells, were isolated by the procedure of Kyewski et al. (26). Briefly, thymi were digested with 0.5 mg/ml collagenase at room temperature, followed by digestion with collagenase/dispase (0.5 mg/ml collagenase, 0.5 mg/ml dispase, 4 μg/ml DNase) at 37°C. Those cells released by the collagenase digestion (T-ros) were collected and kept separate from those released by collagenase/dispase/DNase digestion (epithelial cells). The two groups of cells were subjected to gravity sedimentation over a fetal calf serum gradient four times to enrich for macrophages and dendritic cells or epithelial cells.

Flow Cytometry. Staining was performed with GK1.5-biotin and 53-6.7-FITC. Flow cytometric analysis was performed with a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Fetal Organ Culture. Fetuses (day 15.5 to day 16) were obtained from 12-h timed pregnant BALB/c mice mated in our animal facility. The day of the vaginal plug was considered day 0. Thymi were removed and split into 2–4 pieces each. Individual fragments were placed on a filter (Millipore Corp., Bedford, MA) floating on a gelfoam sponge in complete HL-1 medium. Cultures were carried out in 1.5 ml of media/well in 12-well plates. Fragments were cultured either alone or with 20 μg/ml of H57 (anti-TCR

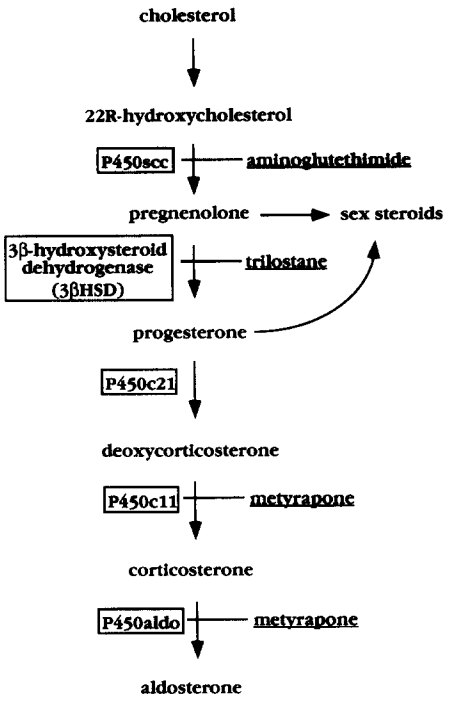


Figure 1. Schematic of some of the steps in steroidogenesis. Many of the steroidogenic enzymes belong to the P450 cytochrome family. Rodents have little or no P450c17 in the adrenal gland, so the major circulating glucocorticoid is corticosterone (49). P450scc and P450c11/P450aldo are located in mitochondria. P450c11 and P450aldo are isoenzymes. Enzymes are boxed, inhibitors are underlined.

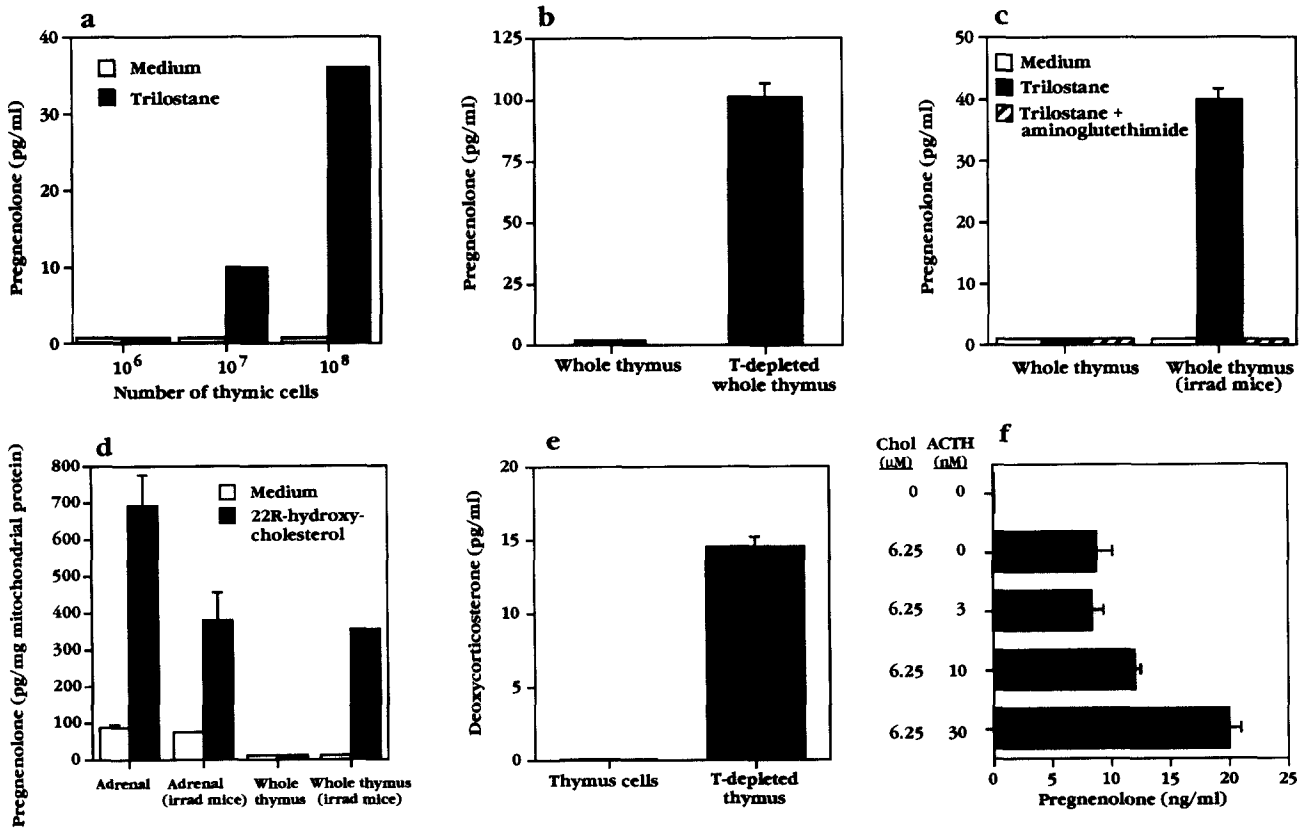


Figure 2. Synthesis of pregnenolone in whole and T-depleted thymus cell populations. (*a*) Thymi were isolated from 3-wk-old BALB/c mice and cultured in triplicate in HL-1 medium supplemented with 6.25 μM 22R-hydroxycholesterol, 10 nM ACTH fragment 1–24, with or without 50 μM trilostane. After 4 d, supernatants were collected and pregnenolone concentrations determined. (*b*) Pregnenolone secretion by 4 × 10^6 cells from collagenase-treated thymus or from collagenase-treated thymus after T cell depletion by an anti-Thy-1.2 antibody, 30H12, plus complement in the presence of 22R-hydroxycholesterol, ACTH, and trilostane. (*c*) 10^6 cells from 3-wk-old BALB/c collagenase-treated normal thymus or thymus from animals sublethally irradiated 4 d previously were cultured for 4 d with 6.25 μM 22R-hydroxycholesterol and 10 nM ACTH, either in HL-1 medium alone or in the presence of 50 μM trilostane, without or with aminoglutethimide, and the supernatants analyzed for pregnenolone. (*d*) Mitochondria were isolated from the indicated tissues from 3.5-wk-old BALB/c mice that had or had not been treated with sublethal γ-radiation 4 d before killing. (*e*) Collagenase-treated thymus cells (4.5 × 10^6) or collagenase-treated thymus cells depleted of thymocytes with anti-Thy-1.2 plus complement (4.5 × 10^6) were incubated for 4 d in HL-1 medium supplemented with 6.25 μM 22R-hydroxycholesterol and 30 nM ACTH. After 4 d, supernatant was collected and assayed for deoxycorticosterone in duplicate. (*f*) 10^8 collagenase-treated thymus cells were cultured for 4 d in the absence or presence of 6.25 μM 22R-hydroxycholesterol, titrated levels of ACTH, and 50 μM trilostane. (*g*) 5 × 10^5 thymic epithelial cells, thymocytes, or T-ros (macrophages and dendritic cells) were cultured with 10 nM ACTH and 6.25 μM 22R-hydroxycholesterol in the presence or absence of 50 μM trilostane for 6 d. Supernatants were harvested and assayed for pregnenolone. Error bars represent SE of triplicate cultures except in *b*, *d*, and *f*, where they represent the standard deviation of assays performed in duplicate.

Cβ), with or without 150 μg/ml of metyrapone for 3 d at 37°C. Fragments were harvested and made into single cell suspensions for staining.

Deletion of H-Y-specific Thymocytes. Preparation of thymic APC monolayers and in vitro induction of apoptosis was performed as previously described by Swat et al. (27). 2 × 10^6 thymocytes from female H-Y-specific D^b-restricted transgenic mice (28) of the H-2^b haplotype were cultured in supplemented Iscove's modified Dulbecco's medium for 36 h in the absence or presence of 10^{-7} M RU-486. Harvested thymocytes were stained with GK1.5-biotin (anti-CD4) and 53-6.7-FITC (anti-CD8). The small, dying $CD4^{lo}CD8^{lo}$ cells were gated out so that the remaining $CD4^{hi}CD8^{hi}$ cells could be better visualized.

Results

Steroid Synthesis by the Thymus. The rate determining step in steroidogenesis is availability of the substrate cholesterol at the inner mitochondrial membrane, where it is metabolized to pregnenolone by the enzyme p450scc (Fig. 1). To determine if the thymus can produce steroids, collagenase-

treated thymus cells were cultured and after 4 d the supernatants were assayed for pregnenolone, the precursor of all other steroids. The cultures were done in serum-free medium to eliminate any exogenous steroids. The cultures were supplemented with the water-soluble cholesterol analog 22R-hydroxycholesterol, to provide a nonlimiting source of cholesterol, and ACTH, which increases mobilization of cholesterol from intracellular stores to the inner mitochondrial membrane and upregulates expression of P450 enzymes (21). Pregnenolone was not detected under these conditions (Fig. 2 *a*). However, in the presence of trilostane, a competitive inhibitor of 3βHSD that prevents further metabolism of pregnenolone, pregnenolone was readily detected and was dependent on the number of cultured cells. To determine the source of the pregnenolone, its production by collagenase-treated thymus cells was determined before and after specific depletion of thymocytes by antibody plus complement. At the cell numbers used (4×10^6 cells/well), no detectable pregnenolone was produced by a population overwhelmingly composed of thymocytes, even in the presence of trilostane. After thymocyte depletion with anti-Thy-1 antibody plus complement, the same number of cells produced easily detectable levels of pregnenolone (Fig. 2 *b*).

Because of the technical difficulties of quantitatively depleting very large numbers of thymocytes with antibody plus complement, further analyses were performed with thymi

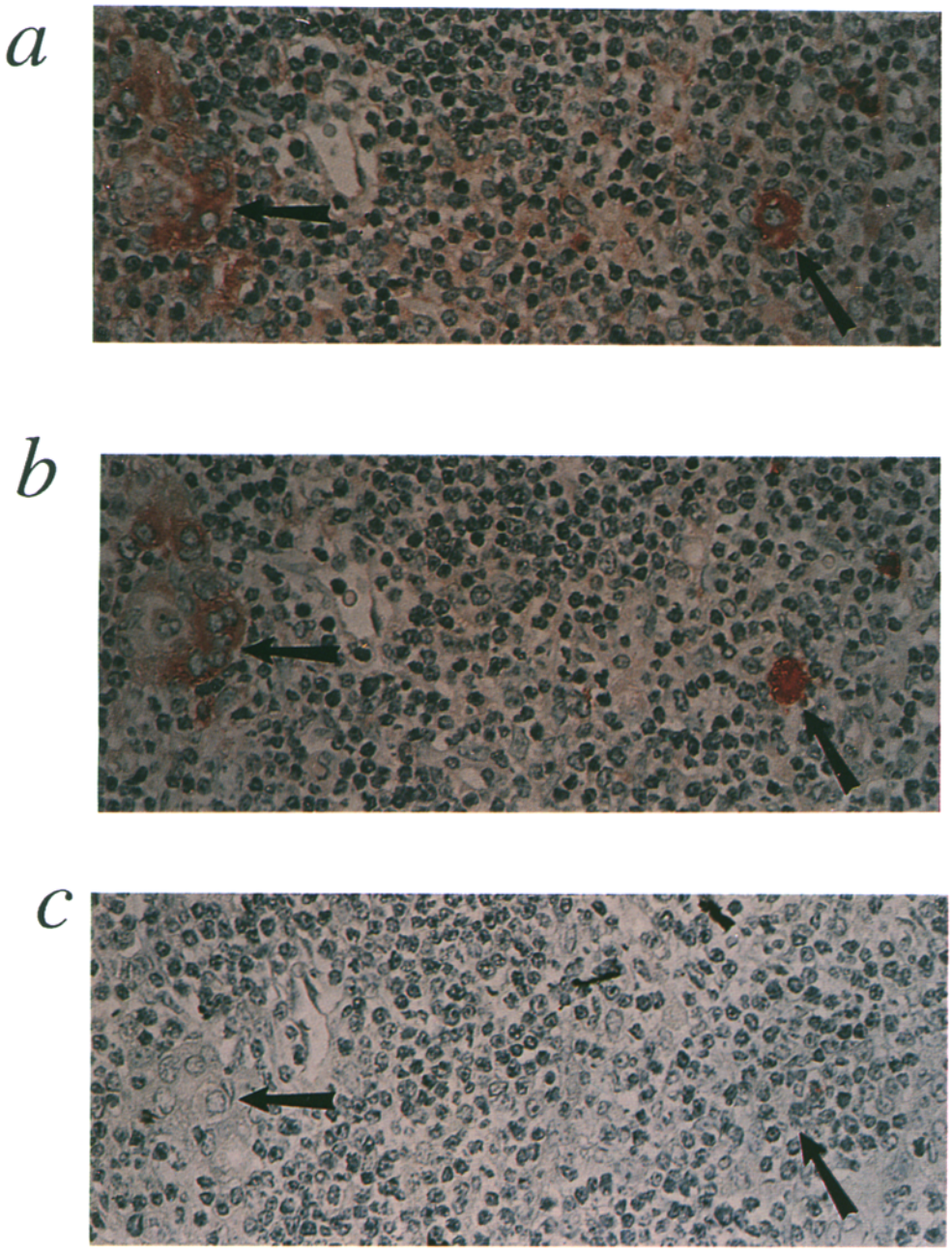
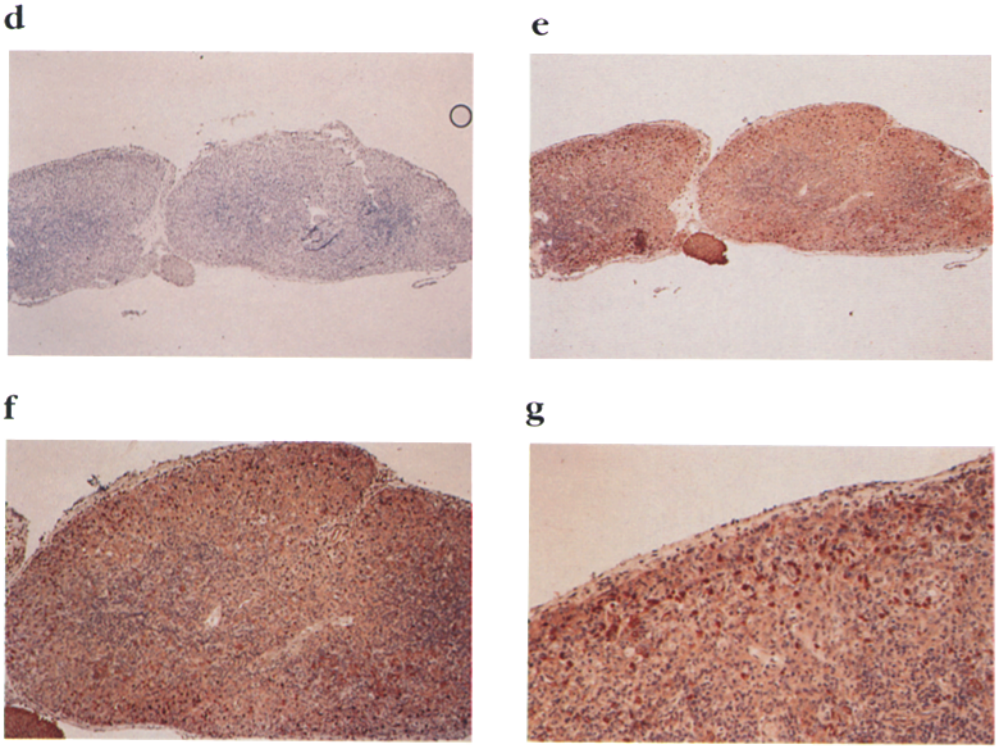


Figure 3. Expression of the steroidogenic enzymes P450scc and P450c11 in mouse thymus. (*a–c*) 400× magnification of serial sections of a thymus from a 3-wk-old BALB/c mouse. Anti-P450scc (*a*), anti-P450c11 (*b*), or control rabbit antibody (*c*) were used to stain serial sections, which were developed with an indirect antibody and peroxidase. The peroxidase reaction results in the formation of a red precipitate. (*d–g*) Expression of steroidogenic enzymes in thymus depleted of thymocytes by sublethal irradiation. Rabbit Ig (*d*, 50× magnification) and anti-P450scc serum (*e*, 50×; *f*, 100×, *g*, 200× magnification). Note that the serial sections are 5 micron in diameter, and so not all cells found in one section will be represented in the adjacent section.

from mice sublethally irradiated (600 cGy) 4 d previously, which resulted in loss of ≥98% of thymocytes. 10^6 cultured collagenase-treated thymus cells did not produce detectable pregnenolone (Fig. 2 *c*). In contrast, pregnenolone was easily detectable in cultures of 10^6 thymus cells that were depleted of T cells by low dose irradiation. Moreover, inclusion of aminoglutethimide, an inhibitor of P450scc (Fig. 1), abolished pregnenolone synthesis. Steroid production was also measured in isolated mitochondria from adrenal and thymus cells. After 16 h of culture with or without 22R-hydroxycholesterol, supernatants were analyzed for the presence of pregnenolone. One representative experiment of five is shown in Fig. 2 *d*. Low but significant levels of pregnenolone were detected in adrenal mitochondria from untreated animals, and increased sharply in the presence of 22R-hydroxycholesterol. Adrenal mitochondria from sublethally irradiated mice produced less pregnenolone than cells from untreated animals, arguing that the irradiation did not itself activate P450scc. No pregnenolone was detected when mitochondria from collagenase-treated thymus cells were assayed, but substantial amounts were found when T-depleted and collagenase-treated thymus cells from previously irradiated animals were used. Since the amount of mitochondrial protein used per group was constant, the detection of pregnenolone in T-depleted thymus is consistent with enrichment of radioresistant epithelial cells. In addition to demonstrating that pregnenolone can be synthesized by radiation-resistant thymic cells, these results indicate that pregnenolone produced in the thymus is further metabolized by 3βHSD, since trilostane caused accumulation of pregnenolone. We also detected deoxycorticosterone in supernatants of collagenase-treated thymus cells after depletion of thymocytes by antibody plus complement (Fig. 2 *e*).

The effect of ACTH on steroid production in the thymus was determined (Fig. 2 *f*). Pregnenolone was produced only when its precursor, 22R-hydroxycholesterol, was provided. Moreover, ACTH caused a dose-dependent increase in steroid biosynthesis. Pregnenolone production was not further increased by ACTH concentrations of >30 nM. Therefore, as for the adrenals, thymic steroid–producing cells are responsive to ACTH.

Expression of Steroidogenic Enzymes in the Thymus. Thymi from 3-wk-old BALB/c mice were sectioned and immunohistochemistry was used to detect expression of the steroidogenesis enzymes P450scc and P450c11, which are responsible for the synthesis of pregnenolone and corticosterone, respectively (Fig. 1). Expression of P450scc is the determining factor in whether a tissue is capable of steroidogenesis; it has been found only in tissues with steroidogenic capabilities, such as the adrenal gland, placenta, sex organs (29–31), and recently the brain (32). The antiserum to P450scc specifically stained a subset of thymic cells (Fig. 3). The large size and abundant cytoplasm suggested that these cells are not thymocytes. Areas of the serial sections that stained brightly with anti-P450scc also stained specifically with anti-P450c11 (Fig. 3 *b*). This was not due to cross-reactivity of the P40c11-specific antiserum with P450scc or other P450 enzymes, since it did not stain MA-10 (33), a mouse tumor Leydig cell line that produces testosterone but not glucocorticoids (data not shown). Visualization of the steroidogenic enzyme-expressing cells was obscured by the large number of enzyme-negative thymocytes. To eliminate these cells, mice were sublethally irradi-



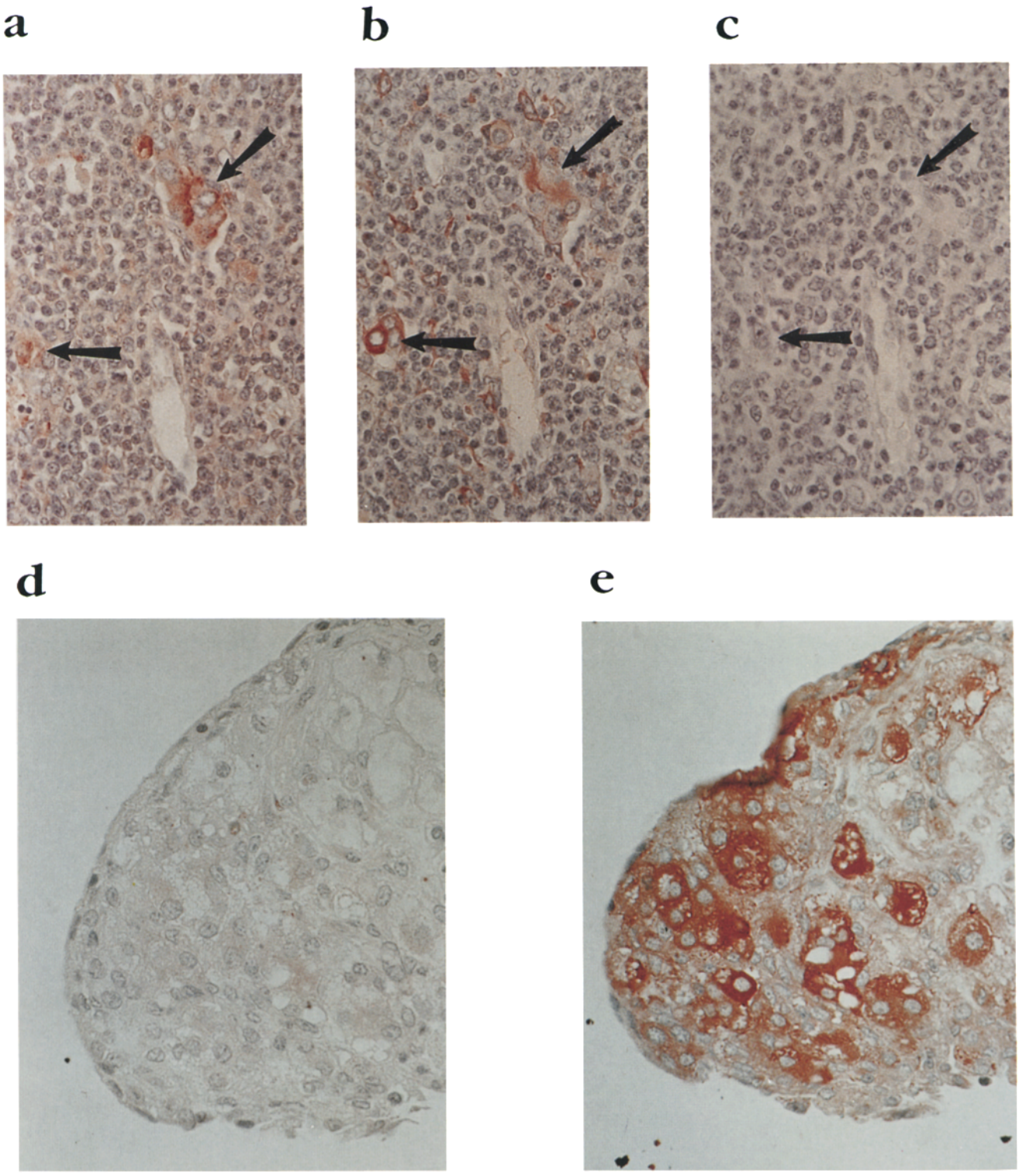


Figure 4. Expression of P450scc in the thymus. Serial sections of thymus from a 3-wk-old BALB/c mouse were stained with anti-P450scc (*a*), anticytokeratin (*b*), or rabbit Ig (*c*). Serial sections of BALB/c fetal thymus (day 19) were stained with rabbit Ig (*d*) or anti-P450scc (*e*). Staining was performed as described for Fig. 3, except that slides stained for cytokeratin (*a–c*) were trypsin-treated for 40 min at 37°C after the glycine incubation. The primary antibodies were used at the following concentrations: rabbit Ig 1:100; anti-P450scc 1:15; anticytokeratin 1:100.

ated and their thymi harvested 4 d later. This procedure dramatically reduced the number of thymocytes and enhanced visualization of the radiation-resistant stromal elements (Fig. 3, *d–g*). Diffuse and specific staining with the anti-P450scc antiserum was seen throughout the thymus, most notably in the cortex. In addition, scattered intensely staining cells were found primarily in the cortex, although a small number were also observed in the medulla. Similar staining was observed with anti-P450c11 antiserum (data not shown). Neither normal rabbit serum antiserum (Fig. 3 *d*) nor an antiserum against P450aromatase (data not shown), an enzyme involved in estrogen but not glucocorticoid biosynthesis, stained these cells. To determine the identity of the radioresistant cells, serial sections of thymus were stained with either epithelial cell-specific anticytokeratin or anti-P450scc antibodies. As shown in Fig. 4, cells positive for P450scc (Fig. 4 *a*) also stained with anticytokeratin (Fig. 4 *b*). As expected, cells that stained with anticytokeratin were ubiquitous in distribution in thymi from sublethally irradiated mice (data not shown), in which visualization of epithelial cells was greatly enhanced by elimination of thymocytes, indicating that the P450scc-positive cells represent a small subset of epithelial cells. In addition, studies with a thymic epithelial cell line, TEC (34), found that it stained specifically with antisera to P450scc and P450c11, but not P450aromatase (data not shown). Thus, enzymes necessary for steroid synthesis are present in the thymus, and appear to be expressed in radioresistant epithelial cells, and at particularly high levels in a subpopulation found predominantly in the cortex, although a small population of P450scc$^+$ nonepithelial cells cannot be excluded.

Steroid Production by Thymic Epithelial Cells. Thymic epithelial cells, especially so-called thymic nurse cells, can be separated from thymic macrophages and dendritic cells (thymic rosettes) and isolated by sedimentation through serum (35). When these populations were cultured for 6 d, a small amount of pregnenolone was detected in cultures of epithelial cells in medium alone, which increased ~10-fold in the presence of trilostane (Fig. 2 *g*). Although T-rosettes had a slightly higher baseline activity, the levels of pregnenolone did not increase with trilostane and were significantly lower than that seen with the epithelial cells. This may be a result of contamination of epithelial cells in the T-rosette cultures and/or a limited but significant ability of cells in the T-rosettes to synthesize pregnenolone. In any case, these results demonstrate that enrichment of thymic epithelial cells also enriches for steroid production.

Ontogeny of Thymic Steroid Production. Circulating levels of glucocorticoids in rats are low to undetectable until 2 wk after birth, increasing thereafter to adult levels by 4 wk of age (14). If steroids play a major role in thymocyte selection, steroid production in the thymus would be expected to differ from that by the adrenals, since thymocyte selection starts during fetal development. To test this, thymi from fetal thymic organ culture were sectioned and stained for the presence P450scc; large, positively staining cells were found (Fig. 4 *e*). The capacity of thymi from mice of different ages to produce steroids was quantitated using thymi taken from mice in four age groups: day 17 of fetal development to 1 d after birth, 10–14 d old, 28 d old, and 84 d old. Pregnenolone was measured from cultures of collagenase-treated, T-depleted thymus cells. Cells from the youngest mice produced detectable levels of pregnenolone that were markedly augmented by the inclusion of trilostane (Fig. 5). Production of pregnenolone was slightly decreased at 2 wk of age, and was 50% lower by 4 wk of age. Similar experiments performed with isolated adrenal cells found the opposite: that production of steroids was initially low and increased after birth (our unpublished observation). Thus, the thymus can produce the greatest amount of steroids during fetal development and early life, and the levels decrease with age.

Thymus-derived Steroids and Activation-induced Thymocyte Deletion. Fetal thymic organ cultures were used to evaluate whether steroids synthesized in the thymus influence TCR-mediated thymocyte selection. Anti-TCR antibodies induce apoptosis in CD4$^+$CD8$^+$ thymocytes, which is thought to mimic TCR-mediated negative selection (36). Day 16 fetal thymi were cultured in serum-free medium with H57-597 (H57), a Cβ-specific antibody (37), in the presence or absence of the P450c11 inhibitor, metyrapone. One representative experiment is shown in Fig. 6, and Tables 1 and 2 contain data from multiple experiments. Culture with H57 alone resulted in a 32% decrease in CD4$^+$CD8$^+$ thymocytes (78.9 → 53.3%). Although at this concentration metyrapone alone had little effect (Table 1), when added with H57 there was a marked decrease in the number of CD4$^+$CD8$^+$ thymo-

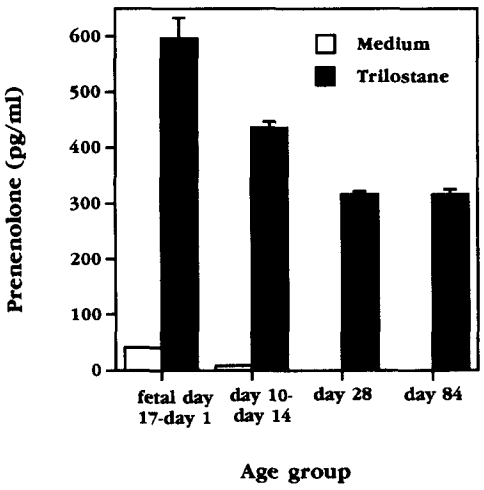


Figure 5. Effect of age on synthesis of steroids in the thymus. Thymi from 25 mice aged fetal day 17–1 d, 10–14 d, 28 d, or 84 d were collagenase-treated and T-depleted. Single cell suspensions were treated with anti-Thy-1.2 plus complement. Such treatment consistently resulted in 0.5–2% recovery of starting cell number. 10^6 cells were cultured for 5 d with 10 nM ACTH and 6.25 μM 22R-hydroxycholesterol in the absence or presence of 50 μM trilostane. Supernatants were harvested and assayed for pregnenolone. Analysis by FACScan® before initiation of cultures revealed equivalent depletion of thymocytes in each culture (<1% of cells stained with anti-CD4 or anti-CD8).

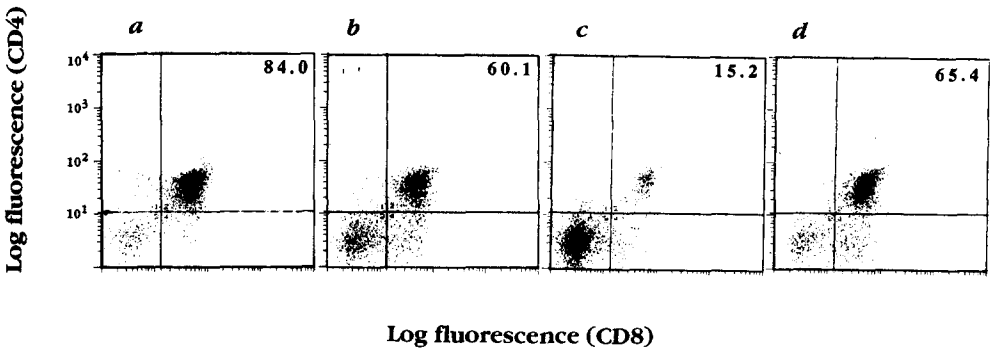


Figure 6. Blockade of steroid biosynthesis and anti-TCR-induced deletion of $CD4^+CD8^+$ thymocytes. Day 15.5–16 fetal thymi were cultured for 3 d in medium alone (*a*), anti-TCR antibody (*b*), anti-TCR antibody plus metyrapone (*c*), and anti-TCR antibody plus metyrapone plus corticosterone (*d*).

cytes of 66% (78.9 → 27.2%) (Table 1, Expt. 1). The enhanced deletion seen in the absence of corticosterone was reversed by inclusion of 10^{-9} M corticosterone (Table 1, Expt. 2); lower concentrations of corticosterone were ineffective (data not shown). Additional experiments were performed with RU-486, a competitive glucocorticoid antagonist (38). Culture of thymocytes in medium or with RU-486 resulted in a normal fraction of $CD4^+CD8^+$ thymocytes (75–80%). Treatment with H57 caused a 25% decrease in $CD4^+CD8^+$ thymocytes (79.0 → 59.2%); addition of RU-486 resulted in a 50% decrease (79.0 → 39.5%) (Table 1, Expt. 3). Similar results were obtained with 145-2C11, an anti-CD3 mAb, and coculture with metyrapone or RU-486 (our unpublished observations). The effect of metyrapone on anti-TCR–induced thymocyte depletion was also evident in terms of cell recovery. As demonstrated in another experiment (Table 3), metyrapone enhanced TCR-mediated deletion by almost 50%, a response that was completely reversed by the addition of corticosterone. Increasing the concentration of metyrapone in the absence of antibodies caused a reduction in the number of $CD4^+CD8^+$ thymocytes and prevented the appearance of $CD4^+CD8^-$ single positive thymocytes; this effect was also reversed by the addition of corticosterone (data not shown).

Culture of thymocytes from H-Y–specific TCR transgenic female mice with male thymic APC monolayers induces apoptosis, as detected by loss of $CD4^+CD8^+$ thymocytes expressing high levels of CD4 and CD8, and the appearance of $CD4^{lo}CD8^{lo}$ cells that are actively fragmenting their DNA (27). Thymocytes from female H-Y–specific TCR transgenic mice were cultured for 36 h alone or with APCs from either B6 female ($H\text{-}Y^-$), BALB/c female ($H\text{-}Y^-$), or B6 male ($H\text{-}Y^+$) mice (Table 2). Culture of female thymocytes alone or with female APC and with or without RU-486 had little effect on the percentage of $CD4^{hi}CD8^{hi}$ cells. In the experiment summarized in Table 2, culture of B6 female thymocytes with B6 male APCs caused a small decrease in

Table 1. *Inhibition of TCR-mediated Thymocyte Deletion by Locally Produced Steroids*

	Percent $CD4^+CD8^+$ thymocytes	
	Medium	Corticosterone
		10^{-9} M
Expt. 1 Medium	78.9 ± 1.7	ND
Metyrapone	74.7 ± 2.4	ND
Anti-TCR	53.3 ± 4.8	ND
Anti-TCR + metyrapone	27.2 ± 6.8	ND
Expt. 2 Medium	69.2 ± 7.6	72.9 ± 0.9
Anti-TCR	53.2 ± 3.1	59.4 ± 2.0
Anti-TCR + metyrapone	13.2 ± 2.2	52.5 ± 3.2
Expt. 3 Medium	79.0 ± 3.4	ND
RU-486	76.0 ± 4.2	ND
Anti-TCR	59.2 ± 3.3	ND
Anti-TCR + RU-486	39.5 ± 5.9	ND

Results are expressed as the arithmetic mean of seven (Expt. 1), four (Expt. 2), or eight (Expt. 3) individual animals, and SEM are shown. To avoid animal-to-animal variability, each thymus was divided into three or four pieces that received the indicated treatments. ND, not determined.

Table 2. *Enhancement of Antigen-driven Thymocyte Deletion by a Glucocorticoid Antagonist*

Thymic APC	Percent $CD4^{hi}CD8^{hi}$ thymocytes		
	Medium	RU-486	RU-486
		(10^{-7} M)	*(5×10^{-7} M)*
B6 female	31.5 ± 1.9	28.7 ± 0.7	32.3 ± 3.8
B6 male	27.0 ± 3.4	18.7 ± 4.4	13.2 ± 4.1
BALB/c female	32.0 ± 1.6	29.0 ± 1.1	26.5 ± 4.3
None	32.3 ± 3.3	30.1 ± 1.3	35.4 ± 1.6

Deletion of H-Y-specific thymocytes was measured as described in Materials and Methods. Each data point is the mean of triplicate cultures, and SEM are shown.

the number of $CD4^{hi}CD8^{hi}$ thymocytes (31.5 → 27.0%) that was greatly enhanced by the addition of RU-486 (28.7 → 18.7% at 10^{-7} M and 32.2 → 13.2% at 5×10^{-7} M RU-486). This result was obtained in three independent experiments, confirming the fetal thymic organ culture results. The adherent thymic APCs specifically stained with the anti-P450scc serum and produced pregnenolone (data not shown). Furthermore, simultaneous fluorescence analysis of CD4, CD8, and transgenic TCR α/β clonotype expression revealed that virtually all of the RU-486–enhanced loss of $CD4^+CD8^+$ cells was from the population that recognizes H-Y/H-2^b (data not shown). Thus, blockade of endogenously produced steroids, most likely corticosterone, enhances antigen-driven and TCR-mediated thymocyte deletion.

Discussion

Antigen-driven positive selection of developing thymocytes is an essential step in the formation of the T cell antigen-specific repertoire. While it is generally believed that it is the avidity of TCRs for self antigen/MHC that determines the fate of immature thymocytes (no selection and death, positive selection, or negative selection), the means by which the cell "interprets" the avidity of the TCR-ligand interaction is unknown. In fact, one of the central paradoxes of thymocyte selection is that occupancy of one receptor can lead to two very different fates, deletion or survival. In its simplest form, the mutual antagonism model holds that signals delivered upon occupancy of TCRs on immature thymocytes initiate a death response, at the same time perhaps promoting differentiation toward a more mature state (28). High avidity TCR/ligand interactions induce an overwhelming death response, while death signals resulting from low-to-moderate avidity TCR/ligand interactions are antagonized by endogenous corticosteroids, allowing these thymocytes to survive and undergo differentiation (positive selection). Thymocytes with very low avidity TCR/ligand interactions fail to survive, perhaps due to the unopposed effects of corticosteroids. The central point of this model is that it is the quantitative balance between TCR and glucocorticoid receptor signaling that determines the fate of a particular thymocyte. The results in this study support the hypothesis that mutual antagonism between TCR-mediated signals and glucocorticoids is one

Table 3. *Inhibition of Corticosterone Synthesis in Fetal Thymic Organ Culture and Effect on Cell Recovery*

Corticosterone	H57	Metyrapone	Cell recovery			
			DP	DN	$CD4^+CD8^-$	$CD4^-CD8^+$
				× 10^{-4}		
–	–	–	75.1 (±0.4)	1.8 (±0.4)	7.3 (±1.0)	1.5 (±0.3)
–	+	–	24.8 (±0.2)	2.3 (±0.2)	2.6 (±0.3)	2.2 (±0.1)
–	+	+	13.9 (±0.7)	2.1 (±0.3)	1.9 (±0.2)	1.3 (±0.1)
+	+	+	25.8 (±0.8)	2.7 (±0.4)	2.9 (±0.3)	2.0 (±0.1)

Results are expressed as the arithmetic mean (± SEM) of 3–5 individual thymic lobes per group. Cultures were performed as described in Materials and Methods. Total cell recovery was determined by microscopic enumeration of cells excluding trypan blue, and fractional recovery of thymocyte subsets by flow cytometery. Corticosterone was added at a concentration of 10^{-9} M. DP, double positive, DN, double negative.

basis for positive selection. First, P450scc and P450c11, enzymes necessary for steroid and corticosterone production, respectively, are expressed in the thymus. Second, radiation-resistant thymic epithelium produces pregnenolone, a precursor for all other steroids, and deoxycorticosterone, an intermediate in glucocorticoid synthesis. Finally, prevention of thymic corticosterone production or blockade of the glucocorticoid receptor greatly enhances TCR-mediated deletion of thymocytes. Recent data indicate that TCR-ligand interactions that normally result in positive selection can be converted to negatively selecting events by increasing their avidity (39, 40), supporting the hypothesis that the outcome of selection (positive vs. negative) depends upon where in the continuum the avidity for self antigen/MHC a particular TCR falls. Glucocorticoid blockade would not be expected to affect TCR avidity, but would alter the balance between the mutually antagonistic stimuli, resulting in unopposed TCR-mediated death signals and narrowing the window of TCR avidity that results in positive selection. Thus, TCR/glucocorticoid mutual antagonism would allow thymocytes with low-to-moderate avidity TCRs to survive and proceed to differentiate towards mature T cells. A recent report found that in vivo injection with RU-486 inhibited anti-CD3–induced deletion of $CD4^+CD8^+$ thymocytes (41), a result seemingly at odds with our observations in fetal organ culture and antigen-specific thymocyte deletion assays. The reason for this difference is not yet clear, but it may be relevant that the TCR specificities of the deleted cells were not analyzed in that study. In vivo injection of anti-CD3, probably due to activation of peripheral T cells and lymphokine secretion, has numerous biological effects (42), which may result in increased adrenal production of glucocorticoids and antigen nonspecific, glucocorticoid-mediated deletion of $CD4^+CD8^+$ thymocytes. By using thymic organ culture we avoided this possibility, and addressed the specificity of the locally produced steroid effect on TCR-mediated deletion by using TCR transgenic mice. Use of a similar approach in vivo, or one using Vβ-specific superantigens, might reconcile the results.

There are a number of reasons why the thymus might produce steroids to supplement those secreted by the adrenals. First, circulating levels of glucocorticoids are very low at birth (14), a time during which the antigen-specific repertoire is being established. The ontogeny of thymus steroid production is inverse to that of the adrenal, being highest in the fetal/neonatal period and declining to adult levels by 4 wk of age. Second, thymic steroid synthesis may be necessary to ensure levels sufficient to fulfill their biological functions. Although serum levels of corticosteroids are in the 500–1,000 nM range, the majority is bound to protein (14, 15). The level of active unbound (free) corticosterone in plasma is much lower, ~20 nM in adult rats (14). It is possible that locally produced steroids, acting as paracrine factors, readily diffuse between contacting cells to achieve functionally significant intracellular levels in thymocytes.

Steroid biosynthesis in the thymus may explain, at least in part, several previous observations. Whereas adrenalectomy before 3 wk has no effect on the thymus, adrenalectomy of adult rats leads to hypertrophy (43). This may be because corticosteroids produced in the thymus regulate thymocyte numbers early in life, but not as endogenous production decreases with age. In addition, activated T lymphocytes can secrete ACTH (44, 45), although the levels are too low to have a significant effect on adrenal steroidogenesis (46). Immunoreactive ACTH has also been detected in thymic epithelial cells (20), and carcinoid tumors that secrete ACTH arise in the thymus (47). Furthermore, CRF, a hypothalamic hormone that induces ACTH production, is synthesized by an adherent cell population in the thymus (19). It is possible that locally produced CRF/ACTH regulates steroid synthesis in the thymus, since P450scc activity in the thymus, as in the adrenal, is enhanced by ACTH (Fig. 2 and our unpublished observations). The finding that thymic epithelial cells produce steroids raises an intriguing possibility. Bone marrow–derived dendritic cells and macrophages in the thymus are primarily responsible for negative selection, whereas epithelial cells mediate positive selection (2, 48). It is possible that antagonism of TCR-mediated death signals is particularly efficient when the APC is a steroid-producing epithelial cell, whereas it is less efficient when the APC is a bone marrow–derived cell that produces no or relatively small amounts of steroids. It should be noted, however, that the finding that epithelial cell–enriched populations make steroids does not exclude the possibility that other radioresistant thymic cells are also capable of steroid synthesis. Further characterization of steroid production by thymus cell subsets is needed to clarify this issue.

Previous in vitro data have demonstrated that glucocorticoids antagonize TCR-mediated T cell hybridoma and thymocyte death. The finding that steroids are produced in the thymic microenvironment, and that disruption of this endocrine/paracrine pathway has a marked effect on TCR-mediated thymocyte deletion, strongly supports the possibility that mutual antagonism participates in determining the fate of thymocytes undergoing antigen-driven selection. Experiments using TCR transgenic animals to evaluate what effect endogenous steroids might have on generation of the antigen-specific repertoire are underway.

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