

In Vivo and In Vitro Clonal Deletion of Double-positive Thymocytes

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Summary

To study the processes of thymic development, we have established transgenic mice expressing an α/β T cell antigen receptor (TCR) specific for cytochrome *c* associated with class II major histocompatibility complex (MHC) molecules. The transgenic TCR chains are expressed by most of the thymocytes in these mice, and these cells have been shown to efficiently mature in association with E^k- and A^b-encoded class II MHC molecules. This report describes a characterization of the negative selection of these transgenic thymocytes in vivo that is associated with the expression of A^s molecules. Negative selection by A^s molecules appears to result in the deletion of a late stage of CD4/CD8 double-positive thymocytes in that there is a virtual absence of transgenic TCR bearing CD4 single-positive thymocytes. This phenotype is accompanied by the appearance of CD4/CD8 double-negative thymocytes and peripheral T cells that are functionally antigen reactive. The process of negative selection has also been investigated using an in vitro culture system. Upon presentation of cytochrome *c* by E^b-expressing nonthymic antigen-presenting cells, there occurs an antigen dose-dependent deletion of the majority of CD4/CD8 double-positive thymocytes. In contrast, presentation of Staphylococcal enterotoxin A by E^b in vitro results in minimal deletion of double-positive thymocytes. In addition, we use this in vitro model to examine the effects of cyclosporin A on negative selection. In contrast to its effects on mature T cells, and the findings of others in vivo, cyclosporin A does not inhibit antigen-induced deletion of double-positive thymocytes. Finally, a comparison of the antigen dose responses for thymocyte deletion and for peripheral T cell activation indicates that double-positive thymocyte recognition is more sensitive than mature T cells to antigen recognition.

The establishment of immunological self-recognition and tolerance is known to occur primarily in the thymus, where thymocytes undergo a program of differentiation leading to mature functional T cells. Thymocytes are specifically selected based on the specificity of their α/β TCR. Most of the information currently available relates to the results of thymic selection and not the selection process itself (1); the actual mechanism for selection has yet to be elucidated. T cells that survive thymic selection and are exported to the peripheral lymphoid organs are not overtly self-reactive, yet have the ability to recognize foreign antigenic peptides bound to MHC molecules that are also expressed in the thymus. The selection events that lead to a naive population of T cells specific for foreign peptides bound to self and not foreign alleles of the MHC molecules are termed positive selection; the deletion of T cells overtly reactive to self-MHC molecules, presumably containing self-peptides, is termed negative selection (2). The distinction between the recognition events that lead to positive selection versus negative selection is not understood.

The developmental pathway followed by thymocytes can

be characterized by the expression of the TCR and the CD4 and CD8 molecules. The earliest T cell precursors in the thymus lack cell surface expression of these proteins (double negative; DN),¹ and in a regulated sequence that includes a productive rearrangement of the β chain and α chain TCR genes, these precursor cells express CD4 and CD8 molecules (double positive; DP) and low levels of the TCR (3, 4). As shown using TCR transgenic mice, the further progression of these DP thymocytes to express high levels of the TCR and either the CD8 or CD4 molecules (single positive; SP) is dependent upon the TCR-mediated recognition of class I or class II MHC molecules, respectively, in the thymus (5–8). Experiments in normal animals using V β -specific antibodies have shown that the deletion of thymocytes reactive with self-encoded superantigens occurs within the transition from a DP to an SP cell (9–13). Results obtained with TCR transgenic animals suggest that deletion can occur at earlier stages of differentiation depending upon the particular self-antigen

¹ Abbreviations used in this paper: CsA, cyclosporin A; DN, double negative; DP, double positive; SEA, Staphylococcal enterotoxin A; SP, single positive.

expressed and the timing of TCR expression. Thus, the presence of the appropriate self-antigen in TCR transgenic animals has been reported to result in the absence of the majority of DP thymocytes in some instances, while in other cases clonal deletion appears to act upon cells only during the transition from a DP to SP maturational stage (7, 14–16). Recently, it has also been shown that DP thymocytes in TCR transgenic animals can be induced to undergo programmed cell death upon administration of the specific antigenic peptide (17). The stage at which physiological clonal deletion occurs is thus unresolved.

In this report we describe the negative selection of thymocytes in cytochrome *c*/I-E^k-specific TCR transgenic mice. Unexpectedly, in the presence of I-A^s, there is a block in the development of CD4 SP thymocytes and a variable decrease in the number of DP thymocytes. These mice also possess a high percentage of DN thymocytes that express high levels of the transgene-encoded TCR. DN T cells also appear in the peripheral lymphoid organs of these mice, and can be shown to be responsive to specific antigen. To better analyze the mechanisms involved in these processes, we have also developed an *in vitro* culture system that appears to mimic many aspects of thymic clonal deletion. We find antigen-specific and dose-dependent cell death of DP thymocytes induced by presentation of a peptide antigen by nonthymic antigen presenting cells. These results are discussed in relationship to the requirements for induction of tolerance as well as the differentiation state and sensitivity of thymocyte subpopulations to clonal deletion.

Materials and Methods

Mice. All mice were bred and maintained in the animal facility at the University of California, San Diego, or Scripps Research Institute. α/β TCR transgenic mice were originally generated as described previously (8). Inbred B10.A, B10.BR, and B10.A(5R) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Preparations. 4–8-wk-old mice were sacrificed by cervical dislocation, and single cell suspensions were obtained from thymus and lymph node.

Antibodies and Flow Cytometry. PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 rat IgG mAbs were obtained from Becton Dickinson & Co. (Mountain View, CA). mAbs to TCR α and β chains were KJ25 (anti-V β 3; reference 18), 1F2 (anti-V α 11; reference 19) and RR-8 (anti-V α 11; a generous gift from Dr. Osami Kanagawa, Washington University, St. Louis, MO). Unconjugated mAbs were detected by FITC-conjugated goat anti-hamster or goat anti-rat IgG (Caltag, South San Francisco, CA). Flow cytometric analysis was performed using a FACScan[®] flow cytometer (Becton Dickinson & Co.) and FACScan[®] Research Software.

T Cell Proliferation Assay. 10⁵ transgenic T cells were cultured in 96-well microtiter plates in the presence of 5 × 10⁵ irradiated spleen cells or confluent monolayers of irradiated FT16.6C5 murine fibroblasts (generously provided by Dr. Ronald Germain, National Institute of Allergy and Infectious Diseases, Bethesda, MD) (20). Various concentrations of pigeon cytochrome *c*, 88–104 COOH-terminal peptide, moth cytochrome *c* 88–103 COOH-terminal peptide, or staphylococcal enterotoxin A were included as indicated. After 48 h, the cultures were pulsed with 1 μ Ci

[³H]thymidine/well (6.7 Ci/mmol; DuPont Co., Wilmington, DE) and harvested after an additional 16 h.

IL-2/IL-4 Assays. 10⁵ transgenic T cells were cultured in 96-well microtiter plates in the presence of 5 × 10⁵ irradiated spleen cells or confluent monolayers of irradiated FT16.6C5 murine fibroblasts (as described above). Various concentrations of moth cytochrome *c* 88–103 COOH-terminal peptide or staphylococcal enterotoxin A were included as indicated. After 48 h, IL-2/IL-4 production was assayed by addition of culture supernatant from the transgenic cell cultures to 10⁴ NK cells, at a final concentration of 20%. The NK cell cultures were then cultured for 24 h, pulsed with 1 μ Ci [³H]thymidine/well (6.7 Ci/mmol; DuPont Co.) and harvested after an additional 16 h.

In Vitro Thymocyte Antigen Culture. Thymocytes were cultured in 24-well tissue culture plates in RPMI (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (Gemini Bioproducts, Calabasas, CA), antibiotics, L-glutamine, and 5 × 10⁻⁵ M 2-ME in the presence of a confluent monolayer of FT16.6C5 fibroblasts, and various concentrations of moth cytochrome *c*, 88–103 COOH-terminal peptide, or Staphylococcal enterotoxin A (SEA), as indicated. Cells were cultured 21 h at 37°C in 5% CO₂. Cells were then harvested by washing wells vigorously, the viable cells counted by trypan blue exclusion, and analyzed by flow cytometry.

Results

Induction of Tolerance in Transgenic Mice That Express A^s. We have produced α/β TCR transgenic mice using α and β chain genes that encode a receptor specific for pigeon cytochrome *c* in association with I-E^{k,s,b} class II MHC molecules (8, 20a). The TCR expressed in these mice is composed of α and β chains derived from the cytochrome *c*-specific T cell clones AN6.2 and 5C.C7, respectively (21, 22); this TCR is termed AND. Previous work with AND transgenic mice demonstrated positive selection of thymocytes mediated by the E^k and, unexpectedly, A^b molecules (8, 20a). Subsequent analysis of mice bearing the H-2^s MHC haplotype revealed a block in development of CD4 SP thymocytes. As illustrated in Fig. 1 A, thymuses from H-2^{b/k} haplotype AND transgenic mice can exhibit as many as 60% CD4 SP thymocytes. In contrast, thymuses from H-2^{s/k} AND transgenic mice lack a significant CD4 SP population despite the presence of the E^k molecules (Fig. 1 b). In addition to lacking CD4 SP cells, thymuses from H-2^{s/k} mice contain a higher percentage of CD4/CD8 DP cells (45%) compared with thymuses from H-2^{b/k} AND transgenic mice (28%). This phenotype suggests that thymocyte development to the CD4 SP stage is blocked at the CD4/CD8 DP stage or at the transition from the DP to the SP stage. Since this lack of CD4 SP thymocyte development is seen in H-2^{b/s} mice (lacking surface I-E molecules), the block in maturation maps to either A^s or the class I molecules K^s or D^s (data not shown). The specificity of the AND receptor for class II MHC molecules suggests that the influence of the H-2^s haplotype maps to A^s. In addition, this reactivity is consistent with the alloreactivity to A^s seen with the T cell clone from which the β chain was derived (22). However, T cells from AND transgenic mice were tested for reactivity to B10.S or

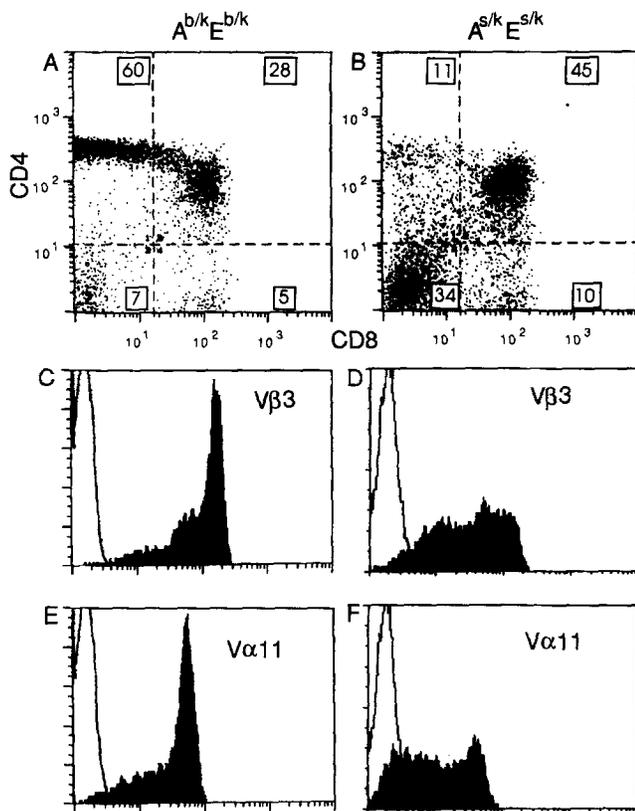


Figure 1. A^s expression in the thymus results in a block in positive selection. Freshly removed thymocytes from H-2^{b/k} (A, C, and E) vs. H-2^{s/k} (B, D, and F) were double stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs; and single stained with anti-Vβ3 (KJ25) and anti-Vα11 (1F2) mAbs as described in Materials and Methods. Cells were subsequently analyzed by flow cytometry. (A and B) CD4/CD8 double staining. Numbers indicate percent thymocytes in each quadrant. (C-F) Vβ3 and Vα11 staining. Solid histogram represents Vβ3 and Vα11 staining; clear histogram represents staining with FITC-conjugated secondary antibody alone.

B10.S(9R) APCs bearing A^s, and no proliferation over background was seen (data not shown).

The distribution of thymocytes according to high vs. low TCR expression is distinctly different in H-2^{b/k} and H-2^{s/k} mice (Fig. 1, c-f). Two-color flow cytometry (anti-Vβ3 vs. anti-CD4) showed that thymocytes from H-2^{b/k} mice separated into two populations, a population that is Vβ3-CD4 dull, which represents the DP population, and Vβ3-CD4 bright, which represents with the CD4 SP population. In contrast, in mice expressing A^s, there is no Vβ3 bright/CD4 bright thymocyte population. The CD4 bright thymocytes lack Vβ3 expression, and conversely, the Vβ3 bright cells are CD4 (and CD8) negative (data not shown).

The populations of T cells in the lymph nodes reflect the MHC-dependent differences in thymic selection seen in AND transgenic mice. In H-2^{s/k} mice, the lymph nodes contain only 20–50% CD3-positive cells, compared with 75–80% in the

lymph nodes from H-2^{b/k} mice (Table 1). In addition, the T cells that are present in A^s-bearing mice are composed, in part, of a large population of CD4/CD8 DN cells, in some cases approaching 50%. The size of the CD4 and CD8 SP populations vary considerably between individual mice; however, there is a dramatic difference between the CD4/CD8 ratios in mice expressing A^s and those that do not. Whereas H-2^{b/k} mice have a large excess of CD4-positive cells, the CD4/CD8 ratios in H-2^{s/k} mice are similar to nontransgenic mice. Two-color flow cytometry analysis of the CD4-positive cells in H-2^{s/k} mice shows that there are very few Vβ3-positive cells compared with H-2^{b/k} AND transgenic mice (9% compared with 77%, data not shown), and most of the Vβ3-positive cells in H-2^{s/k} mice are DN. In other experiments, not presented here, the DN, Vβ3⁺, Vα11⁺ T cells were strongly reactive to pigeon cytochrome *c* presented on APCs from B10.A mice (data not shown). Taken together, these data indicate that the AND TCR expressed by thymocytes reacts with the I-A^s-encoded class II molecule in such a way that a form of negative selection occurs. The net result is that AND TCR⁺, SP CD4⁺ cells appear to be missing from the thymus and peripheral lymphoid organs, whereas functional TCR⁺, CD4⁻ thymocytes and spleen cells are abundant. The interaction between the AND TCR and the I-A^s-encoded ligand, especially in the absence of CD4, may be too weak to result in peripheral T cell activation; thus, self-tolerance is maintained even though the T cells are cytochrome *c* reactive. Alternatively, if deletion is dependent upon a self-peptide associated with A^s, the ligand may only be expressed in the thymus.

Deletion of Transgenic DP Thymocytes in Tissue Culture. To study the stages of thymocyte development that are sensitive to clonal deletion, the biochemical mechanism of clonal deletion, and the efficacy of different ligands in the induction of these events, a culture system that could mimic negative selection was developed. Previous studies demonstrated that the AND receptor recognizes moth cytochrome *c* in association with E^b (20a). We therefore examined the effect of placing transgenic thymocytes in culture with antigen plus the murine L cell, FT16.6C5, that was transfected with the gene encoding E^b (20). The advantage of using the L cell transfectant over other sources of APCs was that they are a uniform population and easily distinguished from thymocytes in flow cytometry analyses by their size and granularity. Thymocytes from an H-2^{b/k} mouse were cultured in the presence of various concentrations of moth cytochrome *c* 88–103 COOH-terminal peptide and a confluent monolayer of FT16.6C5 cells. After 21 h of culture, the viable thymocytes were counted by trypan blue exclusion and analyzed by flow cytometry. Presentation of peptide antigen by E^b-bearing APCs resulted in a dose-dependent and selective depletion of DP thymocytes. Cell culture in the presence of 2.5, 0.25, and 0.025 μM moth cytochrome *c* peptide resulted, respectively, in 72, 55, and 22% reductions in percent DP thymocytes compared with the no antigen control (Fig. 2, a-d).

Table 1. Characterization of Lymph Node Cells from AND TCR Transgenic Mice

Strain	H-2	MHC molecules	Cell # $\times 10^{-7}$	Total LN Cells		CD3 ⁺ Cells			CD4/8 Ratio
				Ig ⁺	CD3 ⁺	CD4	CD8	DN	
				%	%		%		
AND TG	b/a	A ^{b/k} E ^{b/k}	2.0	12	87	94	3	3	31.3
AND TG	b/k	A ^{b/d} E ^{b/k}	3.6	19	77	100	<1	<1	>100
AND TG	b/d	A ^{b/d} E ^{b/d}	3.0	21	75	96	4	<1	24.0
AND TG	b/s	A ^{b/s}	0.4	50	43	44	28	28	1.6
AND TG	s/s	A ^s	1.9	68	23	43	12	45	3.6
AND TG	b/s	A ^{b/s}	2.6	69	28	34	22	44	1.5
AND TG	a/s	A ^{k/s} E ^{k/s}	0.6	50	48	22	32	46	0.7
Littermate	b/s	A ^{b/s}	3.0	10	87	63	37	<1	1.7
Littermate	s/s	A ^{s/s}	0.9	10	87	70	30	<1	2.3
Littermate	b/d	A ^{b/d} E ^{b/d}	4.7	ND	75	63	37	<1	1.7

Lymph node cells from various AND transgenic or normal littermate mice were stained with goat anti-mouse Ig, anti-CD3, or anti-CD4 and anti-CD8. Ig⁺ and CD3⁺ percentages were determined by subtraction of background staining obtained with FITC-conjugated normal rat Ig, using FACScan® Research Software. This control was also used to set gates for determining positive staining. Percentages of total cells that were CD4⁺CD8⁻ and CD4⁻CD8⁺ were determined by quadrant analysis as shown in Fig. 1, and then calculated as a percent of CD3⁺ cells. CD4⁺CD8⁺ cells were <0.5% of total. CD4⁻CD8⁻ cells were calculated as all remaining CD3⁺ cells that were neither CD4⁺ nor CD8⁺ (CD4 = CD4⁺CD8⁻; CD8 = CD4⁻CD8⁺; DN = CD4⁻CD8⁻ cells).

These results were reproduced in nine separate experiments. Cell loss was not merely due to adherence of DP thymocytes to the fibroblast APCs, as thymocyte/APC aggregation was not apparent by microscopic examination of harvested cells. In addition, flow cytometric analysis demonstrated that the APCs (characterized by their forward light scatter pattern) were negative for the thymocyte markers CD4, CD8, and Vβ3, thus indicating the absence of adherent thymocytes (data not shown).

While analysis of thymocyte subpopulations indicates an antigen-dependent loss of DP thymocytes, it is important to note that the antigen culture system results in a significant overall cell loss. Thymocyte recovery after overnight culture can range from 20 to 50% even in the absence of antigen. Analyses of the various subpopulations indicate this gross cell loss is nonspecific. In addition, the culture of nontransgenic thymocytes under these conditions resulted in similar overall cell losses, indicating this result is not unique to the transgenic mice (data not shown). In light of this, thymocyte subpopulation sizes were monitored in all experiments in the event that the decrease in percent DP cells was merely a reflection of expansion or selective maintenance of other subpopulations, particularly the more mature CD4 SP or DN cells, in response to antigen. The actual number of cells recovered in the experiment presented in Fig. 2 are listed Table 2. These data demonstrate that loss of DPs, seen in terms of percent, is a reflection of actual cell loss. At 2.5 μM moth cytochrome *c* peptide, there is an 80% loss of DP thymocytes compared with control cultures. Examination of the CD4 SP and CD4/CD8 DN populations reveals that the DP cells are not converted to either of these other populations.

Negative selection of thymocytes may predominantly occur via a mechanism of programmed cell death, or apoptosis (17, 23–25). One characteristic of apoptosis is a requirement for active transcription and translation. It is therefore possible

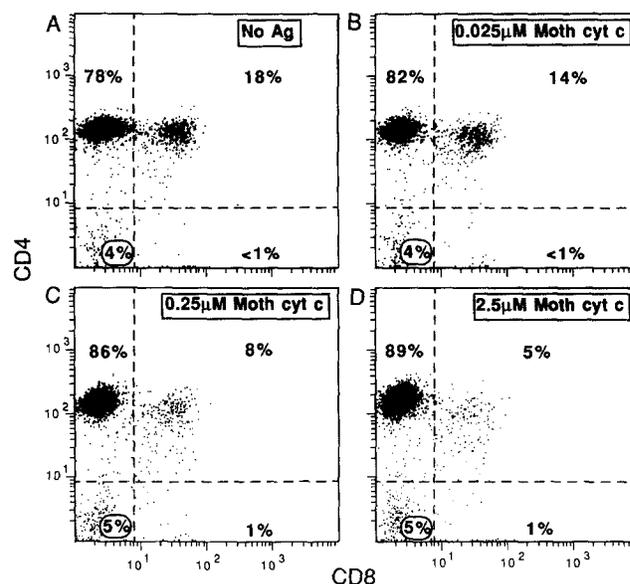


Figure 2. Culturing with antigen results in loss of CD4/CD8 DP transgenic thymocytes. 4×10^6 thymocytes from an H-2^b mouse were cultured in the presence of FT16.6C5, E^b-transfected fibroblasts, and various concentrations of moth cytochrome *c*, 88–103 COOH-terminal peptide as described in Materials and Methods. Cells were harvested after a 21-h culture, counted, and analyzed for CD4 and CD8 surface expression by flow cytometry. Numbers represent percent of cells in each quadrant.

Table 2. Deletion of CD4⁺CD8⁺ Thymocytes Induced by Antigen Presentation

Moth cytochrome <i>c</i>	Cyclohexamide	Total cells recovered	CD4	CD8	DP	DN
μM						
0	-	15	12	0.1	2.6	0.6
0.025	-	23	19	0.2	3.3	0.9
0.25	-	9.4	8.1	0.1	0.7	0.5
2.5	-	11	9.4	0.5	0.1	0.5
0	+	18	13	0.1	4	0.7
0.025	+	19	13	0.5	4	0.8
0.25	+	18	13	0.2	4	0.8
2.5	+	21	15	0.2	5	0.9

After a 21-h culture with various concentrations of antigen (as described in Fig. 5 and Materials and Methods), cells were harvested, and viable cells counted by trypan blue exclusion. The subpopulation sizes were calculated based on overall cell recoveries, and CD4 and CD8 profiles were generated by two-color flow cytometry. The size of the populations are listed multiplied by 10^{-5} (CD4 = CD4⁺CD8⁻; CD8 = CD4⁻CD8⁺; DP = CD4⁺CD8⁺ cells; DN = CD4⁻CD8⁻ cells). Cyclohexamide was added where indicated at 50 $\mu\text{g}/\text{ml}$.

under some circumstances to block the induction of apoptosis with pharmacological agents such as actinomycin D and cyclohexamide (25). To determine whether or not the deletion seen *in vitro* had characteristics of an active cell death, we examined the effects of cyclohexamide on thymocytes cultured in the presence of antigen. Addition of this drug resulted in a complete inhibition of antigen-induced deletion (Table 2). We have also examined the effect of addition of actinomycin

D to cultures, and found that it is equally effective in blocking the death of DP thymocytes (data not shown).

Transgenic mice expressing A^s retain a large number of DP cells in the thymus. It was of interest to determine whether these thymocytes were sensitive to negative selection, or whether they were past the maturational point where clonal deletion could occur. As was seen with thymocytes from H-2^b mice, DP cells from H-2^{s/d} thymuses delete in a dose-dependent manner. Presentation of 2.5, 0.25, and 0.025 μM moth cytochrome *c* peptide resulted in 65, 49, and 5% decreases in the percent DP thymocytes, respectively, compared with the no antigen control (Fig. 3, *a-d*). Analysis of subpopulation sizes confirmed the selective deletion of the CD4/CD8 DP thymocytes.

Because of the large populations of mature CD4-positive thymocytes in these *in vitro* cultures, the possibility remained that the loss of DP cells was a nonspecific bystander effect caused by activation of mature thymocytes with antigen. For example, activation of CD4 SPs could result in the secretion of lymphokines at levels that induce nonspecific death of immature thymocytes under *in vitro* culture conditions. To address this issue, coculture experiments were carried out with thymocytes from a nontransgenic mouse and those from an H-2^{b/k} transgenic mouse. Presentation of peptide antigen to transgenic thymocytes alone resulted in a 57% decrease in absolute numbers of DP cells and had no significant effect on the CD4 or CD8 SP and DN cell subpopulations (Table 3). As expected, the addition of pigeon cytochrome *c* to nontransgenic thymocytes cultured with fibroblast APCs had no specific effect on the viability of any of the subpopulations. Transgenic thymocytes were mixed with an equal number of nontransgenic thymocytes, and the effect of antigen coculture was examined. The mixed cultures were designed to be skewed towards transgenic CD4 SP cells and nontransgenic DP cells. Based on subpopulation sizes seen with the unmixed populations, the ratio of CD4 SP thymocytes would be 5:1,

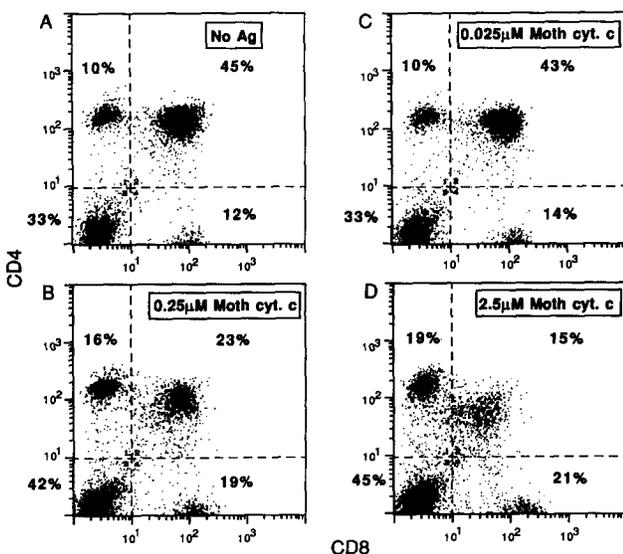


Figure 3. Culturing with antigen results in loss of CD4/CD8 DP thymocytes from an H-2^{s/d} mouse. 3×10^6 cells were cultured, harvested, and analyzed, as described in Materials and Methods. CD4 and CD8 surface profiles are shown after a 21-h culture in the presence of FT16.6C5 cells and various concentrations on moth cytochrome *c*, 88-103 COOH-terminal peptide. Numbers represent percent of cells in each quadrant.

Table 3. Lack of Bystander Deletion

Transgenic thymocytes	Nontransgenic thymocytes	- Ag				+ Ag			
		CD4	CD8	DP	DN	CD4	CD8	DP	DN
+	-	77	4	11	8	83	3	5	8
-	+	14	6	74	6	13	6	76	5
+	+	49	4	40	7	49	5	40	6

Cells were cultured and analyzed as described in Materials and Methods with or without antigen (2.5 mM moth cytochrome *c*, 88-103 peptide). After 21 h, cells were harvested and double stained for CD4 and CD8. Transgenic thymocytes were from an H-2^{b/k} mouse, and nontransgenic thymocytes were taken from a B10.A mouse. The third line represents an equal mixture of H-2^{b/k} transgenic thymocytes and B10.A thymocytes. The numbers indicate the percentage of cells in each subset (CD4 = CD4⁺CD8⁻; CD8 = CD4⁻CD8⁺; DP = CD4⁺CD8⁺ cells; DN = CD4⁻CD8⁻ cells).

transgenic/nontransgenic and the DP thymocytes would be present at a ratio of 1:7, transgenic/nontransgenic. In these mixed cultures there was no evidence for deletion of DP thymocytes upon presentation of antigen despite the presence of a significant population of cytochrome *c*-reactive CD4 SP cells. These data indicate that deletion of DP thymocytes is the direct result of their recognition of the antigen/MHC ligand on the APCs, and not a bystander effect resulting from the activation of mature CD4 SP cells.

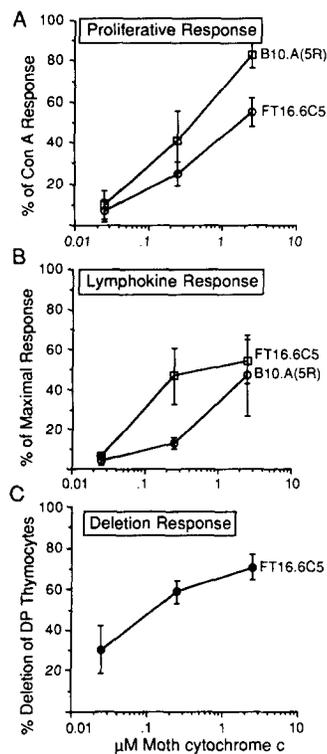


Figure 4. Comparison of moth cytochrome *c* (88-103)/E^b dose responses between antigen-induced deletion of DP thymocytes and lymph node lymphocyte proliferation, and lymphokine production. (A) Proliferation of lymph node lymphocytes from H-2^b AND transgenic mice. Results were generated from three separate experiments, and the error bars represent the SD from the mean. Cells were cultured in the presence of moth cytochrome *c* (88-103) and APCs as indicated, pulsed with [³H]thymidine, and harvested (see Materials and Methods). Response is illustrated as percent of response generated by stimulation with 2.5 μg/ml Con A. (B) Lymphokine response from H-2^b AND transgenic mice. Lymphokine production was measured by monitoring the response of the IL-2/IL-4-dependent NK cell line to culture supernatant from the transgenic cell cultures. Lymphokine responses to cytochrome *c* presented by B10.A(5R) vs. FT16.6C5 were examined. Results are the average

from two experiments and are plotted as percent of NK cell line response to 100 U/ml rIL2. (C) Percent loss of DP thymocytes induced by antigen (conditions as indicated in the legend to Fig. 3). Results are an average of eight separate experiments and are shown as percent loss compared with control culture without antigen.

Comparison of Thymocyte and Splenic T Cell Sensitivity to Antigen. To compare the antigen sensitivity of clonal deletion with that of T cell activation, lymph node cells from H-2^{b/a} and H-2^{b/b} AND transgenic mice were cultured with APCs and various concentrations of moth cytochrome *c* fragment, 88-103 (Fig. 4 A). The proliferative response of the lymphocyte cultures is presented as a percent of Con A response, and plotted against the concentration of antigen added to culture. A comparison of dose-response curves between E^b-expressing APCs indicates that FT16.6C5 fibroblasts induce proliferation approximately fourfold less efficiently than B10.A(5R) splenocytes, as measured by the antigen dose required to achieve 50% of the Con A response.

Previous work has shown that some T cell clones require IL-1 for proliferation, but not for lymphokine secretion (26), and thus a lack of cytokines, or accessory molecules expressed by the fibroblast-presenting cells, could account for the decreased response. We reasoned that a measurement of lymphokine production might be a more appropriate measure of T cell activation. Fig. 4 B illustrates that transgenic lymphocytes show a reduced proliferative dose response to moth cytochrome *c* and FT16.6C5 fibroblasts, but they produce a significant lymphokine response. There is an apparent difference in lymphokine production induced by antigen plus FT16.6C5 cells vs. splenocytes, and this difference may be due to lymphokine depletion in the splenocyte cultures. These lymphocyte responses are particularly interesting when the dose-response curve for antigen-induced deletion of DP thymocytes is considered (Fig. 4 C). Since the normalization values used for the proliferative responses and the lymphokine assays are relatively arbitrary and different from the way that deletion is calculated, it is difficult to directly compare the curves. However, there is an average of almost 30% thymocyte deletion at 0.025 μM peptide, whereas there is reproducibly little T cell proliferation or lymphokine secretion over background at this antigen concentration. This result indicates that T cell activation is less sensitive to antigen than thymocyte deletion.

Examination of the Effects of Cyclosporin A on Lymphocyte and Thymocyte Cultures. Cyclosporin A (CsA) is an immunosuppressant that appears to act by binding to cyclophilin

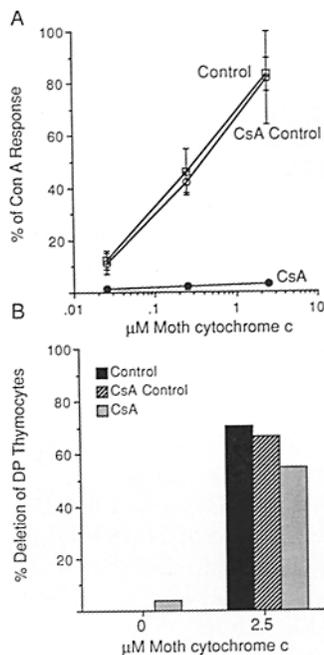


Figure 5. Effect of CsA on AND transgenic lymphocyte proliferation vs. DP thymocyte deletion. (A) Lymph node lymphocytes from H-2^b AND transgenic mice were stimulated with moth cytochrome *c* 88–103 peptide and B10.A(5R) splenocytes in the presence of 10 ng/ml CsA as indicated. CsA control represents addition of equal amounts in the CsA solvent. Results are the average of two separate experiments and are indicated as percent of Con A response. (B) Transgenic thymocytes were cultured with moth cytochrome *c* 88–103 peptide and FT16.6C5 fibroblasts. CsA and CsA control were included as indicated previously. Percent deletion of DP thymocytes was determined as percent DPs compared with control culture without antigen.

and forming a complex that, in turn, binds to and potentially inhibits the activity of calcineurin/calmodulin (27). It has been shown to inhibit T cell proliferation, lymphokine secretion, and to interfere with thymic development both in vivo and in fetal thymic organ cultures (28–33). Examination of the mechanisms by which CsA interferes with T cell activation has demonstrated that the drug blocks the Ca²⁺-dependent activation of transcription factors that are important to IL-2 gene regulation (34, 35). We were therefore interested in the effects of CsA on transgenic lymphocyte proliferation and thymocyte deletion. As expected, addition of CsA to lymphocyte cultures completely inhibited proliferation induced by moth cytochrome *c* and B10.A(5R) splenic APCs (Fig. 5 A). We were surprised, however, to find that addition of this drug under equivalent conditions had a relatively small effect on antigen-dependent deletion of DP thymocytes (Fig. 5 B). In the experiment presented, CsA was added at 10 ng/ml, and subsequent experiments showed that deletion was also insensitive to CsA at 100 ng/ml, 10-fold over the level needed for the inhibition of T cell activation. These results suggest that antigen-induced deletion of DP thymocytes is independent of the CsA-sensitive intracellular events that are necessary for IL-2 secretion and T cell proliferation.

Examination of the Effects of SEA on Thymocyte and Lymphocyte Antigen Cultures. SEA is known to interact with TCRs expressing the V β 3 gene element. This interaction has been shown to result in negative selection of V β 3-bearing thymocytes in vivo, and to induce proliferation of mature T cells in vitro (36, 37). It was therefore of interest to examine the effects of SEA on lymphocyte and thymocyte cultures from AND transgenic mice. Stimulation of transgenic lymph node lymphocytes with SEA and FT16.6C5 fibroblasts results in significant lymphokine production at a range of antigen doses (Fig. 6 A). In contrast, antigen-induced dele-

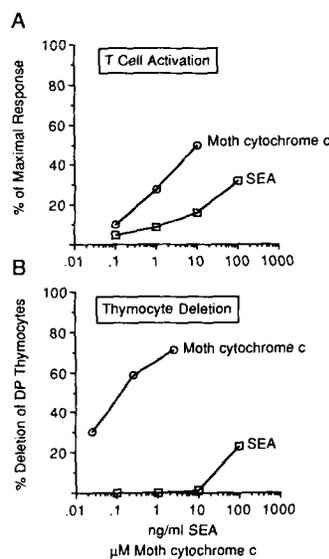


Figure 6. Effect of SEA on AND transgenic lymphocytes vs. thymocytes. (A) Lymph node lymphocytes from H-2^b AND transgenic mice were stimulated with SEA or moth cytochrome *c* 88–103 peptide, and FT16.6C5 fibroblasts. The cultures were assayed for lymphokine production by monitoring the response of the IL-2/IL-4-dependent NK cell line to supernatants from the transgenic cell cultures (see Materials and Methods). Results are shown as percent of response to 100 U/ml rIL2. (B) Transgenic thymocytes were cultured with SEA and FT16.6C5 fibroblasts. Percent deletion of DP thymocytes was determined as percent DPs compared with control culture without antigen.

tion of DP thymocytes by SEA and FT16.6C5 is only induced at the highest antigen dose (Fig. 6 B). Therefore, in contrast to the results seen using moth cytochrome *c* as antigen, presentation of SEA by FT16.6C5 cells stimulates lymphokine secretion in mature T cells at doses that do not induce deletion of DP thymocytes.

Discussion

The Timing and Magnitude of Negative Selection. Negative selection in the thymus has been demonstrated using a variety of experimental models, and through this work it has been suggested that clonal deletion occurs at the CD4/CD8 DP stage of thymic development. Based on the size of the thymus and steady state populations present under different conditions, clonal deletion would appear to take place during a large portion of the DP differentiation state depending upon the ligand or receptor mediating the event (6, 9–13, 17, 38). However, the suggestion was made that the early deletion seen in transgenic mice could be due to the premature expression of the TCR during thymocyte development (16), and that superantigen-mediated deletion, characterized by using V β -specific antibodies, was the more physiological model. We have found that the phenotype of mice transgenic for a particular α/β TCR specific for cytochrome *c* and class II MHC (8) may suggest an alternative view. The presence of the class II MHC-encoded molecule A^s in these mice apparently prevents the maturation of DP thymocytes to CD4 SP thymocytes. Because it is genetically dominant over positive selection mediated by A^b or E^k, this effect is most likely a form of negative selection. The deletion appears to occur late in DP thymocyte development, as indicated by a significant proportion of DP cells in the thymus. This pattern of negative selection in a transgenic mouse is thus similar to that seen with deletion of V β 17a-expressing thymocytes by the I-E-encoded class II molecule (9), V β 6 and V β 8.1 by Mls-1^a (10), and V β 3 by Mls-2^a (18); in these nontransgenic mice, immature DP thymocytes bearing a self-reactive receptor

are present in the thymus while mature SP cells are not. The similarity of the transgenic model presented here to the superantigen models is probably due to the fact that reactivity of the AND TCR with the A^s ligand is very weak. There is no detectable stimulation of T cells from H-2^{k/b} AND transgenic mice with APCs from either B10.S or B10.S(9R) mice (data not shown). A more avid interaction of thymocytes and an antigen/MHC complex is likely to induce deletion at early DP stages (2, 17), perhaps regardless of whether the mouse expresses TCR transgenes. A difference between antigen and superantigen deletion was previously described using a transgenic mouse bearing a receptor specific for lymphocytic choriomeningitis virus (LCMV) and class I MHC, as well as Mls-1^a (14). In this study, Pircher et al. demonstrated that deletion of transgenic thymocytes by Mls-1^a affected mature thymocytes but not immature DP cells. However, these same transgenic mice showed drastically depleted populations of both mature and immature thymocytes when LCMV and appropriate MHC were expressed. Whereas antigens can cause deletion to various levels and at various stages of DP maturation, perhaps superantigens can only cause deletion of SP or transitional thymocytes.

An even more dramatic distinction between antigen and superantigen deletion is seen using the *in vitro* culture system. Relative to the dose response for T cell activation, SEA is extremely inefficient in causing the deletion of thymocytes, as demonstrated by the experiment presented in Fig. 6. This in contrast to the more comparable efficacy of moth cytochrome *c* in inducing T cell activation and thymocyte deletion. These data may relate to the differences between superantigen and antigen/MHC binding to the TCR. For example, staphylococcal enterotoxin and Mls-1^a have been shown to bind a region modeled to be on the side of the TCR molecule away from the proposed antigen/MHC binding site (39, 40). In addition, differences have been seen in phosphoinositide hydrolysis induced by an H-2 alloantigen and an Mls superantigen (41). An implication of these data, consistent with the CsA sensitivity discussed below, is that mature T cells and DP thymocytes are activated by different signaling pathways. This is revealed because the cellular activation events involving superantigens, such as SEA, and antigen/MHC ligands are in some way different. If these results can be extrapolated to all superantigens, then we would argue that the timing and magnitude of deletions caused by endogenous or exogenous superantigens may not be necessarily representative of the physiological negative selection and clonal deletion that occurs in response to self-antigens.

The Mechanisms of Negative Selection. An issue in thymic development is whether negative selection is determined by the function of specialized APCs or whether the determining factor is the stage of thymocyte development. For example, antigen presented on thymic macrophages could induce deletion, whereas antigen presented on epithelial cells could induce further maturation. Alternatively, early DP thymocytes may interpret receptor ligation to result in positive selection, whereas later stage thymocytes may only be sensitive to negative selection. We have demonstrated that DP thymocytes can be deleted by the presentation of specific antigen by non-

thymic APCs. Induction of negative selection thus may not be dependent upon the activity of specialized thymic APCs; however, an important caveat is that the required function, such as cell surface molecules or cytokines, may be present in FT16 fibroblasts. Antigen added to culture can result in up to 80% loss in DP cells compared with thymocytes cultured in the absence of antigen, and deletion is dependent on RNA and protein synthesis. It is therefore evident that the majority, but perhaps not all DP thymocytes, are sensitive to a TCR-mediated signal to undergo a programmed cell death. The magnitude of deletion appears to be dependent on the avidity of the interaction, and this is implied by the antigen dose dependence of the deletion. Furthermore, anti-TCR antibodies have been reported to induce only 50% deletion in fetal thymic organ cultures (42). A recently published report using thymocytes from a TCR transgenic mouse specific for H-Y in association with H-2D^b showed that DP cells are selectively depleted over a period of 24–48 h when cultured with male APCs (43). Consistent with the results presented here, there was a population of thymocytes that were not deleted, even after 3 d in culture. Whether there exists an early DP population that is wholly insensitive to deletion signals is presently unclear. We thus cannot presently distinguish between the above models of selection.

A fundamental difference between *in vitro* antigen-induced clonal deletion and *in vivo* anti-TCR-induced clonal deletion is the sensitivity to CsA. Several reports indicate that CsA is effective in blocking clonal deletion induced *in vivo* by anti-TCR antibodies (29–31), whereas our experiments indicate that the direct, antigen-induced deletion of DP thymocytes is not sensitive to the effects of CsA at doses sufficient for complete inhibition of T cell activation (see Fig. 5). This observation suggests that there are intracellular signaling events necessary for T cell activation or anti-TCR-mediated deletion *in vivo* that are not required for antigen-induced thymocyte deletion. Studies have shown that CsA inhibits Ca²⁺-dependent activation of transcription by NF-AT, NFIL-2A, NFIL-2B, but does not effect Ca²⁺-dependent activation of *c-fos* mRNA; this indicates that CsA blocks some, but not all, Ca²⁺-dependent signaling pathways (44). More recent data indicate that CsA works as a complex with cyclophilin A and inhibits the phosphatase calcineurin (27). Thus, it would appear that deletion of DP thymocytes does not depend on the release of free Ca²⁺ in the same way as T cell activation, and more recent data indicate that it is highly dependent on the activation of protein kinase c (N. Vasquez and S. M. Hedrick, unpublished data). We contend that the results presented here more directly address physiology of antigen/MHC-induced clonal deletion. Another set of studies by Mercep et al. (45) showed that a T cell hybridoma would undergo growth arrest and cell lysis when cultured in the presence of cytochrome *c* and APCs. CsA did not affect growth arrest, but did affect IL-2 production and cell lysis. A conclusion of this study was that signals involved in growth arrest and activation diverge early in the course of the cellular response. The CsA-independent pathway may be analogous to that of thymocyte deletion presented here.

The Sensitivity of Clonal Deletion vs. T Cell Activation. Several

experiments now indicate that the process of clonal deletion is more sensitive to antigen than the process of mature T cell activation. Using TCR transgenic mice, Pircher et al. (46) identified an antigen that could cause negative selection *in vivo*, but not activation of mature T cells in culture. Similarly, in the experiments presented here, we have shown that the presence of A^s in the AND transgenic mice causes a form of negative selection, yet thymic or splenic APCs bearing A^s do not activate CD4⁺ mature T cells from AND-tg H-2^{b/k} mice. Our results inducing clonal deletion *in vitro* directly demonstrate this increased sensitivity of thymocytes vs. T cells. As demonstrated in Fig. 4, there is a significant deletion of DP thymocytes at 0.025 μ M 88-103 moth peptide, whereas virtually no T cell activation is detected at this antigen dose. Thus, although DP thymocytes express fewer receptors than mature T cells, in TCR transgenic and normal mice, they appear to be functionally more sensitive to antigen. We would speculate that this differential sensitivity might result from the different second messengers involved in the two processes. It may have evolved in order to provide a margin of safety to insure that the population of T cells will not respond to self-antigens encountered in the peripheral lymphoid organs.

In conclusion, negative selection appears to be dependent on the affinity/avidity of the TCR and its ligand. Our results suggest that thymocytes are more sensitive to receptor ligation than are mature T cells, and that this increased sensitivity may be mediated by a differential signaling pathway used by immature thymocytes. This hypothesis is additionally attractive because it provides a possible safeguard against autoreactivity. It should be noted that although clonal deletion is easily demonstrable in these cultures, we have found no evidence for the induction of positive selection *in vitro*. Cocultures of thymocytes with various preparations of thymic stromal cells, thymic stromal lines, or L cells expressing class II MHC do not appear to support the transition from DP to CD4⁺ SP thymocytes. There may be some element missing from the *in vitro* cultures that is present in the organismal thymus, fetal thymus organ cultures, or deoxyguanosine-treated thymuses placed in organ culture (3, 4). Understanding the difference in signaling that distinguishes positive selection and negative selection is a major goal of future studies.

We thank Dr. R. Germain for FT16.6C5, I-E^b transfected L cells. We also thank Ms. K. McCambridge-Palmer for her technical support with transgenic animals.

N. Vasquez was supported by a San Diego fellowship, Department of Biology, University of California, San Diego. J. Kaye was supported by the California division of the American Cancer Society (ACS) senior fellowship and by an ACS Junior Faculty Research Award (JFRA-296). S. M. Hedrick was supported by Research Career Development Award K04 AI-00662 from the National Institute of Allergy and Infectious Diseases. This work was supported by National Institutes of Health grants AI-21372 to S.M. Hedrick and AI-31231 to J. Kaye.

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Received for publication 23 September 1991 and in revised form 10 January 1992.

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