

Antigen Compartmentation and T Helper Cell Tolerance Induction

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Summary

The process of antigen recognition depends in part on the amount of peptide antigen available and the affinity of the T cell receptor for a particular peptide-major histocompatibility complex (MHC) molecule complex. The availability of self antigen is limited by antigen processing, which is compartmentalized such that peptide antigens presented by MHC class I molecules originate in the cytoplasm, whereas peptide antigens presented by MHC class II molecules are acquired from the endocytic pathway. This segregation of the antigen-processing pathways may limit the diversity of antigens that influence the development and selection of, e.g., CD4-positive, MHC class II-specific T cells. Selection in this case might involve only a subset of self-encoded proteins, specifically those that are plasma membrane bound or secreted.

To study these aspects of immune development, we engineered pigeon cytochrome *c* for expression in transgenic mice in two forms: one in which it was expressed as a type II plasma membrane protein, and a second in which it was targeted to the mitochondria after cytoplasmic synthesis. Experiments with these mice clearly show that tolerance is induced in the thymus, irrespective of antigen compartmentation. Using radiation bone marrow chimeras, we further show that cytoplasmic/mitochondrial antigen gains access to the MHC class II pathway by direct presentation. As a result of studying the anatomy of the thymus, we show that the amount of antigen and the affinity of the TCR affect the location and time point of thymocytes undergoing apoptosis.

A central mechanism for the establishment of self tolerance is the deletion of developmentally immature CD4/CD8 double-positive (DP)¹ thymocytes. Antigens must satisfy several requirements to affect a state of self tolerance. They must be available for processing and loading onto MHC class I and class II molecules, and they must be processed to peptides that bind and stabilize MHC molecules.

Exogenous and cell surface-associated antigens gain access to the endocytic compartment, where they are degraded and associate with MHC class II (1-4). These MHC class II-peptide complexes are most often recognized by CD4-positive helper T cells. Peptides from endogenous proteins are usually presented on MHC class I and most often recognized by CD8-positive T cells. Most antigens seem to follow this rule. An increasing body of data produced in *in vitro* systems shows, however, that proteins synthesized intracellularly in APC may also associate with MHC class II

(2, 3, 5-12). It therefore seems that cytosolic proteins can also gain access to the MHC class II compartment through pathways that are incompletely characterized. One pathway proposed is autophagy, and this may drive cytosolic components into the late endosomal or lysosomal compartments (13, 14). Another possibility is that endogenous antigens may be directed to the class II pathway in phagocytic cells via the engulfment of apoptotic cells. The relevance of such "alternative" pathways in T cell development is not well characterized, but is of considerable importance since this would mean that endogenous proteins can shape the Th repertoire.

To address these questions, we generated two transgenic mouse lines that express pigeon cytochrome *c* (PCC) endogenously (ePCC) or on the cell surface (mPCC) in the thymus. Cytochrome *c* is normally synthesized in the cytosol and is targeted into the intermembrane space, where it will complex with the respiratory chain proteins located on the inner mitochondrial membrane (15). Using an H-2K^b promoter construct, we expressed PCC representing a cytosolic/mitochondrial neo-self antigen in the thymus of

¹Abbreviations used in this paper: DP, double positive; ePCC, endogenous form of PCC; mPCC, membrane-associated PCC; PCC, pigeon cytochrome *c*; PPD, purified protein derivative; SP, single positive.

transgenic mice. For comparison, PCC was altered by replacing the amino-terminal 19 amino acids with the leader/transmembrane signal anchor sequence from the influenza hemagglutinin type II membrane protein. We predicted that this modification would yield a type II membrane-bound protein on the cell surface and on the inside of an endosomal vesicle. Using these mice together with transgenic mice expressing a TCR specific for PCC peptide 88–104, we analyzed the induction of Th tolerance.

A second issue in the induction of tolerance concerns the timing of deletion. In some models of negative selection, the developing T cells are deleted as early DP thymocytes in the proximal cortex, whereas in other models, the deletion occurs later at the DP to single-positive (SP) stage when the cells are at the cortical-medullary junction. One possibility is that the affinity of the interaction between the TCR and the MHC-peptide complex determines the time point of deletion. Using another PCC-specific TCR transgenic mouse line that expresses a receptor with a reduced affinity, we were able to directly test and visualize the avidity model of negative selection *in vivo*.

Materials and Methods

Transgenic Mice. The synthetic PCC gene was a generous gift from Dr. F. Sherman (University of Rochester, Rochester, NY) (16). It was cloned as a BamHI fragment into the expression vector pES4 (reference 17 and Fig. 2). Vector sequences were removed by a NotI/XhoI digest. Injection of this construct into (C57BL/6 SJL) oocytes yielded the SPK (ePCC) line, which was established to have integrated the PCC gene only on one chromosome. The second PCC construct targeted PCC to the cell surface by replacing the NH₂-terminal mitochondrial targeting sequence with the influenza neuraminidase type II signal anchor (a kind gift from Dr. D. Donoghue, University of California, San Diego). The modified PCC gene was cloned as a BamHI fragment into pES4, and vector sequences were removed by a ClaI/NotI digest. The RO (mPCC) founder line was chosen for further investigation. Transgenic offspring were identified by Southern analysis of tail biopsies. AD10 and AND TCR transgenic mice have been described previously (17, 18).

Northern Analysis. Northern blots were performed as described by Maniatis et al. (19). Total RNA was extracted from the tested tissues using TRIZOL reagent (GIBCO BRL, Gaithersburg, MD), and 20 µg/lane was resolved through agarose/formaldehyde denaturing gels. Autoradiographs were exposed for 8 h. LPS blasts were prepared by incubating spleen cells in Click's EHAA medium supplemented with 10⁻⁶ M 2-ME, penicillin/streptomycin, glutamine, 10% FCS, and 20 µg/ml LPS for 48 h. Blast cells were then purified over a Ficoll gradient.

Immunofluorescence Microscopy. Correct cellular localization of the transgenes was confirmed by transient transfection of Cos7 and HeLa cells with FLAG-tagged PCC gene variants: The 5' end of both PCC gene variants were modified using appropriate PCR primers to clone the genes in frame into the multiple cloning site of the plasmid pCMV-FLAG-2c (Eastman Kodak Co., Rochester NY). This resulted in the NH₂-terminal addition of a DYKD-DDDK peptide FLAG tag that could be detected with the mAb M2 (Kodak). Transcription was driven by the human CMV promoter. Cells were grown on cover slides for 24 h in six-well

plates and transfected with Lipofectamine (GIBCO BRL). After a total incubation time of 48 h, the cells were fixed with 95% ethanol/5% acetic acid, and FLAG-tagged protein was detected using the mAb M2 and secondary FITC-labeled goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Proliferation Assays. For secondary *in vitro* assays, 50 µg of PCC protein (Sigma Immunochemicals, St. Louis) emulsified in CFA were injected into one hind foot pad. After 7 d, the draining popliteal and inguinal lymph nodes were collected and minced into a single-cell suspension. 3–5 × 10⁵ nylon wool-enriched T cells were cocultured in Click's EHAA medium supplemented with 10⁻⁶ M 2-ME, penicillin/streptomycin, glutamine, and 10% FCS. As a source of APCs, 5 × 10⁶ irradiated (3,500 rads) B10.A spleen cells were added. APC were pulsed with the indicated doses of PCC peptide 88–104 or with 50 µg/ml purified protein derivative (PPD) to control for priming. After 3 d, 1 µCi [³H]thymidine was added to the cultures and incubated for another 16 h. Cells were harvested, and the incorporated radioactivity was counted on a liquid scintillation β counter.

FACS[®] Analysis. Stainings were performed in PBS containing 2% FCS, 0.1% sodium azide, and 10 mM EDTA using the following antibodies: anti-CD4 PE, anti-CD8 FITC, and anti-CD8 tricolor (all from Caltag Laboratories, San Francisco, CA). Transgenic TCR chains were detected with KJ25 (anti-Vβ3 [20]) and RR8 (anti-Vα11; a generous gift from Dr. O. Kanagawa, Washington University, St. Louis, MO) culture supernatants. FITC-conjugated goat anti-hamster (for KJ25) and goat anti-rat (for RR8) were used as second stage antibodies. 10,000 live cells were collected on a FACScan[®] (Becton Dickinson & Co., Mountain View, CA) and analyzed using CellQuest software (Becton Dickinson & Co.).

In Situ TUNEL Assays. TUNEL assays were performed as described previously (21). Briefly, thymi were embedded in OCT (Miles, Inc., Kanakee, IL) and frozen tissue sections were prepared onto microscope slides, dried for 2 d at 4°C, fixed in acetone, and endogenous peroxidase was quenched by incubation in 0.01% H₂O₂, 50 mM Tris buffer, 150 mM NaCl. After washing, digoxigenin-labeled dUTP (Boehringer Mannheim Corp., Indianapolis, IN) was incorporated into DNA strand breaks using terminal deoxynucleotidyl transferase (TdT; Promega, Madison, WI). Apoptotic cells were detected with sheep Fab antidigoxigenin (Boehringer Mannheim) followed by incubation with peroxidase-coupled rabbit anti-sheep IgG F(ab)₂-specific antibodies (Jackson ImmunoResearch). Sections were developed with amino-ethyl-carbazole solution and counterstained with Meyer's hematoxylin.

In Situ Hybridization. *In situ* hybridizations were prepared exactly as described previously (22). ³⁵S-labeled antisense and sense riboprobes were transcribed from both directions using the PCC gene cloned into pBluescript SK II. Frozen sections were hybridized with the sense and antisense riboprobes. Exposures were performed at 4°C in the dark for 2 wk before development.

Bone Marrow Chimeras. Bone marrow was collected from the tibia and femur of donors and *in vitro*-depleted of T cells by two rounds of treatment with T24 (anti-Thy1) antibody plus complement. 1–2 × 10⁷ bone marrow cells were injected intravenously into irradiated (1,050 rads) recipients. Recipients were previously injected with 200 µg i.p. of PK136 (anti-NK1.1) ammonium sulfate-precipitated ascites fluid 3 and 1 d before transfusion. Animals were left for reconstitution for the indicated amounts of time with 25 mg/liter neomycin sulfate and 13 mg/liter polymyxin B sulfate supplemented to their drinking water for the first 3 wk. Chimerism of mixed bone marrow chimeras was determined after 3–4 wk by collecting blood from the tail vein into FACS[®] buffer.

Samples were stained with anti-B220-PE and anti-I-E-FITC (both from Caltag). After incubation, red cells were lysed and the degree of chimerism was determined by gating on B220-positive live B cells and monitoring the expression of H-2E.

Results

Generation of Intracellular and Cell Membrane-associated, PCC-transgenic Mice. The wild-type PCC transgene was expressed in lymphoid tissues, including the thymus, using the pES4 expression vector (Fig. 1). A second construct was generated to target PCC to the cell surface: the mitochondrial targeting sequences are located at the amino terminal end and within the protein (15, 23). The NH₂-terminal mitochondrial targeting sequence was replaced with the influenza neuraminidase type II signal anchor. This modified PCC gene was also cloned into the H-2K^b expression vector. SPK mice had integrated the ePCC whereas RO mice were transgenic for mPCC. Northern Blot analysis revealed that the transgenes were expressed in thymus, spleen, and LPS-stimulated B cells (Fig. 2). mPCC mice expressed significantly higher mRNA levels than ePCC mice. Transgenic mRNA was readily detected in LPS blasts and in spleens of ePCC mice after prolonged exposure overnight (not shown).

Cellular Localization. All the antibodies we tested cross-reacted on mouse cytochrome *c*, thus preventing us from directly examining the intracellular localization of transgenic PCC expression. To circumvent this problem, we generated constructs in which the FLAG tag was fused to the NH₂-terminal end of the two PCC variants. Cos7 cells (Fig. 3) and HeLa cells (data not shown) were transiently transfected and analyzed by fluorescence microscopy using the FLAG-specific mAb M2. The staining pattern of FLAG-tagged mPCC-transfected cells was consistent with localization to the cell surface and to an intracellular organelle (Fig. 3 A, see legend). The FLAG-tagged ePCC construct gave rise

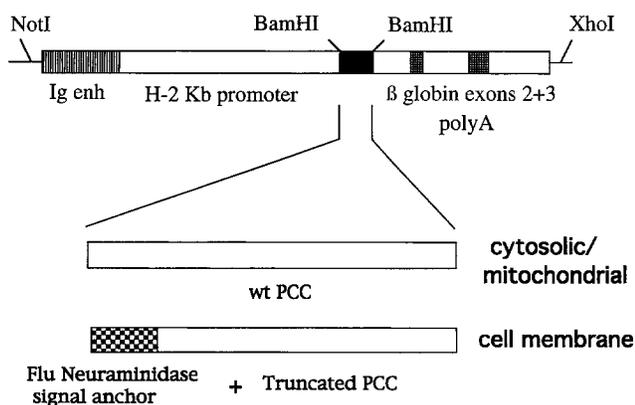


Figure 1. PCC transgene constructs. The synthetic PCC gene (cytosolic/mitochondrial localization) was cloned into the expression vector pES4, providing the H-2K^b promoter, the Ig heavy chain enhancer, human β -globin exons 2 and 3, and a polyadenylation site. In the second PCC gene variant, the NH₂-terminal mitochondrial targeting sequence was replaced by the influenza neuraminidase type II signal anchor to direct PCC to the cell surface.

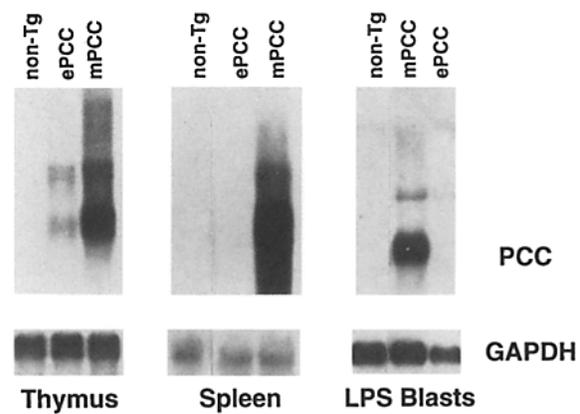


Figure 2. Transgenic PCC mRNA expression. Total RNA from thymus, spleen, and LPS blasts obtained from ePCC, mPCC, and nontransgenic control mice was analyzed by Northern blot. To confirm that equal amounts of total RNA were loaded in each lane, the filters were reprobbed with a glyceraldehyde 3-phosphate dehydrogenase-specific probe.

to a punctate pattern of staining that is typical of mitochondrial localization patterns for ethanol-fixed cells (Fig. 3 B). Thus, the gene products were targeted to the anticipated cellular sites.

Induction of Tolerance in mPCC and ePCC Mice. To investigate whether both forms of PCC were able to induce tolerance, proliferative responses to PCC were measured in secondary *in vitro* cultures. PCC-transgenic mice and control littermates were inoculated with PCC protein emulsified in CFA, and enriched T cells from the draining lymph nodes were explanted to tissue culture 7 d later. Cultures were stimulated with various concentrations of PCC peptide or a single dose of PPD. Proliferation was greatly reduced in ePCC mice and comparable to background levels in mPCC mice (Fig. 4). The response to PPD was equivalent in PCC-transgenic and control mice (see Fig. 4 legend). Thus, ePCC and mPCC were both effective at inducing a state of tolerance. The quantitative difference in the degree of responsiveness presumably resulted from the amount of expressed antigen-MHC complexes on the surface of APCs. We could not determine whether this resulted from the difference in intracellular localization, the level of expressed antigen, or both.

To determine the stage of tolerance induction in the two PCC-transgenic mouse lines, they were crossed with AD10 TCR transgenics that bear a receptor specific for PCC peptide 88-104 on H-2E^k (17, 18). Comparison of thymocytes from AD10×PCC double transgenics with AD10 single transgenic animals revealed that expression of both forms of PCC resulted in a largely reduced population of CD4 SP thymocytes (Fig. 5). Transgenic V α 11 and V β 3 TCR chain expression levels were unaltered on the DP populations (data not shown); however, the CD4 SP thymocytes that escaped clonal deletion displayed biphasic and somewhat reduced patterns of staining for V α 11 and V β 3 (Fig. 5). This is indicative of cells that express endogenous T cell receptor chains. It is even possible that all of the CD4 SP thymocytes express at least one nontransgenic TCR chain. When analyzing

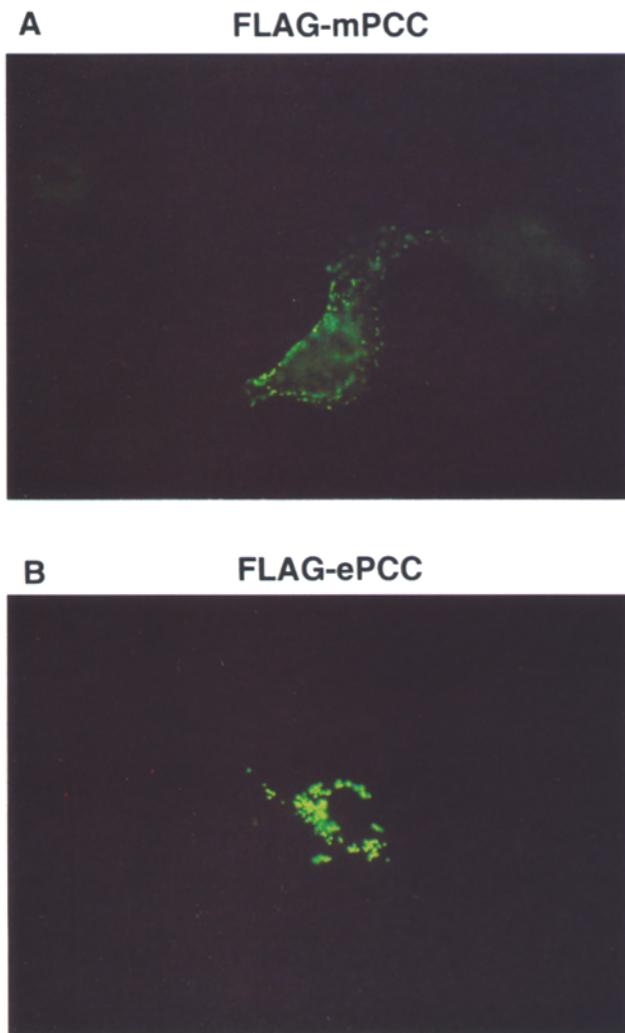


Figure 3. Cellular localization of transgenic mPCC and ePCC. (A) NH₂-terminal addition of the FLAG tag to the mPCC gene variant (*FLAG-mPCC*) resulted in cell-surface staining patterns of transiently transfected Cos7 cells. Punctate staining is cell surface associated and could be distinguished from intracellular staining by scanning through the focal plains. Intracellular diffuse staining peripheral to the nucleus may represent staining of the endoplasmic reticulum. (B) Localization of ePCC to mitochondria was confirmed by detection of an NH₂-terminally FLAG-tagged ePCC (*FLAG-ePCC*) construct. Positive cells shown here are embedded within a confluent cell layer serving as background control staining.

ing peripheral T cells from the lymph nodes of double transgenic mice, we found that percentages of mature CD4 T cells were reduced somewhat more in AD10×mPCC (from 41% in AD10 to 14% in AD10×mPCC) than in AD10×ePCC (from 41% in AD10 to 20% in AD10×ePCC) mice (data not shown). The CD4-positive cells displayed TCR expression patterns similar to the mature thymocytes that had escaped deletion. The cells that had escaped clonal deletion were still responsive in primary in vitro proliferation assays, although 10 (AD10×ePCC) to 100 (AD10×mPCC) times higher PCC doses were required for stimulation comparable to T cells from AD10 TCR single transgenic mice (data not shown). Thus, central tolerance was induced in both

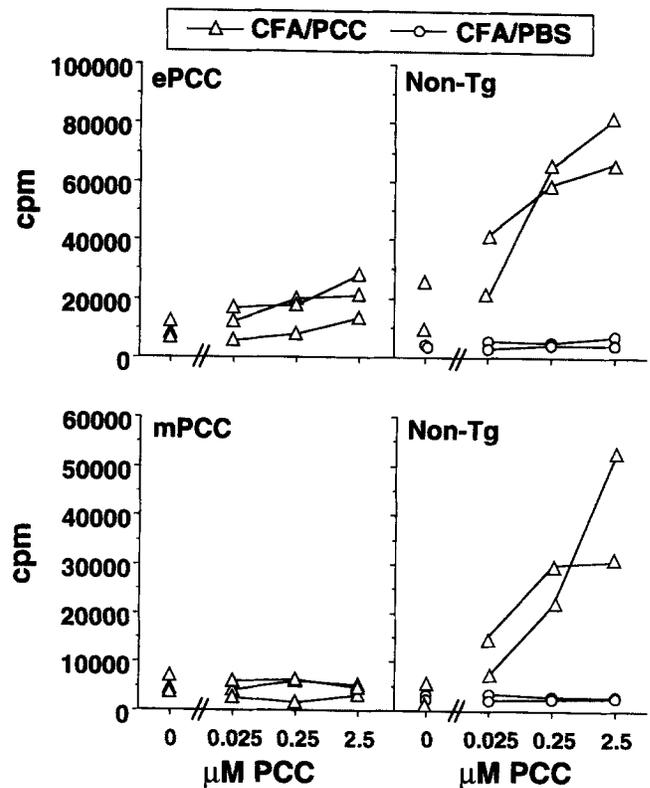


Figure 4. Proliferative responses of ePCC- and mPCC-transgenic mice. Secondary in vitro assays were performed with lymph node cells obtained from ePCC, mPCC, or nontransgenic littermates immunized with PCC protein or PBS as control emulsified in CFA. Responses of ePCC and mPCC mice were measured in two separate experiments at the indicated PCC peptide concentrations. PPD responses measured to control for priming were in similar ranges within experiments: ePCC and controls, 140,000–170,000 cpm; mPCC and controls, 90,000–110,000 cpm.

mouse lines, resulting in a highly diminished population of antigen-reactive CD4 SP cells. There was no evidence of autoimmunity or compromised health in the double transgenic or immunized mice.

Early vs. Late Deletion Correlates with Cortical vs. Cortico-medullary Apoptosis. To more closely define the stage at which self reactive thymocytes were deleted, absolute cell numbers of thymocyte subpopulations were calculated. This revealed a reduction of $61 \pm 13\%$ of DP in AD10×mPCC vs. $4.0 \pm 3.8\%$ of DPs in AD10×ePCC mice, respectively. Since the CD4 SP populations were affected to similar degrees in both lines (deletion of $96 \pm 2\%$ in AD10×mPCC and $83 \pm 4\%$ in AD10×ePCC), we can deduce that DP thymocytes are eliminated at a relatively earlier stage in mPCC compared to ePCC transgenics.

At least two possibilities could account for this difference. First, positioning effects of the transgene could result in the expression of transgenic PCC in different regions of the thymus. Second, toleragen expression levels may dictate time point and site of deletion. To investigate whether expression patterns were responsible for early vs. late deletion and whether the time point of deletion was manifested in the

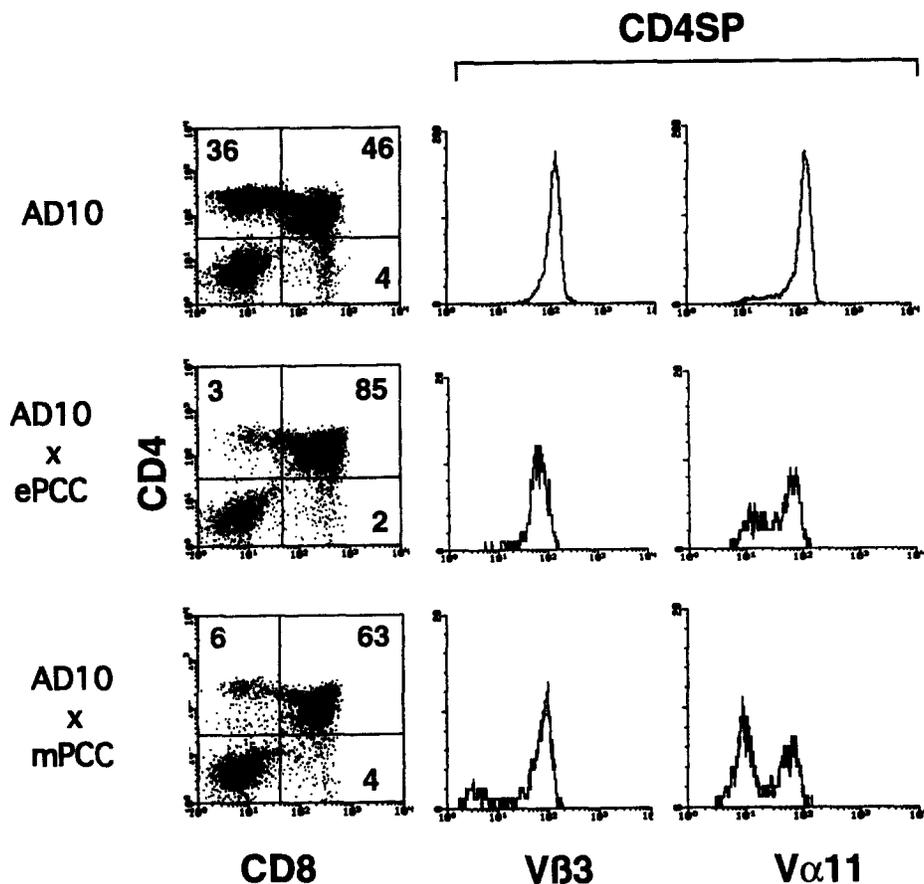


Figure 5. Reduction of CD4SP thymocytes in AD10×PCC double transgenic mice. Thymocyte from AD10 TCR, AD10×ePCC, and AD10×mPCC transgenic mice were triple stained for CD4, CD8, and Vβ3 or Vα11. Transgenic TCR levels were determined by gating on the CD4 SP population. Numbers in quadrants represent percentages of the respective populations. Shown are plots representative of five independent experiments.

site of deletion, we determined where in the thymus self-reactive thymocytes were eliminated. In situ TUNEL assays performed on thymi of double transgenic mice to detect apoptotic thymocytes showed that cells which had fragmented their DNA appeared in the cortex of early deleting mPCC mice, whereas such cells were found at the cortico-medullary junction and in the medulla of late deleting ePCC mice (Fig. 6, *A* and *B*). Thus, relatively early deletion of DP correlated with cortical apoptosis, whereas late deletion paralleled with cortico-medullary and medullary apoptosis.

To determine the anatomical location of PCC expression within the thymus, in situ hybridization studies were carried out using a PCC-specific probe on thymic sections. Fig. 7 *A-C* depicts autoradiographic sections showing that PCC is expressed in the cortex and medulla of both PCC-transgenic mouse lines, albeit at lower levels in ePCC mice. Since thymocytes also express the transgenes, part of the signal seen in this experiment probably originates from thymocytes that presumably do not directly function as APCs. To address this issue further, mPCC and ePCC-transgenic mice were lethally irradiated and reconstituted with bone marrow from normal mice. These mice were analyzed after 4 wk and typed for chimerism. In the thymus of these radiation chimeras, the only cells that could express a PCC transgene message would be epithelial cells and some radioresistant macrophages. The expression patterns were identical to those of unmanipulated transgenic animals. As

expected, however, the overall signal was reduced (data not shown). Compared to the cortical expression levels, medullary expression was lower in both lines and experiments. Thus, at least for the unmanipulated thymus and the radioresistant cells of the thymus, there was no anatomical difference in the expression of ePCC and mPCC.

The above results argue for the second possibility that the level of antigen expression can account for early vs. late deletion. Indeed, RNA expression levels as determined by Northern blot analysis were higher in mPCC than in ePCC mice (see Fig. 2). Taken together, early deletion resulted in apoptosis in the cortex and the medulla, whereas late deletion promoted the elimination of thymocytes at the cortico-medullary junction and in the medulla. Presumably, the time point of deletion was influenced by the levels of PCC-bound MHC class II molecules.

Influence of TCR Affinity on Time Point and Site of Deletion. Our results are consistent with the process of negative selection depending in part on the level of antigen presentation, and thus the avidity of the interaction between the TCR and the peptide-MHC complex. Another component that determines the avidity of the interaction between the TCR and the MHC-peptide complex is the affinity of the TCR itself. AND TCR-transgenic mice bear an α chain that differs in one amino acid in the V-J junction with respect to the original AD10 receptor (17). We have found that 70% of the AD10 DP thymocytes could be

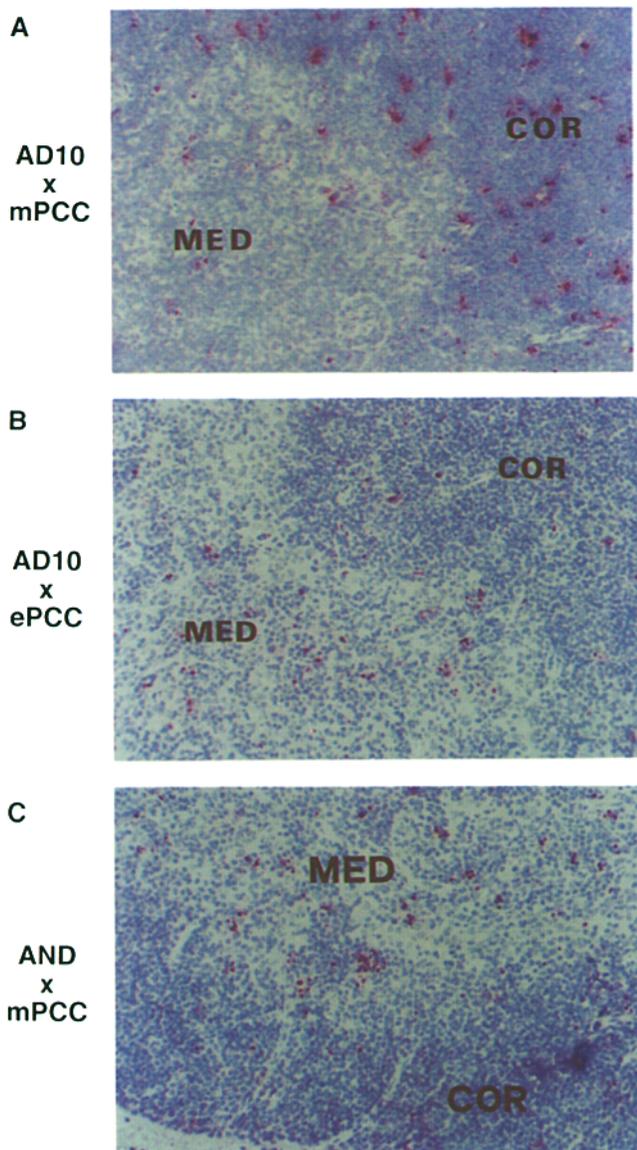


Figure 6. Detection of apoptotic cells in thymic sections by in situ TUNEL assay. (A) AD10×mPCC transgenic thymus section. Dark red staining represents cells that have fragmented their DNA. Most apoptotic cells are detected in the cortex (*cor*), but some are also visible in the medulla (*med*). (B) AD10×ePCC thymic section. Apoptotic cells appear at the cortico-medullary junction and in the medulla. (C) Cortico-medullary apoptosis in AND×mPCC mice. AD10 TCR- and AND TCR-transgenic control sections showed extremely low background staining scattered in the cortex and medulla. Sections represent stainings from three to four different mice. ×200.

deleted after a single injection with 750 μg i.v. of whole PCC protein, whereas not even 1.6 mg of protein was sufficient to detect the deletion of AND DP thymocytes (data not shown). Similar levels of deletion as in AD10 TCR transgenics could be obtained in AND TCR transgenics only after three daily injections of 750 μg of PCC protein. This shows that the AND-transgenic thymocytes are less sensitive to antigen administration than AD10 thymocytes, and would appear to imply that the single amino acid difference in the AND TCR causes a reduction of the affinity

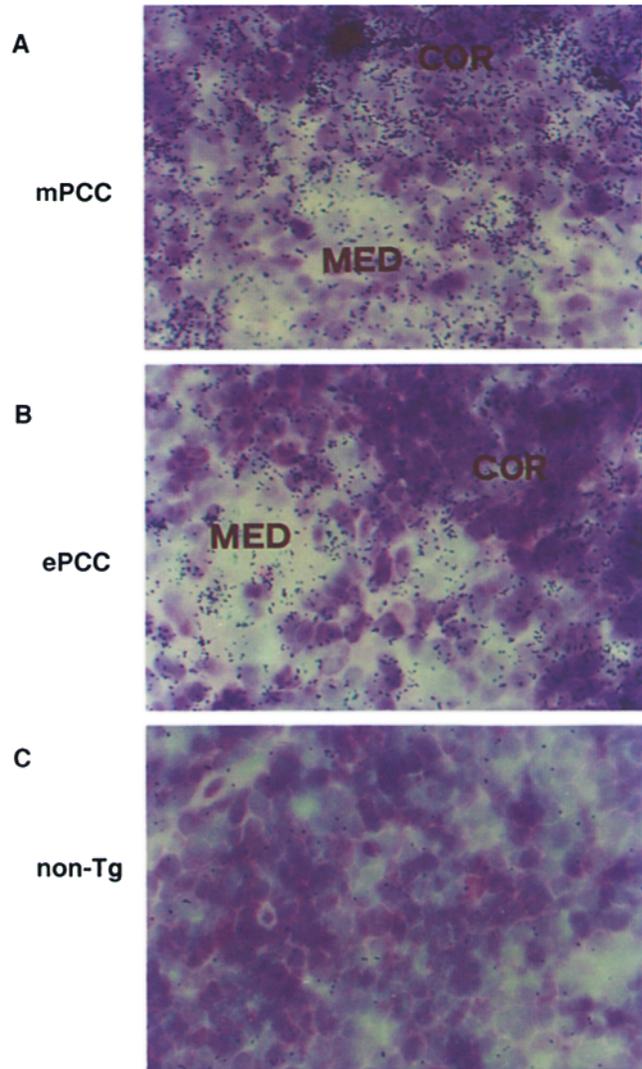


Figure 7. Transgene expression patterns in ePCC and mPCC mice. In situ hybridizations were performed on thymic sections from (A) mPCC and (B) ePCC mice, using a PCC-specific probe. Dark grains represent transgenic mRNA. Transgenic PCC is expressed in the cortex (*cor*) and at lower levels in the medulla (*med*) of both mouse lines. Overall staining is lower in ePCC animals. (C) Nontransgenic littermate. Identical background staining as in C was obtained with the sense riboprobe on all sections. ×1,000.

for MHC/antigen when compared with the AD10 TCR transgenics. To investigate the influence of the difference between AD10 and AND mice on the time point and site of deletion, AND mice were bred with mPCC mice. In such double transgenic animals, negative selection occurred very late in the DP stage (24): deletion of DP thymocytes was marginal in AND×mPCC mice while absolute cell numbers of the CD4 SP population were reduced by 75%. Fig. 6 C shows that apoptotic cells appeared at the cortico-medullary junction in AND×mPCC mice, and not in the cortex as in AD10×mPCC mice (see Fig. 6 A). Thus, the self-reactive TCR expressed by a thymocyte influences the time point and site of deletion.

Endogenous Self Antigen Can be Directly Processed to Induce Negative Selection. In principle, antigen can be processed

within a cell and presented directly, or it can be acquired indirectly via engulfment of apoptotic cells (e.g., thymocytes), cellular debris, soluble shed, or secreted proteins. In particular, we were interested in determining whether ePCC is presented directly to cause negative selection. To investigate this question, the following bone marrow chimeras were generated: H-2E^k-positive ePCC, mPCC, and nontransgenic littermates were irradiated and reconstituted with H-2^b (H-2E^o) AD10 TCR-transgenic bone marrow and analyzed 3–5 wk later (Table 1). In these radiation chimeras, transgenic ePCC can be presented only if it is directly processed and presented by radioresistant macrophages or thymic epithelial cells. Since thymic macrophages are largely MHC class II negative (25, 26), the most likely APC would be epithelial cells. The experiments presented in Table 1 show that the CD4 SP TCR-transgenic thymocyte population was significantly reduced in ePCC and mPCC recipients as compared with controls. Since we find it unlikely that epithelial cells could acquire PCC exogenously in these radiation chimeras, we deduce that ePCC can be directly processed onto MHC class II and effectively presented by thymic epithelial cells (see Discussion).

Reuptake of Self Antigen Results in Clonal Deletion. A second set of bone marrow chimeras were tested for the possibility that PCC can be directed to the endocytic processing pathway by the reuptake of antigen derived from thymocytes undergoing apoptosis (21) or the reuptake of shed antigen. A mixture of two types of bone marrow was injected into PCC-negative, H-2E^k-positive recipients. The first type was taken from mice that expressed PCC and were H-2E^k-negative. This bone marrow provides PCC-transgenic thymocytes that can be engulfed and could serve as a source of shed antigen. Any APCs that develop from this bone marrow, however, will be unable to present PCC because they do not express H-2E^k. The other type of bone marrow was harvested from mice that expressed H-2E^k, but did not ex-

Table 1. Clonal Deletion after Direct Processing of Endogenous PCC

	Donor (I-E ⁻)	Recipients* (I-E ^k)	n	% CD4 SP ± SEM [†] (% deletion)
Experiment 1	TCR	Non-Tg	3	39.7 ± 6.3
	TCR	ePCC	3	17.0 ± 1.5 (56%)
	TCR	mPCC	3	23.3 ± 2.6 (41%)
Experiment 2	TCR	Non-Tg	4	21.9 ± 1.9
	TCR	ePCC	3	11.4 ± 1.1 (48%)
	TCR	mPCC	4	12.2 ± 1.0 (46%)

*H-2E^k-positive recipients were transfused with 10⁷ bone marrow cells obtained from AD10 TCR-transgenic, H-2E⁻-negative donors, and were left for reconstitution for 5 wk in experiment 1 and for 3 wk in experiment 2.

[†]Thymocytes from recipients were stained for CD4 and CD8, and the percentages of CD4 SP thymocytes were determined by flow cytometry. Absolute cell numbers did not vary significantly.

press PCC. This bone marrow provides phagocytes that can engulf and present PCC derived from the first marrow type. Recipients expressed the MHC molecules appropriate for the presentation of PCC. After reconstitution for 8 wk, chimeras were challenged with PCC protein emulsified in CFA, and PCC-specific proliferative responses were measured in vitro. The experiment depicted in Fig. 8 shows that the presence of H-2E^k-negative, PCC-transgenic bone marrow resulted in reduced proliferative responses, regardless of whether ePCC or mPCC marrow was used. Although the degree of chimerism was lower in mice receiving mPCC marrow, the reduction in the response was comparable to ePCC chimeras, probably resulting from higher mPCC expression levels. Thus, reuptake of antigen can participate in the induction of Th tolerance.

This issue was alternatively addressed by producing a similar set of chimeric mice in which all the T cells expressed the AD10 TCR. Tolerance induction could thus be monitored by flow cytometry. Table 2 shows that two out of four recipients that had received ePCC bone marrow showed some reduction in the percentage of CD4 SP thymocytes 5 wk after reconstitution. All recipients that were transfused with mPCC bone marrow showed a small but significant reduction in the CD4 SP population. We interpret these experiments to indicate that reuptake of cell-

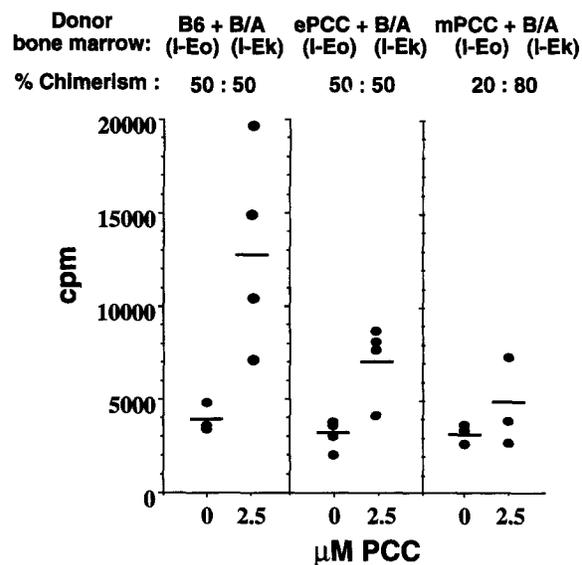


Figure 8. Reduction of proliferative responses in mixed bone marrow chimeras. Lymph node cells from mixed bone marrow chimeras that had been left for reconstitution for 8 wk were injected into the hind footpad with PCC protein emulsified in CFA and assayed in secondary in vitro proliferation assays. Because of the low number of cells recovered, purified cells were stimulated with the one indicated dose of PCC peptide or were left unstimulated for background proliferation. Priming efficiency was determined by measuring the proliferative response to 50 μg/ml PPD and was comparable in all mice tested, ranging from 45,000–65,000 cpm; relatively lower PPD counts did not correlate with corresponding lower PCC-specific proliferation. Points represent results from triplicate wells per mouse. Bone marrow composition and degree of chimerism are indicated. B6, C57Bl/6 bone marrow (H-2E^o); B/A, (C57Bl/6×B10.A)_{F1} bone marrow (H-2E^k).

Table 2. Deletion after Reuptake of Cell-derived Self Antigen

Bone marrow 1 (I-E-)	Bone marrow 2 (I-E+)	Recipient* (I-E+)	% CD4 SP [†] thymocytes (% deletion)	Average \pm SEM
TCR	TCR	1	27.5	25.2 \pm 1.9
		2	29.3	
		3	23.0	
		4	21.0	
(TCR \times ePCC)	TCR	1	26.1	22.5 \pm 2.0
		2	19.5 (23%)	
		3	18.7 (26%)	
		4	25.9	
(TCR \times mPCC)	TCR	1	15.5 (38%)	16.7 \pm 0.5
		2	16.4 (35%)	
		3	17.0 (33%)	
		4	17.7 (30%)	

*Recipients were transfused at a ratio of 1:1 with 10^7 bone marrow cells from each donor and analyzed 5 wk later. Chimerism was 50:50% in all recipients and was determined as described in Materials and Methods.

[†]Thymocytes were stained for CD4 and CD8, and the percentages of CD4 SP cells were determined.

associated or shed self antigen can play a part, albeit possibly minor, in the induction of Th tolerance to self antigens.

Discussion

This study was aimed at investigating parameters governing the induction of Th tolerance, including the cellular localization of self antigen, toleragen expression levels, and TCR affinities, and correlating these effects with the time point and site of deletion *in vivo*.

The data indicate that antigen may be directed into the class II processing pathway primarily via a direct route within the presenting cells and perhaps secondarily via reuptake of apoptotic cells, cell debris, or shed antigen. In the experiment described in Table 1, the only source of PCC are the radioresistant cells of the thymus. The PCC-transgenic mice were irradiated with 1,050 rads, and this dosage has been shown to rapidly eliminate the bone marrow-derived APC of host origin, such as dendritic cells and macrophages (27, 28). Since there are no radioresistant follicular dendritic cells known to reside in the thymus that could trap antigen in antibody-antigen complexes, the only presenting cells remaining after irradiation that express MHC class II molecules would be epithelial cells. These epithelial cells could not harbor peptides acquired from exogenous sources before or as a consequence of irradiation for as long as 5 wk since the half life of MHC class II-peptide complexes has been shown to be 13 h (29). Thus, the simplest interpreta-

tion of these experiments is that both ePCC and mPCC can be presented without secretion or reuptake. Since epithelial cells have previously been shown to mediate negative selection inefficiently (30), the fact that we see negative selection in this experiment is highly significant.

The experiments in which reuptake was needed to present antigen indicated that representation is an inefficient means of inducing negative selection (Table 2). Suhr et al. have recently shown that a large number, if not all, thymocytes that die in the thymus are engulfed by thymic macrophages (21). These macrophages could potentially present PCC, irrespective of its original cellular localization. Thymic macrophages, however, are very poor at inducing tolerance and express low levels of MHC class II (31, 32). Conceivably, cellular material may be released as degradation products from dying thymocytes or thymic macrophages; however, the process of apoptosis is such that the contents of dying cells are not usually released into the tissues (33). Alternatively, peripheral APCs that have engulfed apoptotic immune cells expressing PCC may recirculate into the thymus to induce tolerance.

What is the primary means of presentation in unirradiated ePCC and mPCC mice? The experiments published to date support dendritic cells as the most likely presenting cells to account for negative selection (34-36). Based on the interpretation of the experiments in this report, the most likely route of tolerance is the direct presentation of MHC class II-bound PCC by dendritic cells.

Stockinger et al. produced radiation chimeras in which pro-C5 was expressed endogenously within macrophages, but C5 was not secreted. Pro-C5 was not presented in an antigenic form associated with class II molecules. In these mice, there was no evident tolerance. Assuming that thymic macrophages also expressed pro-C5, these mice would not be expected to exhibit tolerance to C5 for two reasons: macrophages are poor in the induction of tolerance, and endogenously synthesized pro-C5 is not presented by MHC class II. Another likely explanation for the apparent discrepancy between the results presented here and those of Stockinger et al. is that mitochondrial cytochrome *c* and pro-C5 may simply have a different access to the class II pathway. There are numerous examples of cytoplasmic antigens that apparently gain direct access to MHC class II presentation *in vitro*, and this may be protein and expression level dependent (2, 3, 5-12).

The pathway of cytoplasmic or mitochondrial antigen presentation by MHC class II molecules is thus far uncharacterized. Loss et al. have previously reported the existence of an alternative processing pathway that allows endogenously synthesized L^d peptide to traffic onto MHC class II (12). Another potential processing pathway particularly relevant to mitochondria is via the formation of autophagosomes (37, 38). While this is a potential mechanism for transport of mitochondrial or cytoplasmic proteins into the class II pathway, most studies are consistent with an intersection of autophagosomes and lysosomes, a point in the endocytic pathway that is believed to be later than the MHC class II compartment (1).

Toleragen expression levels seem to play a role in determining the developmental stage and corresponding anatomical site of thymocyte deletion. In AD10×mPCC mice, deletion occurred within the DP subset, and this correlated with an increase in apoptosis within the cortex. In contrast, deletion in AD10×ePCC transgenics occurred later, since there was a barely detectable decrease in the number of DP thymocytes, and increased apoptosis was found only at the cortico-medullary junction. There are two possible explanations to account for this difference. One is that the levels of antigen presentation are lower in ePCC mice, and this level is only sufficient to cause deletion in late DPs when the TCR is upregulated. A second possibility is that antigens are expressed in anatomically different sites. We felt we could eliminate the latter possibility, since *in situ* hybridization showed consistent expression throughout the thymus for both transgenes. We thus conclude that a difference in antigen presentation levels accounts for the results. We did not determine whether the diminished antigen presentation is caused by decreased expression, the efficiency of the presentation pathway, or both.

We also observed that mPCC causes a relatively early deletion when crossed with AD10 TCR-transgenic mice and a later deletion when crossed with the AND TCR-transgenic mice. Since we have not detected a difference in the levels of receptor expression in these two TCR-transgenic mouse lines, the difference in the timing of deletion is probably caused by an affinity difference in the interaction

between the TCR and the MHC-peptide complex, the result of a single amino acid difference in the α -chain sequences. Thus, the timing of deletion is a function of the avidity of the thymocyte-APC interaction.

Most previous work in TCR-transgenic mice in which negative selection to nominal antigen was examined revealed deletion at a very early DP stage, whereas studies with negative selection induced by endogenous superantigens have revealed deletion late in the life of DP thymocytes, at the transition from DP to SP. The present experiments show that the timing of deletion in part importantly depends on the presentation of the antigenic determinant. The very same TCR-transgenic mice can show an early or a late deletion, depending on antigen presentation and expression levels. Conversely, the very same antigen can cause early or late deletion, depending on the TCR. This is consistent with the avidity model of negative selection.

Taken together, we were able to show that endogenous cellular proteins can gain access to the endocytic processing pathways *in vivo* to help shape the Th repertoire. The universe of self antigens inducing Th tolerance may therefore be larger than the set of peptides from cell-surface or secreted proteins. Although experiments examining the peptides bound to MHC class II molecules reveal peptides from secreted or membrane-bound proteins (2, 7), these may reflect only the most abundant peptides. In contrast, tolerance induction may require only a small number of peptides presented per cell.

The authors would like to thank Kathleen McCambridge-Palmer for excellent animal care and Dr. Nissi Varki for assistance in the tissue section preparation. We also thank Drs. D. Page and D. Schwarz for critically reading the manuscript.

Stephan Oehen was supported by a senior fellowship (No. S-60-93) from the American Cancer Society. This work was supported by U.S. Public Health Service grant R01 AI21372-11 to S.M. Hedrick. C.D. Surh is a special fellow of the Leukemia Society of America.

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Received for publication 12 December 1995 and in revised form 18 March 1996.

References

1. Neeffes, J.J., and H.L. Ploegh. 1992. Intracellular transport of MHC class II molecules. *Immunol. Today*. 13:179-184.
2. Hunt, D.F., H. Michel, T.A. Dickinson, J. Shabanowitz, A.L. Cox, K. Sakaguchi, E. Appella, H.M. Grey, and A. Sette. 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science (Wash. DC)*. 256:1817-1820.
3. Rudensky, A.Y., S.M. Mazel, and V.L. Yurin. 1990. Presentation of endogenous immunoglobulin determinant to immunoglobulin-recognizing T cell clones by the thymic cells. *Eur. J. Immunol.* 20:2235-2239.
4. Brodsky, F.M., and L.E. Guagliardi. 1991. The cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 9:707-744.
5. Nuchtern, J.G., W.E. Biddison, and R.D. Klausner. 1990. Class II MHC molecules can use the endogenous pathway of antigen presentation. *Nature (Lond.)*. 343:74-76.
6. Malnati, M.S., M. Marti, T. LaVaute, D. Jaraquemada, W. Biddison, R. DeMars, and E.O. Long. 1992. Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature (Lond.)*. 357:702-704.
7. Rudensky, A.Y., P. Preston-Hurlburt, S.C. Hong, A. Barlow, and C.A. Janeway. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature (Lond.)*. 353:622-627.
8. Chen, B.P., A. Madrigal, and P. Parham. 1990. Cytotoxic T cell recognition of an endogenous class I HLA peptide pre-

- sented by a class II HLA molecule. *J. Exp. Med.* 172:779–788.
9. Brooks, A.G., and J. McCluskey. 1993. Class II-restricted presentation of a hen egg lysozyme determinant derived from endogenous antigen sequestered in the cytoplasm or endoplasmic reticulum of the antigen presenting cells. *J. Immunol.* 150:3690–3697.
 10. Jaraquemada, D., M. Marti, and E.O. Long. 1990. An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II-restricted T cells. *J. Exp. Med.* 172:947–954.
 11. Weiss, S., and B. Bogen. 1991. MHC class II-restricted presentation of intracellular antigen. *Cell.* 64:767–776.
 12. Loss, G.E.J., C.G. Elias, P.E. Fields, R.K. Ribaud, M. McKisic, and A.J. Sant. 1993. Major histocompatibility complex class II-restricted presentation of an internally synthesized antigen displays cell-type variability and segregates from the exogenous class II and endogenous class I presentation pathways. *J. Exp. Med.* 178:73–85.
 13. Dunn, W.A.J. 1990. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* 110:1923–1933.
 14. Dunn, W.A.J. 1990. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J. Cell Biol.* 110:1935–1945.
 15. Stuart, R.A., D.W. Nicholson, U. Wienhues, and W. Neupert. 1990. Import of apocytochrome *c* into the mitochondrial intermembrane space along a cytochrome *c*1 sorting pathway. *J. Biol. Chem.* 265:20210–20219.
 16. Hickey, D.R., K. Jayaraman, C.T. Goodhue, J. Shah, S.A. Fingar, J.M. Clements, Y. Hosokawa, S. Tsunasawa, and F. Sherman. 1991. Synthesis and expression of genes encoding tuna, pigeon, and horse cytochromes *c* in the yeast *Saccharomyces cerevisiae*. *Gene (Amst.)* 105:73–81.
 17. Kaye, J., N.J. Vasquez, and S.M. Hedrick. 1992. Involvement of the same region of the T cell antigen receptor in thymic selection and foreign peptide recognition. *J. Immunol.* 148:3342–3353.
 18. Kaye, J., M.L. Hsu, M.E. Sauron, S.C. Jameson, N.R. Gascoigne, and S.M. Hedrick. 1989. Selective development of CD4⁺ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature (Lond.)* 341:746–749.
 19. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 20. Pullen, A.M., P. Marrack, and J.W. Kappler. 1988. The T cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. *Nature (Lond.)* 335:796–801.
 21. Surh, C.D., and J. Sprent. 1994. T cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature (Lond.)* 372:100–103.
 22. Feng, L., Y. Xia, T. Yoshimura, and C.B. Wilson. 1995. Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody. *J. Clin. Invest.* 95:1009–1017.
 23. Nye, S.H., and R.C. Scarpulla. 1990. Mitochondrial targeting of yeast apoiso-1-cytochrome *c* is mediated through functionally independent structural domains. *Mol. Cell. Biol.* 10:5763–5771.
 24. Foy, T., D. Page, T. Waldschmidt, A. Schoneveld, J.D. Laman, S. Masters, L. Tygrett, J.A. Ledbetter, A. Aruffo, E. Claassen, et al., 1995. An essential role of gp39, the ligand for CD40, in thymic selection. *J. Exp. Med.* 182:1377–1388.
 25. Kyewski, B.A., R.V. Rouse, and H.S. Kaplan. 1982. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. *Proc. Natl. Acad. Sci. USA.* 79:5646–5650.
 26. Gordon, S., I. Fraser, D. Nath, D. Hughes, and S. Clarke. 1992. Macrophages in tissues and in vitro. *Curr. Opin. Immunol.* 4:25–30.
 27. Lo, D., and J. Sprent. 1986. Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature (Lond.)* 319:672–675.
 28. Kyewski, B.A., C.G. Fathman, and H.S. Kaplan. 1984. Intrathymic presentation of circulating non-major histocompatibility complex antigens. *Nature (Lond.)* 308:196–199.
 29. Muller, K., J. Schumacher, and B. Kyewski. 1993. Half life of antigen/MHC class II complexes in vivo: intra- and interorgan variations. *Eur. J. Immunol.* 23:3203–3207.
 30. Ramsdell, F., and B.J. Fowlkes. 1990. Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. *Science (Wash. DC)* 248:1342–1348.
 31. Stockinger, B., and B. Hausmann. 1994. Functional recognition of in vivo processed self antigen. *Int. Immunol.* 6:247–254.
 32. Jayaraman, S., Y. Luo, and M.E. Dorf. 1992. Tolerance induction in T helper (Th1) cells by thymic macrophages. *J. Immunol.* 148:2672–2681.
 33. Savill, J., V. Fadock, P. Henson, and C. Haslett. 1993. Phagocyte recognition of cells undergoing apoptosis. *Immunol. Today.* 14:131–136.
 34. Sprent, J., and S.R. Webb. 1987. Function and specificity of T cell subsets in the mouse. *Adv. Immunol.* 41:39–133.
 35. von Boehmer, H. 1990. Developmental biology of T cells in T cell-receptor transgenic mice. *Annu. Rev. Immunol.* 8:531–556.
 36. Marrack, P., D. Lo, R. Brinster, R. Palmiter, L. Burkly, R.H. Flavell, and J. Kappler. 1988. The effect of thymus environment on T cell development and tolerance. *Cell.* 53:627–634.
 37. Heynen, M.J., G. Tricot, and R.L. Verwilghen. 1985. Autophagy of mitochondria in rat bone marrow erythroid cells. Relation to nuclear extrusion. *Cell. Tissue. Res.* 239:235–239.
 38. Knecht, E., A. Martinez-Ramon, and S. Grisolia. 1988. Autophagy of mitochondria in rat liver assessed by immunogold procedures. *J. Histochem. Cytochem.* 36:1433–1440.