CD4 T Cell Tolerance to Human C-reactive Protein, an Inducible Serum Protein, Is Mediated by Medullary Thymic Epithelium

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Summary

Inducible serum proteins whose concentrations oscillate between nontolerogenic and tolerogenic levels pose a particular challenge to the maintenance of self-tolerance. Temporal restrictions of intrathymic antigen supply should prevent continuous central tolerization of T cells, in analogy to the spatial limitation imposed by tissue-restricted antigen expression. Major acute-phase proteins such as human C-reactive protein (hCRP) are typical examples for such inducible self-antigens. The circulating concentration of hCRP, which is secreted by hepatocytes, is induced up to 1,000-fold during an acute-phase reaction. We have analyzed tolerance to hCRP expressed in transgenic mice under its autologous regulatory regions. Physiological regulation of basal levels (<10⁻⁹ M) and inducibility (>500-fold) are preserved in female transgenics, whereas male transgenics constitutively display induced levels. Surprisingly, crossing of hCRP transgenic mice to two lines of T cell receptor transgenic mice (specific for either a dominant or a subdominant epitope) showed that tolerance is mediated by intrathymic deletion of immature thymocytes, irrespective of widely differing serum levels. In the absence of induction, hCRP expressed by thymic medullary epithelial cells rather than liver-derived hCRP is necessary and sufficient to induce tolerance. Importantly, medullary epithelial cells also express two homologous mouse acute-phase proteins. These results support a physiological role of “ectopic” thymic expression in tolerance induction to acute-phase proteins and possibly other inducible self-antigens and have implications for delineating the relative contributions of central versus peripheral tolerance.

Key words: self-tolerance • inducible self-antigens • acute-phase proteins • thymic medullary epithelium • deletion

Tolerance to self is a fundamental property of the immune system. Several mechanisms, including physical elimination (clonal deletion), receptor downregulation, and functional inactivation (clonal anergy), acting both on immature and mature T lymphocytes, have been shown to contribute to tolerance induction in T cells. The individual contribution of each single mechanism to the complex phenomenon of T cell tolerance is difficult to assess. Negative selection of developing T cells apparently constitutes the major mechanism of T cell tolerance. It is estimated that about one half of all positively selectable T cells are removed from the repertoire due to negative selection (1). This process requires constant presentation of a given self-antigen on MHC molecules of thymic APCs, and thus can operate only for those antigens that either are expressed intrathymically (2) or are transported from the tissue of origin into the thymus (3, 4). In addition to central tolerance, peripheral tolerance has been implicated in the case of “tissue-restricted” self-antigens that are neither expressed nor cross-presented in the thymus. A number of model systems have been designed, in most cases with mice expressing an MHC class I-restricted T cell receptor transgene and a tissue-restricted neo-self-antigen, in which distinct mechanisms of peripheral tolerance induction have been described (5–8).

At face value, the prerequisites for tolerance induction to MHC class II-restricted soluble proteins should be less complex. Expression of MHC class II molecules is restricted to professional APCs and thymic epithelium, and as a rule presentation of epitopes on MHC class II requires internalization of a given antigen from the extracellular space. For CD4 T cells, tolerance should be determined primarily...
by the concentration of the circulating protein rather than the cell type of origin. Constitutively secreted self-antigens equilibrate between the vascular and interstitial compartments of lymphoid organs and will be presented by peripheral, as well as thymic, MHC class II–positive APCs (9). Epitopes that are presented above a critical density within the thymus will thus purge the developing T cell repertoire of high avidity self-reactive T cells, whereas epitopes presented below a certain threshold will be ignored both in the thymus and periphery, and thus T cells expressing such TCRs should not pose a danger to self-tolerance (4, 8). An additional safeguard in this delicate balance is provided by the higher sensitivity of central tolerance induction in immature thymocytes versus activation of mature T cells in the periphery (10).

The above considerations apply to MHC class II–restricted epitopes of proteins that are produced constitutively at constant levels either in the thymus or the periphery. However, in the case of inducibly secreted proteins with serum levels that can vary over a wide range, blood-borne antigen may not be sufficient to ensure tolerance imposition on the developing T cell repertoire. Under such circumstances, temporal limitations of intrathymic antigen supply should prevent continuous central tolerization of CD4 T cells in analogy to the spatial limitations imposed by tissue-restricted antigen expression that prevent central tolerization of specific CD8 T cells. Acute-phase proteins (APPs) are a prototypic example of such inducible self-antigens. APPs are a class of liver-derived serum proteins with circulating concentrations that rapidly oscillate between trace amounts in healthy individuals and up to 1,000-fold increased levels in the course of induction under pathological conditions (11).

Several particular features would predict strong immunogenicity of APPs upon induction: (a) the presence of very high serum levels (up to 2 mg/ml); (b) as part of the innate immune response, APPs accumulate at the site of infection and antigen processing; and (c) the uptake of APPs by professional APCs may be facilitated by specific surface receptors (12, 13). How is tolerance maintained under such conditions? To address this issue, we have studied transgenic mice, rearranged V(D)J regions of TCRs from the hCRP-specific T cell clones TCRP6 and TCRP2 were cloned into the cassette vectors pTCRcass and pTCRcass (20, provided by D. MATHIS and C. BENOIST, I.G.B.M.C., STRASBOURG, France). Both CD4 T cell clones were derived from a C57BL/6 mouse immunized with hCRP. The TCR from clone TCRP6 (dominant epitope [Dep] TCR) recognizes the immunodominant epitope (amino acid 89–101) of hCRP, and the TCR from clone TCRP2 (subdominant [Sep] TCR) recognizes the subdominant epitope (amino acid 80–90) of hCRP, both in the context of MHC class II I-A^d (21).

The variable region of the Dep TCR contains rearranged V_Tc26 and V_Ps1.1/D1/Jb16 elements. The rearranged regions were amplified by PCR from genomic DNA of the T cell clone TCRP6 using the oligonucleotides 5'-GAG GAT CCC GGG GAT TGG ACA GGG GCC-3' (sense) and 5'-CAG GCC GGC TGG TGT ACC ACC-3' (antisense) for the a chain, and 5'-ATC GAC TCG AGA GGA AGC ATG TCT AAC-3' (sense) and 5'-CGG ACA AAC CCA GGG TCA TCC AAC ACA AAC G-3' (antisense) for the b chain. Both PCR fragments were digested with appropriate restriction enzymes (a chain: PsaI/NotI; b chain: XhoI/SstII) and cloned into plasmid vector SK+ (Stratagene, Heidelberg, Germany). After verification of the correct sequence, the fragments were subcloned into the cassette

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**Materials and Methods**

Animals. C57BL/6 and transgenic mice were kept under specific pathogen-free conditions in the animal facilities at the German Cancer Research Center (Heidelberg, Germany). TCR and hCRP transgenic mice were bred as heterozygotes.

Generation of TCR transgenic mice. To generate TCR transgenic mice, rearranged V(D)J regions of TCRs from the hCRP-specific T cell clones TCRP6 and TCRP2 were cloned into the cassette vectors pTCRcass and pTCRcass (20, provided by D. MATHIS and C. BENOIST, I.G.B.M.C., STRASBOURG, France). Both CD4 T cell clones were derived from a C57BL/6 mouse immunized with hCRP. The TCR from clone TCRP6 (dominant epitope [Dep] TCR) recognizes the immunodominant epitope (amino acid 89–101) of hCRP, and the TCR from clone TCRP2 (subdominant [Sep] TCR) recognizes the subdominant epitope (amino acid 80–90) of hCRP, both in the context of MHC class II I-A^d (21).

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14 Abbreviations used in this paper: APP, acute-phase protein; DC, dendritic cell; Dep, dominant epitope; DN, double negative; DP, double positive; hCRP, human C-reactive protein; RIP, rat insulin promoter; mSAP, mouse serum amyloid P component; Sep, subdominant epitope; SP, single positive; Tag, large T antigen.
vectors pTαcass and pTβcass resulting in constructs termed pTα1CRP P6 and pTβ1CRP P6. The variable regions of the Sep TCR are encoded by Vα4/ Jα17 and Vβ3.3/D/Jβ16 elements. The following oligonucleotides were used to amplify these rearrangements: 5′-GAG CAC CCG GGA ATA CCA CTC TGA AC-3′ (sense) and 5′-TCA TCC GGC GCC AAA ATA ACC CAC ACA C-3′ (anti-sense) for the α chain, and 5′-GCA TAC TCG AGT CGC GAG ATG GGC TCC-3′ (sense) and 5′-CCA AGA CGG CCG TCA TCC AAC ACA G-3′ (anti-sense) for the β chain. The amplification products were cloned into pSK+ and then into the respective TCR expression cassettes as described above, yielding the vectors pTα3CR P2 and pTβ3CR P2.

The constructs were functionally tested in vitro after electroporation into the TCR-negative T cell hybridoma BW58 (see reference 22). Recognition of the specific peptide-MHC complex by the transfected TCR was assessed by IL-2 production upon stimulation with BL/6 splenocytes and the respective peptide.

Before microinjection, the pTαcass and the pTβcass vectors were digested with Sall and KpnI, respectively, to remove prokaryotic regions. The corresponding α and β chain constructs were cotransfected into C57BL/6 × C3H.JF mice. Transgenic founders were backcrossed to C57BL/6 for at least 5 generations. The resulting mouse lines were termed Sep TCR-α and Sep TCR-β according to expression of the TCR specific either for the subdominant or dominant epitope of hCRP.

Reverse Transcriptase PCR Analysis of Acute-phase Gene Expression.

Reverse transcriptase/PCR analyses were performed using 1/10 of this material as template in the PCR reaction. The following oligonucleotides were used: for hCRP: hCRP 2 (sense) 5′-GAG CAC CCG GGA ATA CCA CTC TGA AC-3′ and hCRP 3 (antisense) 5′-GAG CAC CCG GGA ATA CCA CTC TGA AC-3′ (306 to 324), and hCRP 1 (antisense) 5′-CCA AGA CGG CCG TCA TCC AAC ACA G-3′ (448 to 464); for mouse sap: mSAP 1 (sense) 5′-CAA ATG GGC TCC-3′ and mSAP 2 (antisense) 5′-CAA ATG GGC TCC-3′ (448 to 464); for mouse rnap: mRPa 1 (sense) 5′-CAA ATG GGC TCC-3′ and mRPa 2 (antisense) 5′-CAA ATG GGC TCC-3′ (448 to 464); for mouse amyloid P component (mSAP): mSAP 1 (sense) 5′-CAA ATG GGC TCC-3′ and mSAP 2 (antisense) 5′-CAA ATG GGC TCC-3′ (448 to 464); for mouse se-rum amyloid P component (mSAP): mSAP 1 (sense) 5′-CAA GCA TGG AGA AGC TG-3′ and mSAP 2 (antisense) 5′-CAA GCA TGG AGA AGC TG-3′.
Results

Transgenic Expression of α/β-TCRs Recognizing Two Epitopes of hCRP: Development and Selection. CD4 T cells of C57BL/6 mice respond against two epitopes of hCRP when immunized with complete protein or peptides corresponding to each epitope (16, 21). Transgenic mice expressing hCRP as a neo-self-antigen under control of its autologous regulatory elements are tolerant to the immunodominant and reactive to the subdominant epitope of hCRP. Responsiveness to the subdominant epitope is only revealed by peptide but not by protein immunization. This differential tolerance is independent of basal serum levels that can vary by three orders of magnitude. To address the underlying tolerance mechanisms at the clonal level, we generated two strains of TCR transgenic mice.

The transgenic line termed Dep expresses a TCR that is specific for the dominant epitope of hCRP-spanning residues 89–101 and encodes a Vα11 and a Vβ5.1 region. Expression and selection of the Dep TCR during development was followed by costaining for the transgenic Vα and Vβ chains. Selection of double positive (DP) thymocytes into the CD4 lineage in H-2b mice is highly efficient with a CD4/CD8 ratio of 10:1 and ~85% of peripheral CD4 T cells expressing both transgenic TCR chains at high levels. These cells are activated in vivo upon intravenous injection of peptide hCRP 89–101, as reflected by their phenotype 24 h thereafter (CD69+; HSAhi, CD62Llo; data not shown).

The second TCR transgenic line, termed Sep, expresses a TCR that is specific for the subdominant epitope of hCRP-spanning residues 80–94 and encodes a Vα14 and a Vβ8.3 region. Based on the analysis of the Vβ8.3 epitope, the Sep TCR shows a similar expression pattern as the Dep TCR, i.e., early expression on double negative (DN) thymocytes, a physiological density of CD3 complexes on SP

| Dep | Dep × hCRP |
|-----|------------|
| BL/6 | (120 ± 31) × 10⁶ | (12 ± 5) × 10⁶ |
| Dep | (80 ± 24) × 10⁶ | 38 ± 17 × 10⁶ |

Figure 1. Positive and negative selection of CD4 thymocytes specific for the dominant epitope of hCRP. Thymocytes of BL/6, TCR single transgenic Dep, and Dep × hCRP female mice were stained for coexpression of CD4 and CD8 (a) and the transgenic TCR chains Vα11 and Vβ5.1 (b), and were analyzed by four-color fluorescence. Note the efficient positive selection of TCR transgenic CD4 T cells in single transgenic mice and their deletion at the DP stage in noninduced Dep × hCRP mice. The total number of thymocytes (mean ± SD of at least three animals) and the percentages of subsets per quadrant are indicated. Male mice displayed an identical phenotype. Fluorescence intensity is shown on a four-decade logarithmic scale. 6-10-wk-old mice were analyzed.
Peripheral (also results in intrathymic deletion. The thymus of
stage. Likewise, several observations indicate that expres-
sion of the TCR × hCRP strains indicate that both T cell specificities are subject to intrathy-
mic deletion. How do these results comply with responsiv-
ness to the subdominant epitope of the polyclonal repertoire in hCRP single transgenic mice?
The observation that only 15% of the CD4 T cells emerging in the D-ep × hCRP mice express the transgenic β chain suggests that coexpression of an endogenous α chain in addition to the transgenic chains is not sufficient to rescue D-ep-expressing cells from deletion. In contrast, >70% of peripheral CD4 SP cells in the Sep × hCRP mice still do express the transgenic β chain. Thus, negative selec-
tion of the transgenic TCR for the subdominant epitope seems to be less stringent, allowing for exit of low affinity/ avidity T cells from the thymus. However, we cannot ex-
clude that subtle differences in the precise timing or level of expression of these two transgenic TCR influence the ob-
served differences in β chain allelic exclusion.

The notion of differential tolerance is supported by ob-
servations at the functional level. Immunization with pep-
tides corresponding to the antigenic epitope of each TCR revealed that tolerance by intrathymic deletion is complete only in D-ep × hCRP mice, whereas Sep × hCRP mice, despite deletion of the transgenic TCR, show a vigorous proliferative response (Fig. 3). Again these differences were sex independent within each transgenic line. Thus, T cell tolerance to hCRP is dictated by epitope hierarchy rather than circulating levels of antigen.

Central tolerance is due to ectopic expression of hCRP in
the thymus. Based on previous reports, the basal levels of hCRP in male mice of ~5 × 10^-7 M are compatible with intrathymic deletion due to entry and presentation of blood-borne antigen (4, 18). The at least 500-fold lower levels in female mice (~10^-9 M) are more difficult to rec-
Oncile with the observed phenotype of early and complete intrathymic deletion. Moreover, pronounced deletion oc-
curred in fetal thymus organ cultures (FTOC) of D-ep × hCRP thymi in the absence of blood-borne antigen supply (data not shown). We thus considered an additional intrathy-
mic source of hCRP leading to continuous and suffi-
cient presentation of hCRP epitopes in the thymic mi-
croenvironment irrespective of circulating levels. Two major cell populations, hemopoietic and radio-resistant ep-
ithelial cells, were assessed as a putative source of ectopi-
cally expressed hCRP.

In view of previous reports on a membrane-bound form of CRP being expressed by subsets of macrophages in hu-
man and rat (13, 37, 38), we first assessed the influence of hCRP transgenic hemopoietic cells on the development of D-ep cells. When lethally irradiated B6 mice were reconsti-
tuted with bone marrow cells from D-ep × hCRP mice, thereby generating animals in which only hemopoietic cells carried the hCRP transgene, no deletion of the D-ep TCR was observed. The central and peripheral compartments of these mice were identical to those of B6 mice reconstituted with D-ep single transgenic bone marrow, i.e., mice lacking

**Figure 2.** Positive and negative selection of CD4 thymocytes specific for the subdominant epitope of hCRP. Thymocytes of BL/6, TCR single transgenic Sep, and Sep × hCRP female mice were stained for coexpression of the transgenic TCR chain Vbetab3.1, CD4, and CD8, and were ana-
lyzed by three-color fluorescence. CD4 versus CD8 expression (a) and Vbetab3 expression gated on the CD4 SP subset (b) are shown. Note the strong bias towards CD4 SP T cells in TCR single transgenic mice and its reversion in noninduced Sep × hCRP mice. The total number of thymocytes (mean ± SD of at least three animals) and the percentages of sub-
sets per quadrant are indicated. Male mice displayed an identical phenome-
type. 6–10-wk-old mice were analyzed.
the hCRP transgene (Fig. 4). Bone marrow–derived cells (macrophages, DCs, and T and B cells) thus do not provide a source of thymic hCRP that would lead to deletion of specific T cells. This conclusion is also supported by the observation that hCRP mice reconstituted with D\textsuperscript{ep} TCR bone marrow, mice in which all cells except hemopoietic cells carry the hCRP transgene, exhibit the same degree of deletion as D\textsuperscript{ep} \times hCRP mice (Fig. 4).

To assess the role of thymic epithelial cells in tolerance to hCRP, we constructed chimeric animals in which only radiosensitive thymic stromal cells, i.e., epithelial cells, carry the hCRP transgene. Thymectomized B6 mice were grafted with an irradiated (5 Gy) hCRP transgenic thymus and reconstituted with D\textsuperscript{ep} TCR bone marrow. In these animals, early and profound deletion of the D\textsuperscript{ep} TCR was observed (Fig. 5 a) indicating that thymic epithelial cells do produce hCRP and that this source is sufficient to induce negative selection of specific T cells in the absence of liver-derived antigen. We next asked whether epithelium-derived hCRP is also necessary for negative selection. Endogenous hCRP transgenic thymus in male and female hCRP transgenic mice was replaced by fetal B6 thymi and, after reconstitution with D\textsuperscript{ep} bone marrow cells, the differentiation of D\textsuperscript{ep} TCR T cells was followed. In male hCRP transgenic mice deletion was seen within the B6 thymus graft (Fig. 5 a). Interestingly, the onset and extent of this deletion was indistinguishable from that observed in double transgenic mice or in a B6 animal grafted with an hCRP transgenic thymus (see Figs. 1 and 4). Thymus-derived antigen and high levels of blood-borne antigen thus cause the same phenotype of intrathymic deletion. In contrast, in female hCRP mice carrying a B6 thymus, no intragraft deletion was observed (Fig. 5 a). The cellular composition of these grafts was identical to B6 grafts carried by nontransgenic recipients. Immunization of these chimeras resulted in a strong proliferative response of peripheral D\textsuperscript{ep} T cells (Fig. 5 b) and simultaneous central deletion of D\textsuperscript{ep} thymocytes (data not shown). The latter is most likely caused by increased serum levels of hCRP due to concomitant induction of an acute phase.

In summary, these data show that ectopic expression of hCRP by thymic epithelial cells is (a) sufficient to cause deletion of a T cell repertoire largely consisting of hCRP–specific cells and (b) necessary to ensure tolerance at low basal hCRP serum levels in female mice, which correspond to the levels found in healthy humans.

Transgenic hCRP mimics expression of endogenous murine APPs and of hCRP in humans. The thymus transplantation experiments did not formally rule out the possibility of antigen carry-over by intrathymic APCs. Liver-derived antigen might have been taken up by MHC class II–positive cells from the circulation of the fetal donor and presented at tolerogenic levels during the experimental period of \( \sim 10 \) wk. Given the turnover of peptide–MHC complexes on thymic APCs (39), and the turnover of thymic DCs (40), this explanation is unlikely. To directly demonstrate the intrathymic origin of hCRP, the expression pattern of hCRP was analyzed by reverse transcriptase PCR. As expected, hCRP mRNA was readily detectable in the liver of hCRP transgenic male mice (Fig. 6 a). A weaker signal was reproducibly obtained with RNA extracts from the thymus of these mice. Under the same conditions all other organs tested (spleen, brain, heart, kidney, and lung) did not yield a signal. Further amplification of the PCR products with nested primers revealed additional signals in brain, kidney, and lung (data not shown). The signal in liver and thymus of noninduced female hCRP transgenic animals was weaker and sometimes undetectable, but upon induction of an acute-phase response, the hCRP–specific signals strongly increased in liver and thymus (data not shown).

Since ectopic expression of transgenes is often attributed to positional effects, we asked whether intrathymic expres-
sion of the hCRP transgene mimics the tissue-specificity of endogenous APPs. The expression analysis was extended to two endogenous murine APPs with homology to hCRP, namely mouse CRP (mCRP) and mSAP. Messenger RNA of mCRP could be detected by reverse transcriptase PCR in liver and thymus of male and female mice irrespective of experimental induction of an acute phase (Fig. 6a). This constitutive expression is in accordance with CRP being a minor APP in mice, which is only weakly induced during the course of an acute-phase response. Similarly, expression of mSAP, the major murine APP, was detectable in the liver and thymus of noninduced male and female animals (Fig. 6a). Upon induction, these signals increased in strength in the liver, as expected for a major APP, and also in the thymus (data not shown). Analysis of various human tissues also revealed ectopic expression of hCRP in the postnatal human thymus (age 3 mo; Fig. 6b). Thus, the organ-specific expression and the induction pattern of the hCRP transgene closely resemble those of its functional murine homologue mSAP and of hCRP in humans.

Prenatal Onset of Ectopic Expression. Since central deletion of Dep T cells already occurred during the fetal period (data not shown), intrathymic expression of hCRP during ontogeny was assessed. In accordance with the prenatal onset of tolerance induction, hCRP mRNA was detectable by reverse transcriptase PCR in fetal thymus of E15 embryos (earliest time point tested; Fig. 6d). Likewise, mSAP and mCRP were expressed in thymus without any gender difference (data not shown). Thus, both murine APP and the human transgene are expressed in the thymus throughout the pre- and early postnatal period, when the bulk of the T cell repertoire is generated.

Medullary Epithelial Cells Express APPs. As shown above, an hCRP source in radioresistant thymic stromal cells rather than in bone marrow–derived cells was responsible for deletion of Dep T cells in the absence of liver-derived hCRP (Figs. 4 and 5). To delineate this cell type more precisely, we enriched hemopoietic cells (mostly thymocytes), monocytes (macrophages and DCs), cortical epithelial cells, or medullary epithelial cells using appropriate cell surface markers. These cell subsets were enriched either sequentially or separately (see Materials and Methods). Since the marker used for medullary epithelial cells (G8.8) is also weakly expressed on cortical epithelial cells (31), it was important to deplete cortical epithelial cells before selection of G8.8-positive cells. Irrespective of the enrichment procedure, reverse transcriptase PCR analyses on cDNA prepared from these thymic cell fractions revealed expression of transgenic hCRP as well as endogenous mCRP and mSAP exclusively in medullary epithelial cells (Fig. 6d).

Discussion

The surprising finding of early and profound central deletion of T cells specific for both epitopes of hCRP even at very low levels of circulating protein, and the striking similarity of intrathymic T cell fate and peripheral reactivity irrespective of 500-fold differing serum levels prompted us to reanalyze the tissue specificity of hCRP expression.
Transplantation experiments as well as reverse transcriptase PCR analyses revealed expression of hCRP in medullary epithelial cells of the thymus. We consider this “ectopic” expression of the neo-self-antigen to be physiological for several reasons. (a) The hCRP transgene spans a region of 31 kb containing all known autologous 5′ and 3′ cis-acting elements (15). Liver-specificity (as formerly assessed by northern blotting) and inducibility of hepatic gene expression indicate that the trans-acting factors are conserved in mouse and humans (14). (b) We show that ectopic expression of hCRP is exclusively restricted to medullary epithelial cells of the thymus. Gene-regulation in this cellular subset of the thymus is comparable to that in hepatocytes with regard to onset during ontogeny, and, surprisingly, inducibility and sexual dimorphism. (c) Importantly, we demonstrate that hCRP is also expressed in the human thymus and that the homologous APPs of the mouse (mSAP and mCRP) show a cell type-specific expression pattern, identical to the hCRP transgene. It is noteworthy that hCRP and mSAP, two species-specific major APPs, are inducible in medullary cells of the thymus (data not shown), indicating the presence of all components of the signaling cascade (cytokine receptors, second messengers, and transcription factors) that hitherto were thought to be confined to hepatocytes and a macrophage subset (38).

Several neo-self-antigens under direction of putatively tissue-specific promoters have been found to be expressed in the thymus of transgenic mice. These include the promoters of rat insulin II (41, 42), rat elastase I (43), guinea pig α-lactalbumin, human beta globin (44, 45), keratin-IV (46), and metallothionein (47). Thymic expression of different model antigens driven by these heterologous regulatory elements was often variable and usually found in some but not all transgenic lines, consistent with the notion that the integration site and/or the copy number influence expression. In this context it is noteworthy that a truncated version of the hCRP promoter directing expression of an MHC class I alloantigen was active in hepatocytes but not in the thymus (as assessed by selection of specific T cells in chimeras and PCR analysis; reference 48). In view of the variability and unpredictability of the thymic activity of these hybrid transgenes, it was initially difficult to assess the biological significance of this ectopic gene expression. However, recent studies revealed a diverse group of endogenous “peripheral” antigens to be expressed in the thymus of rodents or primates, including pancreas-specific genes (41), components of the myelin sheath (myelin basic protein and myelin proteolipid protein; references 49–51), S-100β (52), acetylcholine receptor (53), retinal proteins (arrestin and interphotoreceptor retinoid-binding protein; reference 54), and neuro-endocrine hormones (55). Thus, thymic expression of “tissue-specific” genes seems to be a common occurrence and to be part of physiological expression patterns. This should result in a more diverse presentation of “self” within the thymus than has been appreciated previously. Although a role of intrathymic
expression of “nonthymic” proteins in the establishment of self-tolerance has been proposed, it has not been formally demonstrated. Nevertheless, intriguing correlations between the thymic expression level of self-antigens and the propensity to spontaneously develop or succumb to experimental induction of autoimmune disease have been recently reported for insulin (56, 57) and two retinal proteins (54). Our analysis demonstrates for the first time that thymic expression of a secreted neo–self-antigen under its autologous regulatory elements confers tolerance upon the developing T cell repertoire by deletion of specific T cells with remarkable efficiency.

Expression of several “peripheral” antigens has been assigned to the thymic medulla by histological analysis (50, 52, 58), although delineation of the precise cell type has been difficult. Expression of hCRP and mouse APP is clearly confined to radio-resistant stromal cells as shown by transplantation experiments, and more precisely to medullary epithelial cells as shown by cell separation and subsequent reverse transcriptase PCR. Medullary epithelial cells have been previously implicated in tolerance induction both by deletion or anergy induction (46, 59–61). How does the restriction of hCRP expression to the medulla comply with the profound deletion phenotype, in particular the lack of immature DP thymocytes, most of which reside in the cortex? We suggest that hCRP is secreted by medullary epithelial cells and subsequently presented by MHC class II-positive APCs, including DCs and medullary and cortical epithelial cells. Such intercellular antigen transfer and “cross presentation” within the thymic microenvironment has been described for an MHC class II-restricted membrane protein (62). Recognition of specific peptide–MHC complexes on cortical epithelial cells would result in deletion of hCRP-specific cells at the transition from the DN to DP stage as soon as they become susceptible to apoptotic signals via the TCR (63; note that DN thymocytes prematurely express the thymic TCR at high levels [Fig. 1]). Interestingly, an identical phenotype of early intrathymic deletion is observed when hCRP is derived either exclusively from epithelial cells (as in hCRP grafts in B6 mice; Fig. 5 a) or from the circulation (as in B6 grafts in male hCRP transgenic mice; Fig. 5 a), arguing for efficient antigen presentation on cortical cells in both instances. In the latter case, hCRP may gain access to the cortex via blood vessels or the capsule (64). Indeed, cortical epithelial cells have been shown to be accessible to bloodborne proteins (9) and to mediate deletion of immature thymocytes in vitro and in fetal thymus organ cultures (25, 27). In support of this notion, hCRP protein can be directly visualized within the cortical parenchyma by immunohistology in hCRP transgenic mice after experimental induction (data not shown).

The proposed intrathymic “antigen spread” may explain why deletion of hCRP-specific thymocytes is remarkably efficient when compared with other experimental models in which ectopic expression of heterologous transgene-constructs was observed. Ectopic expression in the thymus of various model-antigens under control of the rat insulin promoter (RIP), for instance, yielded divergent results. No central deletion of TCR transgenic CD4 cells specific for the large T antigen (Tag) of SV 40 was observed in RIP-Tag × TCR mice (22), yet tolerance of CD4 and CD8 T cells could be demonstrated at the polyclonal level in RIP-Tag single transgenic mice (58). Ectopic expression of the nuclear protein of LCMV or the allo-MHC antigen Kb driven by the RIP lead to partial tolerance among CD8 T cells (42, 65). In the latter study, deletion of only those Kb-specific T cells that express a transgenic TCR at high density was described. Deletion of CD8 T cells was also incomplete when ectopic Kb expression was directed by the promoters of guinea pig α-lactalbumin or human beta globin (45). Intrathymic expression of TAg under the rat elastase I promoter or MHC class I-Kb under the keratin IV promoter resulted in anergy induction of specific CD8 T cells rather than deletion (43, 46). Several reasons may account for these different outcomes, namely antigen availability (secreted versus intracellular antigens), the type of APC (bone marrow–derived versus epithelial cell), and the affinity/avidity of the TCR–MHC interaction (66, 67). Given this variability, it will be important to test the contribution of the thymic activity of each of these promoters to tolerance induction in their native genetic context.

Tolerance to a major murine APP has recently been documented (68). Is ectopic expression of APPs necessary for tolerance induction? Serum levels of hCRP in female transgenic animals closely mimic hCRP levels in humans. These basal levels of circulating hCRP are insufficient to induce intragraft deletion and tolerance in transgenic female mice grafted with a B6 thymus. Upon induction of an acute phase, hCRP levels in females rise 500-fold, and are now sufficient to activate peripheral T cells (naive CD4 and CD8 T cells) when transferred into hCRP transgenic animals. In the latter study, expression of APPs in the pre- and postnatal thymus results in presentation of both dominant and subdominant epitopes at levels sufficient to confer functional tolerance irrespective of an acute phase. Ectopic expression by thymic medullary epithelial cells thus seems to be a physiological device to safeguard tolerance to APPs.

Sensitive detection methods reveal the expression of a sensitive nuclear protein of LCMV or the allo-MHC antigen Kb driven by the RIP lead to partial tolerance among CD8 T cells (42, 65). In the latter study, deletion of only those Kb-specific T cells that express a transgenic TCR at high density was described. Deletion of CD8 T cells was also incomplete when ectopic Kb expression was directed by the promoters of guinea pig α-lactalbumin or human beta globin (45). Intrathymic expression of TAg under the rat elastase I promoter or MHC class I-Kb under the keratin IV promoter resulted in anergy induction of specific CD8 T cells rather than deletion (43, 46). Several reasons may account for these different outcomes, namely antigen availability (secreted versus intracellular antigens), the type of APC (bone marrow–derived versus epithelial cell), and the affinity/avidity of the TCR–MHC interaction (66, 67). Given this variability, it will be important to test the contribution of the thymic activity of each of these promoters to tolerance induction in their native genetic context.
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