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*J Immunol* 1999; 163:4851-4858; ;
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Thymocyte Antigens Do Not Induce Tolerance in the CD4+ T Cell Compartment

Kanchan G. Jhaver,* Phillip Chandler,† Elizabeth Simpson, † and Andrew L. Mellor2*

Thymocytes fail to tolerate the developing T cell repertoire to self MHC class I (MHC I) Ags because transgenic (CD2Kb) mice expressing H-2Kb solely in lymphoid cell lineages reject skin grafts mismatched only for H-2Kb. In this study, we examined why thymocytes fail to tolerate the T cell repertoire to self MHC I Ags. The ability of CD2Kb mice to reject H-2Kb skin grafts was age dependent because CD2Kb mice older than 20 wk accepted skin grafts. T cells from younger CD2Kb mice proliferated, but did not develop cytotoxic functions in vitro in response to H-2Kb. Proliferative responses were dominated by H-2Kb-specific, CD4+ T cells rather than CD8+ T cells. Representative CD4+ T cell clones from CD2Kb mice were MHC II restricted and recognized processed H-2Kb. TCR transgenic mice were generated from one CD4+ T cell clone (361) to monitor development of H-2Kb-specific immature thymocytes when all thymic or lymphoid cell lineages only expressed H-2Kb. Thymocyte precursors were not eliminated and mice were not tolerant to H-2Kb when Tg361 TCR transgenic mice were intercrossed with CD2Kb mice. In contrast, all thymocyte precursors were eliminated efficiently in thymic microenvironments in which all cells expressed H-2Kb. We conclude that self MHC I Ags expressed exclusively in thymocytes do not induce T cell tolerance because presentation of processed self MHC I Ags on self MHC II molecules fails to induce negative selection of CD4+ T cell precursors. This suggests that some self Ags are effectively compartmentalized and cannot induce self-tolerance in the T cell repertoire. The Journal of Immunology, 1999, 163: 4851–4858.

A ccording to the postulates of the clonal selection hypothesis, self-reactive lymphocytes do not mature because they are eliminated during maturation in primary lymphoid organs (1). However, data from many experimental systems reveal that self-reactive T and B cells are present in the mature lymphocyte repertoire and contribute to the onset and maintenance of autoimmune disease. In particular, self-reactive CD4+ T cells have been implicated in early events that gradually overcome tolerance in the rest of the lymphocyte repertoire before overt symptoms of autoimmune disease manifest (2–4). Unraveling the complex etiology of autoimmune diseases depends on elucidating the origin of self-reactive T cells that evade molecular and cellular interactions leading to negative selection of thymocytes.

Negative selection ensures that most self-reactive thymocytes are eliminated when they recognize MHC/peptide complexes on the surface of cells they encounter in the thymus (5, 6). Efficient negative selection requires high avidity interactions between TCR and coreceptors (CD4, CD8) on thymocytes with MHC/peptide complexes displayed on the surface of presenting cells (7–9). Self peptides associated with MHC molecules on the surface of thymic cells may originate from endogenous proteins produced within the presenting cells themselves or from exogenous proteins produced by other cells that are taken up, processed, and presented by thymic cells.

The efficiency of self protein uptake, processing, and association with MHC molecules depends on whether proteins originate from endogenous sources or are acquired from exogenous sources as cell-associated or soluble (serum) proteins. In addition, the outcome of interactions between thymocytes and T cells and APCs may depend on whether peptides originate from endogenous or exogenous sources (10). The cellular and biochemical processes that mediate protein (Ag) transfer between cells in the thymic microenvironment leading to presentation of MHC/peptide complexes are not well understood, as they are difficult to assess directly. Failure to expose self peptides to thymocytes would, in effect, conceivably allow potentially autoreactive thymocytes to evade negative selection and become mature T cells. These considerations are of particular significance for tolerance induction in the CD4+ T cell compartment because some thymic cells do not express MHC II and, hence, cannot present self peptides directly to thymocyte precursors of CD4+ T cells. Moreover, the preference for MHC II association with peptides produced by processing of exogenous Ags may inhibit access of processed endogenous self proteins to MHC II expressed by some thymic cells, effectively rendering endogenous Ags invisible to developing thymocytes. Indeed, evidence that different thymus cell types expressing MHC II have distinct effects on the outcome of thymocyte selection has been obtained from several experimental systems (11–13). In another study, targeted expression of MHC II to cortical epithelial cells using a keratin promoter resulted in thymocyte evasion of negative selection because large cohorts of autoreactive CD4+ T cells matured in these mice (14). Consequently, for some thymic cell types, the correlation between self protein expression and ability to induce negative selection is poor.

Targeting expression of self Ags in transgenic mice has been a productive strategy to determine how self Ags induce, or fail to induce, T cell tolerance (9, 11, 14–18). In most cases, mice were
tolerant to the self Ag encoded by the transgene even though transgene expression was limited to certain thymic cell types. For example, transgene-targeted expression of MHC I to thymocytes resulted in efficient elimination (negative selection) of thymocyte precursors of allogeneic CD8 T cells in several experimental systems (19–21). These experiments demonstrated that thymocytes could mediate negative selection directly, but did not address whether thymocyte self Ags requiring processing and presentation in association with MHC II could also affect negative selection, thereby inducing functional T cell tolerance.

To examine this issue, we assessed the tolerance status of transgenic (CD2K b) mice expressing H-2K b under the control of promoter elements from the human CD2 gene mice. In a previous study, we demonstrated that most CD2K b mice rejected skin grafts on which H-2K b was the only alloantigen, even though thymocyte precursors expressing two different TCR clonotypes conferring recognition of native H-2K b molecules were eliminated efficiently in thymus via negative selection (22). In this study, we demonstrate that the T cell repertoire of CD2K b mice is not tolerized to processed H-2K b by characterizing H-2K b-specific T cells in CD2K b mice and analyzing thymocyte development in transgenic mice carrying rearranged TCR genes isolated from a H-2K b-specific, CD4 T cell clone.

Materials and Methods

Mice

CBA/Ca, C57BL/6 (B6), BALB/c, and transgenic mice used in these studies were bred and maintained in a barrier facility within the Medical College of Georgia Transgenic Unit and were free of all common murine pathogens including Murine hepatitis virus. Transgenic CBK (23) and BM3 (24) mice have been described previously. Transgenic CD2K b mice used in this study were from line 4 described previously (22). All mice used in these studies were age 3–9 wk, unless otherwise stated. TCR transgenic Tg361 mice were mated with CD2K b and CBK mice, and double transgenic progeny were used for experiments.

Flow cytometry and mAbs

Analytical flow cytometry was conducted using a FACS Calibur (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest Software. A minimum of 10,000 gated events was collected for each analysis. Three-color staining of single cell suspension of thymocytes or splenocytes was performed using FITC, PE, and biotin-conjugated Abs followed by streptavidin-RED 670 (Life Technologies, Gaithersburg, MD). PE-conjugated anti-CD4 (H129.19), FITC-conjugated anti-CD8a (53–6.7), and biotinylated anti-Vβ10 (B21.5) Abs were purchased from PharMingen (San Diego, CA). FITC-conjugated anti-H-2K b (MM601) was obtained from Caltag (South San Francisco, CA). Purified mAbs against H-2A b (11.5.2), H-2E b (17.3.3), H-2K b (11.4.1), H-2D b (15.5.5), and H-2K b (28.8.6) were purchased from Pharmingen and dialyzed against PBS before use at various concentrations in proliferation assays.

Skin grafts

Skin grafting was performed by transplanting tail skin (~0.5 cm²) from donor mice onto the flank of recipient mice, as described previously (25). Plaster bandages were removed on day 10, and grafts were inspected daily for signs of rejection.

Isolation of T cell clones

Nylon wool-purified splenocytes and lymph node cells from mice that had rejected CBK skin grafts or mice immunized with CBK splenocytes (10⁷) were cultured with irradiated CBK splenocytes in IMDM (Cellgro) containing 5% FCS (Sigma, St. Louis, MO), glutamine, penicillin-streptomycin, and 2-ME (referred to as complete medium) for 2 wk. Cells were restimulated with fresh irradiated CBK stimulator cells every 12 to 15 days for three cycles. After this, cells were plated in 96-well flat-bottom plates at limiting dilution in the presence of irradiated stimulator cells in complete medium containing 5% IL-2 supernatant (IL-2 supernatant generated from a murine cell line secreting IL-2; kind gift from Dr. S. Vukmanovic, New York University Medical Center, New York, NY). Clones obtained were expanded in 24-well plates in complete medium containing IL-2 and stimulated with irradiated CBK splenocytes every 2 wk before setting up proliferation assays. In experiment 2 (Table II), clones were stimulated for 10 to 12 days with irradiated CBK splenocytes, then washed and cultured in 1% IL-2-containing medium for another 10 to 12 days before use in proliferation assays. In experiment 3 (Table II), clones were stimulated for 15 days with irradiated splenocytes from B6 mice before use in proliferation assays. In all cases, results shown are representative of at least three separate experiments.

T cell proliferation assays

Assays were conducted in 96-well flat-bottom plates (Falcon). T cell clones maintained as described above were harvested, washed, and used at a final concentration of 1 × 10⁶/well/100 µl. Irradiated stimulators (100 µl/well) as indicated were added at various concentrations in complete medium containing 1% IL-2 supernatant. Assays were terminated after 72 h by pulsing with [3H]thymidine (1 µCi/well; Amersham, Arlington Heights, IL) for the final 8 h and harvested onto glass fiber filters. Incorporation of radioactivity was determined by liquid scintillation counting. In mAb-blocking experiments, irradiated CBK splenocytes were preincubated for 1 h with anti-MHC Abs (as described) before adding them to cocultures. For responders from single or double TCR (Tg361) transgenic mice, nylon wool-enriched splenocytes were cocultured with irradiated splenocytes from CBA, CBK, or B6, as described. After 40 h, cocultures were incubated for an additional 8 h with [3H]thymidine (1 µCi/well), and radioactivity incorporated was assessed as described.

DNA constructs for TCR transgenic mice

TCR transgenes were constructed essentially as described (15). TCRα and TCRβ segments expressed by T cell clones were identified by staining 3 CD4⁺ and 3 CD8⁺ T cell clones with a range of murine anti-TCR mAbs and subjecting them to flow-cytometric analysis (Abs were kindly provided by Dr. L. Ignatowicz, Institute of Molecular Medicine and Genetics, Medical College of Georgia). RNA from clones was isolated using RNA STAT 60 solution (Tel-Test, Friendswood, TX) and was converted to cDNA using the RT system (Promega, Madison, WI). Purified TCRα and TCRβ DNA sequences were amplified from cDNA using PCR C region primers and Vα/Vβ-specific primers (26). Based on these analyses, CD4⁺ T cell clone Tg361 was selected for further analysis. Full-length cDNAs encoding productively rearranged TCRs for clone 361 (Vα5 and Vβ10) genes were recloned into a lymphoid-cell-specific transgene expression cassette (CD2-VA) (27). TCRα and TCRβ transgenes were co-microinjected into fertilized oocytes from inbred CBA/Ca mice by the MCG Transgenic Unit. Transgenic founder mice obtained were screened by PCR for transgene transmission. Transgene expression was assessed by flow cytometry after staining thymocytes or splenocytes with an anti-TCR/Vβ10 mAb. Mice from two independent transgenic (Tg361.17 and Tg361.20) lines gave comparable results when analyzed phenotypically by flow cytometry and functionally in assays for T cell proliferation and specificity.

Results

CD2K b mice are not tolerant to H-2K b

In our earlier study, we reported that most, but not all, CD2K b transgenic mice rejected skin grafts from other transgenic mice (CBK) expressing H-2K b ubiquitously under control of the H-2K b promoter (22). These mice share identical genetic backgrounds as CBK, B6, and transgenic mice used in these studies were age 3–9 wk, unless otherwise stated. TCR transgenic mice rejected skin grafts or mice immunized with CBK splenocytes (10⁷) and did not address whether thymocyte self Ags requiring processing and presentation in association with MHC II could also affect negative selection.
ages. All 11-wk-old CD2Kb recipient mice rejected CBK grafts with a mean graft survival time of 38 days (Table I). As expected, all skin grafts applied to control CBA recipients were rejected (mean graft survival time = 15 days), while all CBK recipients accepted their grafts indefinitely. We conclude that CD2Kb mice did not acquire T cell tolerance to H-2Kb by 11 wk of age. The discrepancy between our current and previous results became clear when CBK grafts were applied to older CD2Kb mice (>20 wk) that accepted grafts indefinitely (Table I). Thus, tolerance to H-2Kb was acquired as mice age, presumably, due to tolerogenic processes that occur, over time, in the peripheral, extrathymic compartment.

As well as the different rates of rejection, the appearance of skin grafts during rejection differed depending on whether they were applied to CD2Kb or control CBA recipients. Grafts applied to CD2Kb recipients gradually became shriveled, but were not shed, whereas grafts applied to CBA recipients became necrotic and were shed shortly afterwards. These data suggested that the H-2Kb-specific T cell repertoire in CD2Kb and CBA mice was profoundly different.

### H-2Kb-specific T cell clones from CD2Kb mice recognize processed H-2Kb

The H-2Kb-specific T cell repertoire of CD2Kb mice was analyzed by isolating T cell clones from immunized 9-wk-old mice using stimulator cells from CBK mice. Clones that proliferated in response to CBK but not CBA stimulators were selected and analyzed. The majority (31/47) of clones isolated were CD4+ T cells, while the rest were CD8+ T cells, an unusual bias because alloreactive CD8+ T cells usually dominate responses directed against native MHC I alloantigens. This outcome was consistent with our previous finding that precursors of H-2Kb-specific, CD8+ T cells were eliminated efficiently in CD2Kb thymus (22). Thus, most of the H-2Kb-specific CD8+ T cells that usually dominate T cell responses to MHC I alloantigens were purged from the T cell repertoire in CD2Kb mice.

To determine whether H-2Kb-specific T cell clones recognized native H-2Kb directly or as processed forms of H-2Kb presented by other MHC molecules, clones were stimulated with cells from C57BL/6 (B6, H-2b haplotype) mice. All three CD4+ clones tested failed to proliferate in response to B6 cells (Table II). Similar results were obtained when clones were restimulated (Expt. 2) or cocultured with B6 splenocytes (Expt. 3) for 15 days in the presence of IL-2 before assay ing proliferation. In contrast, these treatments resulted in significantly enhanced responsiveness to CBK stimulators. Two CD8+ T cell clones tested in the same assays also failed to proliferate when cocultured with B6 cells (Table II). These data show that five of five H-2Kb-specific T cell clones failed to recognize native H-2Kb molecules expressed by cells from B6 mice. The most plausible explanation is that H-2Kb-specific responses were provoked by processed H-2Kb presented in the context of MHC molecules of H-2b haplotype origin. A less likely explanation is that H-2Kb-specific T cell clones from CD2Kb mice recognized a CBA strain-specific processed Ag presented in the context of native H-2Kb, which is not presented by cells of B6 origin.

To address this issue further, CD4+ T cell clones were cocultured with mixtures of splenocytes from B6 and CBA mice (Fig. 1, A–C). These mixtures elicited proliferative responses from all three CD4+ T cell clones tested, and the magnitude of the response increased as more B6 cells were added, while the number of CBA cells was kept constant. Similar results were obtained when the number of CBA cells was varied while the number of B6 cells was kept constant (data not shown). B6 and CBA cells added separately gave similar (background) results in this assay, and responses elicited by mixtures of CBK + CBA or B6 + B6 cells were comparable (data not shown). In contrast, mixtures of cells from CBA + BALB/c (H-2d haplotype) mice did not elicit proliferative responses (data not shown), eliminating the possibility that responses to cell mixtures were induced nonspecifically. Taken together, these data support the hypothesis that H-2Kb-specific, CD4+ T cell clones from CD2Kb mice recognize processed Ag (H-2Kb) from B6 cells presented in the context of MHC II molecules expressed by APCs of CBA origin (i.e., H-2Ak, H-2Eε). CD8+ T cell clones did not respond to mixtures of B6 and CBA splenocytes (data not shown). Therefore, it is unclear whether these clones recognize processed H-2Kb or specific peptides from a protein expressed in CBA, but not in B6 mice that is presented on H-2Kb.

To determine whether responses by CD4+ T cell clones were specific for CD2Kb mice restricted, anti-MHC mAbs were added to cocultures containing CBK stimulator cells (Fig. 1, D–F). Proliferation of CD4+ T cell clones 361 and 418 was inhibited in the presence of a mixture of two mAbs specific for H-2Aε and H-2Eε, whereas a mixture of two Abs specific for H-2Kb and H-2Dd had relatively little effect on T cell proliferation (Fig. 1, D and E, respectively). Furthermore, proliferation of T cell clone 361 was inhibited when an anti-H-2Aε Ab was added to cocultures, while addition of an anti-H-2Eε Ab had relatively little effect on T cell proliferation (Fig. 1F). Addition of an anti-H-2Kb mAb had little effect on proliferation of these CD4+ T cell clones (data not shown). These data demonstrate that responses by H-2Kb-specific,

### Table II. H-2Kb-specific T cell clones recognize processed not native H-2Kb

<table>
<thead>
<tr>
<th>Precultured with</th>
<th>Stimulator Cells</th>
<th>CD4+ Clones</th>
<th>CD8+ Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 135</td>
<td>No. 361</td>
<td>No. 418</td>
</tr>
<tr>
<td>CBK (Expt. 1)</td>
<td>CBK</td>
<td>465 ± 76b</td>
<td>594 ± 8</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>510 ± 55</td>
<td>792 ± 55</td>
</tr>
<tr>
<td>(Expt. 2)</td>
<td>CBK</td>
<td>286 ± 72</td>
<td>271 ± 22</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>347 ± 61</td>
<td>434 ± 70</td>
</tr>
<tr>
<td>(Expt. 3)</td>
<td>CBK</td>
<td>2,308 ± 33</td>
<td>2,598 ± 179</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>205 ± 20</td>
<td>188 ± 23</td>
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</tbody>
</table>

* See Materials and Methods for details.

* Results are shown as mean cpm of triplicate cultures ± 1 so.

* Figures in bold are >1.5× background responses to CBA cells.
CD4+ T cell clones isolated from CD2Kb mice are MHC II restricted and identify H-2Aβ as the restriction element used by clone 361. These findings provide further support for our hypothesis that H-2Kb-specific precursors of CD4+ T cells evaded negative selection in CD2Kb mice because thymic MHC II+ cells either do not present processed H-2Kb or are incompetent mediators of negative selection (22).

Positive selection of H-2Kb-specific thymocytes occurs in CD2Kb mice

TCR transgenic mice were generated to test the validity of our hypothesis by monitoring the fate of thymocyte precursors of H-2Kb-specific precursors of CD4+ T cells. Tg361 TCR transgenic mice were generated by microinjecting fertilized oocytes from inbred CBA mice with DNA constructs containing productively rearranged TCRα (Vα5) and TCRβ (Vβ10) cDNA sequences isolated from T cell clone 361. Two transgenic founder mice were obtained, and each was mated with CBA partners to establish independent lineages of Tg361 (CBA) mice in which thymocyte development and selection, as well as T cell functional status, could be assessed in the absence of H-2Kb expression.

Mean thymus cellularity in Tg361 (CBA) mice was comparable with results from control CBA mice, an indication that unanticipated interactions did not impede thymocyte development in the absence of H-2Kb expression. More significantly, flow-cytometric analyses of thymocyte populations revealed that selection of mature CD4+CD8- thymocytes was enhanced relative to CBA controls, and high levels of transgenic Vβ10 (Vβ10high) were detected on all mature CD4+CD8- thymocytes from Tg361 (CBA) mice (Fig. 2). Consistent with these observations, large cohorts of CD4+ T cells were detected in spleens of Tg361 (CBA) mice. Relatively small cohorts of CD8+ T cells were also detected in spleens and expressed transgenic Vβ10 molecules at levels comparable with CD4+ T cells. Most likely, productive rearrangements of endogenous Vα-chain genes may give rise to CD8+, Vβ10+ T cells with other specificities. However, because Abs specific for the Tg361 TCR clonotype or transgenic Vα5 molecules were unavailable, the reason that Vβ10high, CD8+ T cells develop in Tg361 (CBA) mice is uncertain. Nevertheless, these data revealed that thymocytes expressing transgenic Tg361 clonotypic TCR molecules evaded negative selection and underwent positive selection in Tg361 (CBA) mice.

To determine whether thymocytes expressing the Tg361 TCR clonotype were subject to negative selection when thymic cells expressed H-2Kb, Tg361 (CBA) mice were mated first with CBK transgenic mice. Double transgenic Tg361 × CBK offspring were selected for phenotypic analyses to assess the fate of thymocytes. Thymus from Tg361 × CBK mice exhibited a drastic, 10-fold reduction in overall cellularity relative to Tg361 (CBA) control mice (Fig. 2, see legend). Flow-cytometric analyses of the few remaining thymocytes in Tg361 × CBK mice revealed relatively large cohorts of CD4+CD8- and mature CD4+CD8+ thymocytes and smaller cohorts of CD4+CD8- and mature CD4+CD8- thymocytes. Furthermore, CD4+ and CD8+ T cells were virtually undetectable in spleens from Tg361 × CBK mice. These data showed that negative selection occurred predominantly before the development of CD4+CD8- thymocytes. Moreover, none of the remaining mature thymocytes expressed transgenic Vβ10, suggesting that their survival depended on their failure to express the Tg361 TCR clonotype. These data suggest that thymocytes expressing the Tg361 clonotype were subject to negative selection at an early developmental stage in thymic microenvironments in which all cell types expressed H-2Kb.

To evaluate whether restricting H-2Kb expression to thymocytes leads to reduced efficiency of negative selection of H-2Kb-specific
thymocytes, Tg361 (CBA) mice were mated with CD2Kb mice, and double transgenic Tg361 × CD2Kb offspring were analyzed, as described previously (Fig. 2). Mean thymus cellularity in Tg361 × CD2Kb mice was reduced only ~2-fold relative to Tg361 (CBA) control mice. Moreover, thymocyte development was not blocked at an early developmental stage because flow-cytometric analyses revealed the presence of two overlapping thymocyte subpopulations consisting of immature CD4+CD8+ and mature CD4+CD8- thymocytes (Fig. 2). Very few CD4+CD8- thymocytes and virtually no mature CD4-CD8+ thymocytes were detected. When compared with staining profiles obtained from control Tg361 (CBA) samples, levels of CD4 and CD8 expressed by CD4+CD8- thymocytes from Tg361 × CD2Kb mice were more heterogeneous. For example, the gated CD4+CD8- subpopulation consistently exhibited weak CD8 staining relative to Tg361 (CBA) controls, indicating that a higher proportion of thymocytes was CD4+CD8low in Tg361 × CD2Kb mice.

Mature CD4+CD8- thymocytes expressed transgenic Vβ10 and CD3 (data not shown) molecules at comparable levels in Tg361(CBA) and Tg361 × CD2Kb mice. Slight decreases in mean fluorescence intensities for Vβ10 staining were detected in double transgenic mice. This may result from secondary (endogenous) TCR gene rearrangements. However, a significant proportion of thymocytes in this population expressed Vβ10 at high levels in both double and single mice, indicating that mature CD4+CD8- thymocytes were selected in CD2Kb thymus. Moreover, annexin V did not stain CD4+CD8- thymocytes (data not shown), showing that these thymocytes were not undergoing apoptosis. Vβ10 and CD3 expression levels were lower (by ~0.5 logs) on immature CD4+CD8- thymocytes in double and single transgenic mice than on mature CD4+CD8- thymocytes. Taken together, these data reveal that negative selection was not an efficient process in Tg361 × CD2Kb mice, while positive selection favored maturation of CD4+CD8- thymocytes.

Analyses of splenic T cells revealed the presence of distinct populations of CD4+ and CD8+ T cells expressing Vβ10 molecules in Tg361 × CD2Kb mice. Thus, thymocyte precursors of H-2Kb-specific, CD4+ T cells evade negative selection and exit the thymus when H-2Kb is expressed exclusively on thymocytes. CD4:CD8 ratios observed in Tg361 × CD2Kb mice were less than in Tg361 (CBA) mice, suggesting that some thymocyte precursors of CD4+ T cells were eliminated. Alternatively, encounters with APCs displaying processed H-2Kb in the extrathymic compartment might induce down-modulation of CD4 or deletion of peripheral CD4+ T cells. CD8+ T cells expressed a ~2–3-fold lower level of transgenic Vβ10 than CD4+ T cells in Tg361 × CD2Kb spleen. The origin of these Vβ10low, CD8+ T cells is not known, especially as mature CD4-CD8+ thymocytes were virtually undetectable in thymus. A possible explanation is that CD8+ T cells develop from CD4-CD8low thymocytes that reexpress CD8 and down-regulate CD4 as they exit the thymus. These data, together with the observation that CD4+ and CD8+ T cells were absent in Tg361 × CBK control mice, show that exclusive expression of H-2Kb in thymocytes allows H-2Kb-specific T cell precursors to evade negative selection.

The T cell repertoire of Tg361 × CD2Kb mice is not tolerant to H-2Kb

As a further test of our hypothesis, functional analyses and skin grafting experiments were performed to assess T cell responsiveness and the tolerance status of Tg361 × CD2Kb double transgenic mice.
THYMOCYTE ANTIGEN-SPECIFIC TOLERANCE

Table III. *Tg361 mice with CD2K<sup>b</sup> backgrounds are not tolerant to H-2K<sup>b</sup>*

| Graft Recipient<sup>a</sup> | No. Rejected/No. Grafted | Graft Survival Time
<table>
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<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Tg36 (CBA)</td>
<td>3/3</td>
<td>14 [13–15]</td>
</tr>
<tr>
<td>Tg36 × CD2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5/5</td>
<td>15 [14–17]</td>
</tr>
<tr>
<td>Tg36 × CBK</td>
<td>0/6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BM3 × CD2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/3</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

<sup>a</sup> All mice were grafted with tail skin from CBK donor mice.

Discussion

CD2K<sup>b</sup> transgenic mice exhibit an unexpected nontolerant status because they reject skin grafts expressing H-2K<sup>b</sup>, even though H-2K<sup>b</sup> is a self Ag expressed at high levels in thymocytes and T cells. In our earlier study, we discovered that mice from only one line (lineage 3) of four original lines of CD2K<sup>b</sup> transgenic mice were tolerant to H-2K<sup>b</sup>. This confirms that allospecific CD8<sup>+</sup> T cells, which normally dominate responses to MHC I alloantigens, are eliminated efficiently via negative selection in CD2K<sup>b</sup> mice (22). Taken together, these data provide unequivocal evidence that processed H-2K<sup>b</sup> Ag derived exclusively from thymocytes does not mediate negative selection of H-2K<sup>b</sup>-specific thymocytes and allows them to mature into functionally active, peripheral T cells capable of rejecting tissue grafts.

The failure of thymocytes expressing H-2K<sup>b</sup> to tolerate the T cell repertoire in CD2K<sup>b</sup> mice raises a number of issues relating to cell biological and biochemical processes that generate peptide/MHC ligands on thymic cells capable of inducing negative selection. Not all cells express MHC II molecules in thymus, and this raises the important question of whether proteins expressed only in MHC II<sup>+</sup> cells are transferred to MHC II<sup>+</sup> cells in thymus to affect negative selection. Our results with CD2K<sup>b</sup> mice demonstrate that H-2K<sup>b</sup> expressed in murine thymocytes (MHC II<sup>+</sup>) is not transferred to MHC II<sup>+</sup> cells capable of presenting processed forms of H-2K<sup>b</sup> to affect negative selection. Cells with this capability are present in thymus because all thymocytes expressing the Tg361 clonotype are eliminated efficiently in mice with CBK backgrounds in which all cells express H-2K<sup>b</sup>. The conclusion that thymocyte Ags are not transferred to MHC II<sup>+</sup> cells is surprising because large numbers of apoptotic thymocytes are engulfed, and their contents degraded, by thymic macrophages (28).

Intuitively, it seems likely that these processes would provide a route by which thymocyte Ags could tolerate the developing CD4<sup>+</sup> T cell repertoire. However, this would occur only if phagocytic macrophages...
expressed MHC II and their proteolytic processing pathways diverted partially processed exogenous Ags to endosomal compartments in which they associated with MHC II molecules and were transported to the macrophage surface. Our results imply that one, or more, of these processes either do not occur or are inefficient following thymocyte engulfment by macrophages. The inability of thymic macrophages, or any other thymic MHC II cells, to present processed H-2Kb is also surprising in light of evidence that exogenous self Ags can be presented to CD4+ T cells (10). Indeed, exogenous complement C5 protein from serum is processed and presented to affect negative selection in thymus (29). This suggests that uptake and/or processing of exogenous thymocyte-associated and serum Ags have different effects on T cell tolerance induction.

Another potential explanation for our findings is that phagocytic thymic macrophages cannot mediate negative selection, even if they can process and display processed thymocyte Ags in association with MHC II. For example, they might not express coreceptors or adhesion molecules that contribute to high avidity interactions necessary for negative selection to occur. Interestingly, thymic macrophages were the only MHC II+ cell type from thymus that failed to bring about negative selection of thymocytes specific for the serum protein C5 when injected into fetal thymus organ cultures (12). This suggests that thymic macrophages, even when they do express MHC II, are unable to present processed exogenous Ags to bring about negative selection. Thus, thymic macrophages may have evolved to destroy thymocyte Ags completely via proteolytic degradation in lysosomal compartments rather than divert some partially degraded proteins to endosomal compartments. This would reduce the diversity of self peptides displayed to developing thymocytes, effectively lowering the number of thymocytes subjected to negative selection. Perhaps the need to maximize the complexity of thymocytes that survive negative selection outweighs the risks associated with allowing potentially autoreactive T cells to evade negative selection. Similar speculations have been proposed to explain why macrophage engulfment of apoptotic cells in peripheral tissues might be necessary to limit exposure of peripheral T cells to processed self Ags as a means of minimizing the risk of initiating T cell autoimmunity (30). Presumably, under normal conditions in CD2Kb mice, the presence of CD4+ T cells expressing H-2Kb and T cells capable of recognizing processed H-2Kb presented on MHC II molecules would be of no consequence unless some cells constitutively presented processed H-2Kb in the context of MHC II. However, the potential exists for eliciting H-2Kb-specific T cell responses during inflammatory responses when T cells are destroyed (3). Thus, segregation of MHC II and H-2Kb expression in CD2Kb mice reveals that Ags expressed in MHC II+ cells can compartmentalize and, in effect, conceal self Ags that could be recognized by CD4+ T cells and their thymocyte precursors.

Most, if not all, alloreactive H-2Kb-specific, CD8+ T cells are absent in CD2Kb mice because their precursors are eliminated efficiently in thymus (22). Almost certainly, thymocytes themselves mediate negative selection directly in these cases, as has been reported previously (19–22). However, segregated transgene expression does not readily explain the origin of the residual H-2Kb-specific, CD8+ T cells detected in CD2Kb mice because murine thymocytes express MHC I. Some residual CD8+ T cells may evade negative selection because they recognize H-2Kb-restricted peptides derived from proteins expressed only in extrathyMIC cells of CBA origin or because processed peptides derived from endogenous H-2Kb and associated with H-2Kb or H-2Dd are not effective mediators of negative selection. Both scenarios would explain why B6 cells did not stimulate responses in vitro and why thymocyte precursors were not eliminated in vivo. Alternatively, residual CD8+ T cells might recognize exogenously processed forms of H-2Kb that are not presented (indirectly) by other thymic cells in CD2Kb mice. These explanations for the origin of H-2Kb-specific, CD8+ T cells in CD2Kb mice are currently under investigation.

CD2Kb mice also exhibit the unusual characteristic of acquiring tolerance to H-2Kb skin grafts after an initial period of exhibiting immunity to identical grafts. Presumably, tolerogenic processes act upon peripheral H-2Kb-reactive T cells as CD2Kb mice age. One possible mechanism, currently under investigation, is that peripheral APCs gradually tolerate H-2Kb-specific T cells, presumably by displaying processed H-2Kb in ways that are tolerogenic. For example, B cells, which express H-2Kb at barely detectable levels in CD2Kb mice (22, and unpublished results), may display processed H-2Kb on MHC II to tolerize H-2Kb-specific T cells. However, peripheral APCs from CD2Kb mice do not elicit proliferative responses when cocultured with T cells from Tg361 (CBA) mice (unpublished results). Whatever mechanisms regulate H-2Kb-specific T cell responses in CD2Kb mice, it is clear that a delicate balance between tolerance and immunity exists that resolves, as mice age, in favor of tolerance to self H-2Kb. The finding that immunization of young mice with splenocytes or skin grafts from CBK mice prevents the onset of tolerance in older mice further testifies for the plasticity of the immunity/tolerance status of CD2Kb mice (our unpublished results).

Extrapolating from the results presented in this study, we predict that other self Ags expressed exclusively in thymocyte/T cell lineages do not tolerize the murine T cell compartment. This provides a novel explanation for the origin of some autoreactive T cells in mice. Self proteins expressed by other MHC II cell types may also fail to tolerize the T cell compartment for similar reasons. Autoreactive T cells that recognize processed forms of thymocyte/T cell lineage-specific Ags might encounter their cognate MHC/peptide ligand at sites of inflammation in which T cells are destroyed. This raises questions about the potential of these encounters to promote T cell activation and the eventual outcome of T cell responses elicited. In the CD2Kb system, H-2Kb-specific effector T cells capable of mediating graft rejection are activated following engraftment of skin from CBK mice. In contrast, the gradual imposition of T cell tolerance to H-2Kb as mice grow older suggests that encounters between H-2Kb-specific T cells and APCs can promote tolerance induction. Thus, complete segregation of H-2Kb and MHC II expression in distinct cell lineages in CD2Kb mice explains why young CD2Kb mice display the unusual functional characteristic of being able to mount effective immune responses to grafts expressing a self Ag while not developing autoimmune.

Acknowledgments

We thank Doris McCool, Anita Wylds, John Iacomini, and Meral and Levent Keskinettepe for assistance with various aspects of the experimental studies described in this manuscript. We thank Leszek Ignatowicz, Demetrios Moskophidis, Makio Iwashima, David Munn, and other members of the IMMAG Molecular Immunology Program for constructive advice and discussions.

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