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A Threshold for Central T Cell Tolerance to an Inducible Serum Protein

Dipica Haribhai,* Deborah Engle,2† Michelle Meyer, † David Donermeyer, ‡ J. Michael White, § and Calvin B. Williams*3

We report an inducible system of self Ag expression that examines the relationship between serum protein levels and central T cell tolerance. This transgenic approach is based on tetracycline-regulated expression of a secreted form of hen egg lysozyme, tagged with a murine hemoglobin (Hb) epitope. In the absence of the tetracycline-regulated transactivator, serum levels of the chimeric protein are extremely low (≤0.1 ng/ml) and the mice show partial tolerance to both Hb(64–76) and lysozyme epitopes. In the presence of the transactivator, expression increases to 1.5 ng/ml and the mice are completely tolerant. Partial tolerance was further investigated by crossing these mice to strains expressing transgenic TCRs. At the lowest Ag levels, 3.L2tg T cells (specific for Hb(64–76)/I-Ak) escape the thymus and ~10% of CD4+ splenocytes express the 3.L2 TCR. In contrast, 3A9 T cells (specific for hen egg lysozyme(46–61)/I-Ak) are completely eliminated by negative selection. These data define a tolerogenic threshold for negative selection of Ag-specific T cells by circulating self proteins that are 100-fold more sensitive than previously demonstrated. They suggest that partial tolerance at extremely low levels of self Ag exposure is the result of a restricted repertoire of responding T cells, rather than a simple reduction in precursor frequency; tolerogenic thresholds are T cell specific. The Journal of Immunology, 2003, 170: 3007–3014.

Cell tolerance to self Ags is a dynamic process, involving central and peripheral mechanisms (1, 2). These processes must eliminate or render inactive all T cells with receptors that are activated by self peptide/MHC complexes. Many proteins, such as those generated during an inflammatory response (acute phase proteins), have rapidly changing levels, posing a special challenge (3, 4). Developmentally regulated proteins may also fluctuate between tolerogenic and nontolerogenic levels. At low levels of circulating self protein, the relevant self peptide/MHC complexes in the thymus could be limiting, allowing some self-reactive T cells to escape negative selection. When protein levels are induced, the number of peripheral self peptide/MHC complexes could become sufficient to activate these T cells, resulting in autoimmunity (5). In general, there are two central mechanisms that are thought to prevent autoimmunity under these conditions. First, the increased sensitivity of thymocytes relative to mature T cells may provide a sufficient margin of safety by eliminating self-reactive T cells when the number of selecting complexes is low (6). Second, there is some evidence to suggest that ectopic thymic expression of tissue-specific and acute phase proteins may increase the number of relevant peptide/MHC complexes in the thymus and play an important role in T cell tolerance (reviewed in Refs. 7 and 8). Tolerance to inducible, circulating proteins is therefore likely to involve many factors, making it difficult to correlate serum levels with tolerance and to dissect the relative contributions of the mechanisms involved.

Several experimental systems have addressed these important issues with varying results. One estimate for the minimum serum concentration of any self protein required for negative selection comes from data generated by immunizing metallothionein-hen egg lysozyme (MT-HEL) mice. These transgenic animals express HEL under the control of the metallothionein promoter, resulting in a serum concentration of HEL between 13 and 20 ng/ml (~1 × 10−9 M). Immunization studies in these animals demonstrate that T cells are partially tolerant to HEL (9). However, when MT-HEL mice were bred to 3A9 TCR transgenic mice (specific for HEL(46–61)), complete negative selection of 3A9 T cells was observed (5). It has been estimated that as few as three peptide/MHC complexes are needed for this to occur, highlighting the sensitivity of this process for some T cells (10). Tolerance in the MT-HEL system contrasts sharply with studies using an Ig L chain as the self Ag (11). Although clonal deletion of specific T cells was achieved, the serum concentration of Ig needed was greater than 100 μg/ml (>10−6 M). In another system, negative selection of CD4+ T cells specific for C5 was observed at the physiologic concentration of 50 μg/ml (10−7 M) (4). Tolerance to an inducible serum protein was examined in transgenic mice expressing human C-reactive protein (hCRP) under autologous regulatory region control. It was determined that female mice were tolerant at basal hCRP levels (10−9 M), but were also found to express the hCRP transgene in the thymic medullary epithelium (3). Indeed, ectopic or targeted thymic expression leading to tolerance has been reported in several transgenic systems (12–16). In one report, this expression was localized to rare medullary cells that also contained transcripts for tissue-restricted genes. This finding led the authors to postulate that these rare thymic peripheral Ag-expressing cells played an important role in central tolerance (17).

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We have designed an inducible system to test the hypothesis that there is a threshold for central tolerance to secreted self proteins and to examine the consequences of a subthreshold stimulus. This system uses a tetracycline (tet)-regulated transactivator (tTA) (18) to control expression of an epitope-tagged version of HEL (secreted HEL (sHEL)/hemoglobin (Hb)). This secreted, chimeric protein circulates at levels ranging from ≤0.1 ng/ml (≤1 × 10^{-11} M) to 1.5 ng/ml (1–10^{-10} M) and contains two well-characterized H-2^k-restricted epitopes, HEL(46–61)/I-A^d and Hb(64–76)/I-E^k. The transgenic protein is widely expressed in many tissues, including the thymus. We find that mice expressing the lowest levels of protein are only partially tolerant. When immunized with HEL protein or Hb(64–76) peptide, these mice make a diminished T cell response. The mechanism of partial tolerance was investigated at the clonal level by breeding sHEL/Hb mice to TCR transgenic mice. These later studies demonstrate that partial tolerance results from the complete elimination of some clones and partial elimination of others. Thymic transplantation studies confirm that the circulating self protein alone is sufficient for tolerance induction. Together these data define a lower limit for central tolerance to an inducible, secreted protein. They suggest that these limits are likely to be T cell specific and that the repertoire of responding T cells in partially tolerant animals is restricted.

**Materials and Methods**

**Transgenic mice**

We have used tet-regulated expression of a neo-self Ag to study T cell tolerance in transgenic mice (18, 19). For this system, we produced two new lines of mice. The tTA line was generated from a 2.7-kb XhoI/PstI fragment derived from plasmid pUGH 15-1. The fragment contains the human CMV promoter and enhancer (bases –3008 to –76) peptide, these mice make a diminished T cell response. The mechanism of partial tolerance was investigated at the clonal level by breeding sHEL/Hb mice to TCR transgenic mice. These later studies demonstrate that partial tolerance results from the complete elimination of some clones and partial elimination of others. Thymic transplantation studies confirm that the circulating self protein alone is sufficient for tolerance induction. Together these data define a lower limit for central tolerance to an inducible, secreted protein. They suggest that these limits are likely to be T cell specific and that the repertoire of responding T cells in partially tolerant animals is restricted.

**Production, purification, and analysis of sHEL/Hb**

The sHEL/Hb portion of the transgene was cloned into the baculovirus expression plasmid pVL1393 and expressed in sf9 cells using standard techniques (BD PharMingen, San Diego, CA). The protein was purified from culture supernatants by affinity chromatography using the anti-HEL Ab 154.1B12 bound to cyanogen bromide-activated Sepharose 4B. The bound material was eluted with 50 mM glycine and 150 mM NaCl, pH 2.5, directly into neutralization buffer (1 M Tris-HCl, pH 8.6). The fractions containing protein were pooled and dialyzed against PBS. The purified protein obtained was analyzed on a 15% polyacrylamide gel containing SDS. The purified protein was also analyzed for β-4 glycosidase activity (25). In this assay, a 25 mg/ml solution of lyophilized Micrococcus lysi- deiticus (Sigma-Aldrich, St. Louis, MO) was prepared in 50 mM NaPO4 buffer, pH 6.2. To this suspension, 2 mg of either HEL or sHEL/Hb was added, and the absorption at 540 nm was measured at 1-min intervals. The rate of decrease in A540, as bacterial cell walls are enzymatically digested, is a measure of enzyme activity. The sHEL/Hb protein was also analyzed for its ability to stimulate T cell hybridomas, as outlined below.

**T cell hybridomas and hybridoma assays**

T cell hybridomas were used to detect the presence of stimulatory ligands, as previously described (24). Briefly, 1 × 10^5 T cell hybridomas were mixed with 5 × 10^5 APCs in each well of a 96-well plate. To this mixture, increasing amounts of a peptide or peptide complex were added, and the plates were incubated for 24 h at 37°C in 5% CO2. Triplicate wells were performed at each Ag concentration. Stimulation of each hybridoma was then determined by measuring the level of IL-2 produced using the IL-2-dependent cell line CTLL-2. The data shown represent one typical assay, and each assay was repeated at least three times with similar results. The T cell hybridomas used in these assays were YOI.6, which is specific for Hb(64–76)I-E^k (26), and 3A9 (27), which is specific for HEL(46–61)/I-A^d. CH27 cells were used for Ag processing and presentation. In some assays (Fig. 1E), the CH27 cells were lightly fixed with 1% paraformaldehyde in HBSS for 15 min at room temperature. The cells were then washed three times in HBSS to remove the paraformaldehyde before use.

**Flow cytometry**

Single cell suspensions of thymocytes or splenocytes were stained in FACs buffer (PBS supplemented with 0.5% BSA and 0.1% sodium azide) using the following protocol. Aliquots of cells (1 × 10^6/sample in 100 μl FACs buffer) were placed in polypropylene culture tubes (12 × 75 mm; VWR, West Chester, PA) and incubated on ice for 1 h with the biotinylated or directly labeled Abs. Cells were then washed once with 3 ml of FACs buffer and incubated for 30 min on ice with the streptavidin-fluorochrome conjugate where appropriate. Cells were washed again, fixed for 18–24 h in PBS-azide (0.1%) plus 1% paraformaldehyde, and analyzed on a FACScan (BD Biosciences, Mountain View, CA) analytical flow cytometer using CellQuest (BD Biosciences) software. Samples were gated on live cells, and 100,000 live cell events per sample were collected.

**Peptides**

The peptides used in this study were synthesized, purified, and analyzed, as previously described (29). The peptide sequences, in single-letter amino acid code, are: GKKVITAFNEGLK (N72/ wild type); NTDDGSTDYGILQSNR (HEL(46–61)).

**Antibodies**

The Abs used were PE-conjugated anti-mouse CD4 (BD PharMingen); FITC-conjugated anti-mouse CD8a (BD PharMingen); Fc block (BD PharMingen); biotinylated 3.2L clonotypic Ab (CAB); biotinylated 3A9 CAB; and 154.1B12 anti-HEL Ab (9). Cells stained with biotinylated Abs were subsequently incubated with Tricolor streptavidin (Caltag, San Francisco, CA).

**Lymph node proliferation assays**

Mice were immunized s.c. with 20 nmol of either HEL or Hb(64–76) emulsified in CFA. After 10 days, the draining lymph nodes were removed and a single cell suspension was prepared. The suspension was washed, and 5 × 10^6 lymph node cells in a volume of 100 μl were added to wells in a 96-well plate. Appropriate dilutions of HEL protein or Hb(64–76) peptide were then added in a volume of 100 μl. RPMI 1640 with 5% normal mouse serum was used as the culture medium. The plates were incubated at 37°C for 72 h, pulsed with 0.4 μCi/well of [3H]Tdr for 18 h, and harvested. Proliferation was measured as cpm incorporated (mean of triplicate wells). Results from several experiments were then averaged to obtain the data shown in Fig. 4.

**ELISA for sHEL/Hb**

Immune 1b plates were coated overnight at 4°C with 100 μl of the anti-HEL mAb 154.1B12 at a concentration of 5 μg/ml in carbonate coating buffer (15 mM NaCO3, 35 mM NaHCO3, pH 9.6). After coating with the capture Ab, the plates were washed five times with wash buffer (0.01 M Na phosphate, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) using an EL 404
microplate washer (Bio-Tek Instruments, Winooski, VT). Nonspecific binding was blocked by adding 100 μl/well of RPMI plus 10% FCS for 1 h at room temperature, and the plates were washed as above. Standards of known concentration were prepared with HEL (Sigma-Aldrich), and serum samples from the mice were analyzed neat or diluted 1/2 to 1/4. All dilutions were performed with RPMI 1640 plus 10% FCS. Samples and standards were added to triplicate wells in a volume of 100 μl, and the plates were incubated overnight at 4°C. Following Ag capture, the plates were washed as above, and 100 μl of rabbit antisera to HEL diluted 1/1000 was added to each well (9). The plates were then incubated for 1 h at room temperature and washed as above. Next, 100 μl of HRP-coupled goat anti-rabbit antisera (Tago, Burlingame, CA) diluted 1/2000 was added to each well, and the plates were incubated for 1 h at room temperature and washed. Ag was detected with 2.2’-azino-di-3-ethylbenzthiazolinesulfonate (Roche Diagnostics, Mannheim, Germany) in substrate buffer (0.06 M citric acid, 0.09 M Na2HPO4, pH 4.2). H2O2 to a final concentration of 0.1% was added to the substrate just before use. The plates were incubated in the dark at room temperature for 30 min, and the results were read on a microplate reader (Elx800; Bio-Tek Instruments) at a wavelength of 415 nm.

**Thymic transplants**

Thymectomy was performed on B6.AKR neonatal mice on day of life 5, as follows. A midline thoracic skin incision was made, and the thoracic cavity was exposed by a bilateral incision of the thorax. The thymus was exposed, and a sterile cotton tip previously wet with cold sterile PBS was placed under the organ. The thymus was then lifted, gently cut from the thoracic cavity, and a sterile cotton tip previously wet with cold sterile PBS was placed outside the cavity with a curved hemostat placed under the kidney. Using a microdissection microscope, a 3- to 5-mm longitudinal incision was made on the kidney capsule, which was lifted gently with a fine point jewelers forceps. One thymic lobe was placed under the kidney capsule and away from the incision point. The kidney was returned to the abdominal cavity and the abdominal muscles were closed with 4-0 Vycril. The skin was closed with 4-0 Ethilon suture, and Enrofloxacin was administered i.m. Recipient mice were kept warm until recovery. Endogenous and transplanted thymus were examined 30 days after transplantation.

**Results**

**Generation and characterization of a neo-self Ag**

Our system is based on regulated expression of a neoprotein carrying an epitope derived from murine Hb, a well-characterized self Ag (26, 28–30). Briefly, there are two allelic variants of the Hb minor β-chain described in the inbred mouse population, d and s (31). The product of the d allele is recognized by s mice as allo-Ag, with most of the response directed toward Hb(64–76)/I-Ek. To study T cell tolerance to this immunodominant epitope, we created a chimeric gene by inserting the codons for Hb(64–76) into a cDNA for HEL. Creation of an epitope-tagged form of Hb(64–76) was desirable for a number of reasons: 1) expression of free (secreted) Hb requires coordinated expression of both the α- and β-chain; 2) free Hb is nephrotoxic (32); 3) the well-characterized HEL(46–61) epitope adds versatility to the system. To test stability and immunogenicity of the epitope-tagged protein, the coding region of the construct shown in Fig. 1A was cloned into the baculovirus expression plasmid pVL1393. This construct was then transfected into sf9 cells, which secreted the chimeric protein (3009The Journal of Immunology)}
(rHEL/Hb) into culture supernatants at a concentration of 1 μg/ml. (Note that rHEL/Hb refers to the protein produced in culture by insect cells, and sHEL/Hb refers to the secreted protein made by transgenic mice.) The rHEL/Hb protein was purified by affinity chromatography with an anti-HEL Ab and analyzed by acrylamide gel electrophoresis. Results show a single band running slightly larger than the 14.6-kDa band seen with unmodified HEL (Fig. 1B).

Next, stability, processing, and presentation of the rHEL/Hb protein were assessed. Using a modified muramidase assay (25), the hydrolytic activity of rHEL/Hb was demonstrated by mixing either HEL or rHEL/Hb with a suspension of UV-killed, lyophilized M. lysodeikticus. Addition of either HEL or rHEL/Hb resulted in cleavage of the β-1,4 glucosidic bonds found in the peptidoglycan layer of the bacterial cell walls and clearing of the suspension (Fig. 1C). It is important to note that the substrate-binding site in HEL consists of six subsites that are brought together in the properly folded molecule (33). Based on the x-ray structure (34), the additional amino acids inserted into rHEL/Hb are predicted to form a random-chain external loop, which should permit folding, but could interfere with substrate binding. Although rHEL/Hb is not as active as HEL, this demonstration of enzyme activity strongly implies both proper folding and stability of the rHEL/Hb protein.

Previously, we demonstrated that both HEL(46–61) and Hb(64–76) were processed and presented from a membrane-bound form of HEL/Hb (24, 28). The same is true for the recombinant protein. When varying concentrations of either the native Hb protein or rHEL/Hb were mixed with the hybridoma YO1.6 and APCs, the hybridoma was stimulated and produced IL-2 (Fig. 1D). Furthermore, the proliferative responses to Hb protein and rHEL/Hb were equivalent. The T cell hybridoma 3A9, which recognizes HEL(46–61) in the context of I-Ak, was also stimulated by the rHEL/Hb protein (data not shown). Finally, fixing the APCs prevented Ag processing and eliminated the ability of both Hb protein and rHEL/Hb to stimulate the hybridomas, as in Fig. 1E. In summary, these results clearly demonstrate that the chimeric rHEL/Hb protein is stable, retains enzyme activity, and, when processed by APCs, allows recognition of Hb(64–76)/I-Ek and HEL(46–61)/I-Ak complexes by Ag-specific T cells.

**Generation of tet-responsive sHEL/Hb transgenic mice**

Tet-controlled expression of the neo-self Ag was achieved using two independently derived transgenes: a tTA and a reporter (secreted or sHEL/Hb). In our modification of this binary system, originally developed by Bujard and colleagues (18), the TTA transgene was generated from the 2.7-kb XhoI-PflMI fragment of pUHG 15-1. This transcripational activator is a fusion protein containing the tet repressor and the HSV VP16 transactivator (22). Binding of tet to the tet repressor induces a conformational change, eliminating binding at the tet operator and transactivation (35). Expression of the tTA transgene is controlled by the major immediate-early human CMV promoter and enhancer, which together have been shown to efficiently drive expression of transgenes in multiple tissues of epithelial and endothelial origin (20, 21). The sHEL/Hb reporter was derived from a 3.2-kb BamHI-Eco fragment of pBJ1.7 (Fig. 1A). Expression of the sHEL/Hb transgene is controlled by a minimal 52-bp thymidine kinase promoter, preceded by seven tet operator sequences. In double transgenic mice, tTA should bind to the tet operator and drive transcription of the sHEL/Hb transgene. In the presence of tet or in single transgenic (sHEL/Hb only) mice, the minimal 52-bp thymidine kinase promoter alone should be unable to support transcription of sHEL/Hb. Both the tTA line and the sHEL/Hb line were generated independently in B6 mice. Founders were obtained and bred onto the congenic B6.AKR (H2k) background, which is necessary for presentation of Hb(64–76) and HEL(46–61). RT-PCR data from sHEL/Hb mice demonstrated very low levels of transcripts from many tissues, including liver, gut, testes, and thymus (data not shown).

**Inducible expression of the sHEL/Hb transgene**

The HEL and sHEL/Hb proteins can reliably be detected by ELISA down to a concentration of 0.05–0.10 ng/ml (Fig. 2A). The capture Ab for the ELISA detects both native and denatured forms of the protein (unpublished data). When serum from single transgenic mice (sHEL/Hb only) was tested for the presence of the chimeric protein, most mice expressed levels ≤ 0.1 ng/ml. Given the sensitivity of the anti-HEL ELISA, the value shown in Fig. 2B represents the upper limit of the range of protein expressed in these mice. Double transgenic mice, produced by crossing the tTA and sHEL/Hb founder lines, express ∼1.5 ng/ml. At minimum, this represents a 15-fold induction of sHEL/Hb expression. Placing double transgenic mice on tet in the drinking water resulted in suppression of sHEL/Hb expression to baseline levels by 48 h (data not shown). Removal of tet resulted in protein re-expression with similar kinetics. In any event, the single and double transgenic mice have circulating levels of self protein well below the levels achieved in other models of self tolerance.

**The threshold for tolerance to the circulating self protein sHEL/Hb**

To determine whether these low levels of circulating sHEL/Hb protein were sufficient for tolerance induction, we performed...
The mechanism for tolerance induction was investigated at the clonal level by crossing the sHEL/Hb mice to mice expressing transgenic TCRs. We selected the 3.L2tg and 3A9 TCR transgenic lines, which are specific for Hb(64–76) and HEL(46–61), respectively. Overall, analysis of thymocyte development in 3.L2tg x sHEL/Hb mice shows a decrease in CD4 single-positive (CD4-SP) thymocytes, a decrease in double-positive thymocytes, and an increase in the double-negative population, all consistent with negative selection (Fig. 4). When the level of circulating protein is increased in 3.L2tg x sHEL/Hb, tTA mice, a similar phenotype is observed. Increasing the level of circulating self protein still further, as in 3.L2tg x Hbβ2 mice, has little additional effect on the major thymic compartments.

In each case, thymocytes with high levels of the 3.L2 TCR escape negative selection and can be found in the periphery. FACS analysis of splenocytes from 3.L2tg x sHEL/Hb mice with a CAB reveals that 9.2% CD4+ cells fall within the CABhigh gate, in contrast to the 50% seen in normal 3.L2tg mice. The percentage of CD4+, CAB+ splenocytes decreases to 5.2% in induced mice (3.L2tg x sHEL/Hb, tTA), reflecting the increase in circulating levels of sHEL/Hb protein. The relative number of CD4+, CAB+ splenocytes falls still further in 3.L2tg x Hbβ2 mice, demonstrating the effect of natural levels of the Hb(64–76) epitope achieved by processing of endogenous Hb. Also included in Fig. 5 are 3.L2tg x mHEL/Hb mice. The mHEL/Hb transgene is expressed

FIGURE 4. Negative selection of 3.L2tg T cells correlates with the level of circulating self protein. Top four panels. Show the effect on thymic development when 3.L2tg mice were bred to mice expressing either the sHEL/Hb protein at 0.1 ng/ml, the sHEL/Hb protein at 1.5 ng/ml (induced), or the native Hbβ2 protein. FACS analysis for CD4 and CD8 demonstrates a reduction in CD4-SP and double-positive compartments as the circulating concentration of sHEL/Hb is increased (top right compared with middle left). The CD4-SP compartment is reduced even further when 3.L2tg mice are bred to Hbβ2 mice. These mice express the native Hb upon which the system is based (middle right panel). These results should be compared with 3A9 x sHEL/Hb mice (lower right panel), which show complete elimination of all Ag-specific 3A9 T cells at the lowest circulating concentration of sHEL/Hb. The 3.L2tg mouse bred to a membrane form of the HEL/Hb protein expressed in all class II+ APCs (lower left panel) is also shown for comparison. The percentage of thymocytes in each quadrant is indicated, and the numbers represent the mean values derived from 8–20 mice per group. The average thymus size is as follows: 3.L2tg, 108 ± 9 × 106; 3.L2tg x sHEL/Hb, 56 ± 6 × 106; 3.L2tg x sHEL/Hb x tTA, 38 ± 7 × 106; 3A9 x sHEL/Hb, 68 ± 3 × 106.

FIGURE 5. The number of transgenic Ag-specific T cells in the periphery reflects the efficiency of negative selection. CD4+ splenocytes from the mice used in Fig. 4 were examined by FACS for expression of the transgenic 3.L2 or 3A9 TCR. The data are shown as histograms, with the heavy filled line representing the experimental condition, the thin filled line the negative control (B6.AKR), and the dashed line the positive control (3.L2tg or 3A9). The marker indicates the percentage of CD4+ splenocytes that express high levels of the clonotypic TCR.

Graded tolerance to Hb(64–76) by negative selection

lymph node proliferation assays. Single and double transgenic mice were immunized s.c. with either 20 nmol of Hb(64–76) peptide or 20 nmol of HEL protein emulsified in CFA. After 10 days, the draining lymph nodes were harvested, and the cells were restimulated in culture with either the Hb(64–76) peptide or HEL protein. The lymph node cultures were incubated for 72 h, and the proliferative response was determined as a measure of tolerance. Results of two experiments, with three mice per group in each experiment, are summarized in Fig. 3. The data show that primed lymphocytes from mice with 1.5 ng/ml of circulating sHEL/Hb protein fail to proliferate under these conditions and are completely tolerant to both the Hb(64–76) peptide and the HEL protein. However, single transgenic mice with ≤0.1 ng/ml of circulating self protein are only partially tolerant. After immunization with Hb(64–76), T cells from sHEL/Hb mice are ~30 times less sensitive to restimulation than B6.AKR controls. Similarly, T cells from sHEL/Hb mice immunized with HEL are 30–100 times less sensitive to restimulation with HEL than B6.AKR mice.
as an integral membrane protein in all MHC class II cells and is likely to provide the greatest number of I-Ek/Hb(64-76) complexes per APC (10, 36). As expected, these mice are the most efficient in eliminating 3.L2tg T cells.

In normal 3.L2tg mice, ~9% of splenocytes are CD4+, and about one-half of these are CAB+. It is not surprising that elimination of 3.L2tg T cells by negative selection also results in an overall decrease in the percentage of splenocytes that are CD4+.

FIGURE 6. The proliferative response of 3.L2tg T cells upon stimulation with Hb(64-76) peptide correlates with relative number of 3.L2tg T cells/1×10⁶ splenocytes and demonstrates that the cells detected by FACS are functional. A. The relative number of clonotypehigh T cells per 1×10⁶ splenocytes. Results from several experiments were averaged to obtain the data shown: 3.L2tg (n = 20, □), 3.L2tg × sHEL/Hb (n = 19, ○), 3.L2tg × sHEL/Hb, tTA (n = 8, △), 3.L2tg × Hbβ² (n = 6, ●), 3.L2tg × mHEL/Hb (n = 8, □), and 3A9 × sHEL/Hb (n = 6, ▽).

FIGURE 7. Transplanted nontransgenic (B6.AKR) thymi show negative selection in 3.L2tg, sHEL/Hb and 3A9, sHEL/Hb recipients. A. Thymic lobes from neonatal B6.AKR mice were transplanted under the kidney capsule of 3.L2tg (left panels) and 3.L2tg, sHEL/Hb (right panels) mice. In the absence of sHEL/Hb (left panels), 3.L2tg T cell development proceeds normally after 4 wk, with 60–70% of CD4-SP thymocytes expressing the 3.L2 TCR. When the 3.L2tg mice also express low circulating levels of the sHEL/Hb protein, equivalent negative selection occurs in both the native and transplanted thymi. Percentages of cells in each quadrant are the averages from 3 experiments and 12 transplant recipients. *, Due to the small number of events, the number of CD4-SP clonotypehigh cells was divided by the number of CD4-SP clonotypehigh cells in the 3.L2tg control to obtain these percentages. B. Thymic lobes from neonatal B6.AKR mice were transplanted under the kidney capsule of 3A9 (left panels) and 3A9, sHEL/Hb (right panels) mice. Analysis was performed as in A above. Percentages of cells in each quadrant are the averages from 2 experiments with 14 transplant recipients.
The magnitude of this overall decrease reflects the efficiency of negative selection. The average number of CD4+ B cells per 10⁵ splenocytes is shown in Fig. 6A. The average size of the spleens was similar (3.1L2tg, 159 ± 13 × 10⁶; sHEL/Hb × 3.1L2tg, 139 ± 15 × 10⁶; sHEL/Hb × tTA × 3.1L2tg, 142 ± 13 × 10⁶), and these estimates therefore accurately reflect the total number of B cells in each group. Importantly, when these splenocytes are stimulated by Hb(64–76) peptide in vitro, the magnitude of the proliferative responses also reflects the predicted number of CD4+ cells (Fig. 6B). Experiments with plate-bound CAB used to cross-link the 3.1L2 TCR yielded similar results (data not shown), implying that the observed Hb(64–76) response is not due to the transgenic 3.1L2 TCR β-chain pairing with endogenous TCR α-chains and creating Hb(64–76)-reactive T cells. Together, these data demonstrate that the remaining CD4+ cells in each cross are functional and that the number of cells correlates with the serum level of the sHEL/Hb protein. This graded negative selection of 3.1L2tg T cells stands in contrast to negative selection of 3A9 T cells. Even at the lowest Ag dose achieved in our system, all thymocytes with the 3A9 TCR were eliminated, resulting in an absence of clonotype+ cells in the periphery and in complete tolerance to HEL in 3A9 × sHEL/Hb mice (Figs. 5 and 6B).

Negative selection is due to the circulating self protein
Because low levels of sHEL/Hb RNA could be detected in the thymi of single transgenic mice by RT-PCR (data not shown), it was possible that tolerance in this system was due to local secretion and reuptake of the sHEL/Hb protein within the thymic cortical or medullary epithelium and not reflective of the circulating levels. To evaluate this possibility, we transplanted B6.AKR thymi under the kidney capsule of 3.1L2g × sHEL/Hb, 3A9 × sHEL/Hb, or control mice. The development of transgenic T cells within the transplanted and native thymi was analyzed by FACS 1 mo after transplantation, and the results are shown in Fig. 7. Importantly, the B6.AKR thymi showed negative selection of transgenic T cells equivalent to that seen in the native thymi. Because the sHEL/Hb protein is not expressed in the donor B6.AKR thymi, negative selection must result from low-level expression of this circulating self protein in recipient mice. The role of cross-presentation of sHEL/Hb and recirculation of recipient dendritic cells in the negative selection observed in donor thymi cannot be determined in these experiments (37). Control 3.1L2g and 3A9 mice demonstrate complete repopulation of transplanted B6.AKR thymi with transgenic T cells after 1 mo, with no difference between the transplanted and native thymi.

Discussion
We have developed an inducible transgenic system controlling expression of a neo-self Ag to investigate the mechanism and efficiency of tolerance to low levels of circulating self proteins. In this system, transcription of the neo-self Ag sHEL/Hb is controlled by the tet-responsive transactivator tTA. Serum levels of the sHEL/Hb protein are 0.05–0.1 ng/ml in single transgenic sHEL/Hb mice, and 1.5 ng/ml in bigenic sHEL/Hb, tTA mice. We find that when circulating levels of sHEL/Hb protein are ≤0.1 ng/ml (≤1 × 10⁻¹¹ M), tolerance is incomplete and mice immunized with HEL or Hb(64–76) are capable of a T cell response, albeit diminished one. These data imply that at low Ag levels, some T cells escape central and peripheral tolerance mechanisms and are available for self-specific T cell responses. At higher levels of circulating self protein (1.5 ng/ml or 1 × 10⁻¹⁰ M), tolerance was complete. As such, these data define a tolerogenic threshold for sHEL/Hb, which correlates with the level of circulating protein.

This concept of a tolerogenic threshold was investigated further at the clonal level by breeding bigenic sHEL/Hb, tTA mice to 3.1L2tg TCR transgenic mice, which are specific for Hb(64–76). In the progeny of this cross, negative selection is graded within the range of protein concentrations examined. When circulating sHEL/Hb levels are ≤0.1 ng/ml, many 3.1L2tg T cells escape negative selection and accumulate in the periphery, where they comprise 9.2% of all CD4+ splenocytes (as opposed to 50% of all CD4+ splenocytes in the absence of negative selection). These surviving 3.1L2tg T cells generally have lower levels of the clonotypic receptor than normal 3.1L2g T cells, implying that low Ag doses negative selection preferentially eliminates cells with the highest levels of the 3.1L2tg TCR (12). Alternatively, 3.1L2g thymocytes may actively down-regulate their TCR in response to this level of stimulation. Higher Ag doses in the L.5 ng/ml (1 × 10⁻¹⁰ M) range eliminate more, but not all, 3.1L2tg T cells.

In this transgenic system, we can detect sHEL/Hb transcripts in the thymus by RT-PCR, but have not examined thymi for sHEL/Hb protein expression and do not directly determine the potential contribution of thymic sHEL/Hb to central tolerance. Transgenic and peripheral proteins expressed at very low levels in the thymus do induce central tolerance in other systems, even in the apparent absence of mRNA expression (12–15). Nevertheless, based on the immunization and thymic transplantation studies presented in this work, we can conclude that the circulating sHEL/Hb is sufficient to induce the observed phenotypes.

Importantly, Ag-specific, clonotype+ 3.1L2tg T cells do escape the thymus in mice expressing the lowest levels of sHEL/Hb protein, and are found in the periphery in significant numbers. Some of these are L-selectinlow and CD44high, implying that they are no longer naive and are turning over in the periphery (unpublished data). Presumably, this activation is in response to the endogenous Hb(64–76)/I-E<sup>+</sup> complexes found on peripheral APCs, as a direct consequence of the processing and presentation of circulating sHEL/Hb. Peripheral tolerogenic mechanisms must therefore operate to limit expansion of this self-reactive population.

It is also possible that 3.1L2tg T cells escape central tolerance by expressing a second TCR, comprised of an endogenous β-chain paired with the transgenic α-chain. Many, if not all, CBBhigh 3.1L2tg and 3.1L2tg × sHEL/Hb T cells possess a second receptor (38) (data not shown). This observation raises the possibility that some cells could be positively selected on one TCR and activated in the periphery on the second TCR. In one scenario, dual receptor T cells escape normal central tolerance mechanisms and are activated by self Ags in the periphery, predisposing to autoimmunity (39–41). From another point of view, dual receptor T cells rescue nonfunctional T cells in the thymus and are activated by foreign Ag in the periphery, thereby expanding the T cell repertoire (42). Regardless of their role in immune responses, T cells bearing two receptors may not explain all the data, as non-TCR transgenic mice also showed partial tolerance at low Ag levels. The influence of a potential second receptor on tolerance mechanisms was not directly examined in these investigations.

The 3.1L2tg T cell data stand in direct contrast to the experiments with HEL(46–61)-specific 3A9 T cells, which appear more sensitive to the same low level of circulating self protein. The 3A9 T cells are efficiently eliminated in the thymus of sHEL/Hb × 3A9 mice, and these mice are completely tolerant. Taken together, these transgenic T cell studies support the immunization data by illustrating the spectrum of T cell responses available. In other words, they demonstrate that for self proteins circulating in the serum at low levels (1 × 10⁻¹¹ M), tolerance may be incomplete due to the inability to eliminate all self-reactive T cell clones in the thymus. Clearly, many factors not directly examined in this study are likely to influence the tolerogenic threshold at the clonal level, including TCR ligand kinetics (t₁/₂ of TCR-MHC/peptide complexes) and
the number of MHC/peptide complexes available. In regard to the latter factor, it has been estimated that as few as three MHC/peptide complexes per APC are sufficient for complete negative selection of 3A9 T cells (10). Although no such estimate has been possible for 3L2tg T cells, the number is likely to be considerably larger. This speculation is based on experiments involving a membrane form of HEL/Hb expressed in all thymic APCs, which failed to completely tolerate 3L2tg mice (28).

It is important to note that in models of transplantation tolerance, proteins do not need to reach the thymus and thymic APCs via the circulatory system to induce central tolerance. Serum proteins can be picked up by bone marrow-derived dendritic cells in the periphery, which then migrate to the thymus and induce negative selection (43–45). Furthermore, there is recent evidence to suggest that both myeloid and lymphoid precursors give rise to all the different dendritic cell populations, including those found in the thymus (46). These observations suggest that central tolerance via the recirculation of peripheral dendritic cells through the thymus is at least plausible, although the relevance of these findings to our model remains to be established. The participation of multiple tolerogenic mechanisms would not be surprising, given the essential nature of this process.

Acknowledgments

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