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In Vivo Expression of a TCR Antagonist: T Cells Escape Central Tolerance But Are Antagonized in the Periphery

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Transgenic 3.L2 T cells are stimulated by Hb(64–76)/I-Ek and are positively selected on I-Ek plus self-peptides. To this pool of self-peptides we have added a single, well-defined 3.L2 TCR antagonist (A72) in vivo. We find that mice expressing both the 3.L2 TCR and A72 have a minimal loss of T cells expressing the clonotypic TCR in the thymus and spleen. Importantly, the proliferative response of 3.L2 × A72 splenocytes is significantly reduced compared with splenocytes from 3.L2 mice. This reduced response can be attributed to peripheral antagonism. Thus we have identified a new class of self-ligands whose predominant effect is constitutive peripheral antagonism rather than negative selection. The net effect of these ligands is to avoid potential self-reactivity while maintaining as large a repertoire as possible. The Journal of Immunology, 1998, 161: 128–137.

Peripheral T cells are stimulated to proliferate and produce cytokines when their Ag-specific receptor engages an antigenic peptide bound to a MHC protein (1, 2). These same T cells also have flexibility in their recognition of MHC/peptide ligands, in that they can productively interact with peptides containing specific substitutions at TCR contact sites (3). Interaction of mature T cells with these altered peptide ligands (APLs)4 can lead to a variety of functional responses, including differential cytokine production, antagonism, and anergy (4–7). The biochemical hallmark of this partial response is differential phosphorylation of the TCR-ζ and lack of Zap-70 activation. These phenomena have led us and others to conclude that the T cell signaling triggered by these APLs is both qualitatively and quantitatively different from signaling with the antigenic ligand (8–11).

Many other investigations strongly imply that both positive and negative selection of developing thymocytes involves similar recognition events (12–22). For example, in a class I-restricted system, the data show that antagonists promoted positive selection of OVA-transgenic T cells in fetal thymic organ culture (FTOC) (12). In a class II-specific system, FTOC results implicate antagonists in negative selection (21). Others have used an in vitro cell culture system to examine the role of suboptimal ligands in thymic selection. Their data implicate antagonists in negative selection, and suggest that negative selection of thymocytes as a measure of peptide activity is a more sensitive assay than antagonism of peripheral T cells (22).

Using a TCR β-chain only transgenic mouse, we previously reported the effect of an endogenous TCR antagonist on T cell development and peripheral tolerance (23). The data showed that antagonist peptides could promote the negative selection of high avidity thymocytes. Peripheral antagonism was not observed unless the number of antagonist peptide/MHC complexes was increased by adding exogenous peptide (24). In summary, APL/MHC complexes clearly play a role in thymic selection. The rules governing their influence in vivo remain to be established.

In the present study, we describe the functional consequences for T cells when an endogenous antagonist is expressed in the thymus and the periphery. We have utilized the 3.L2 TCR-transgenic mouse (3.L2tg), which is specific for hemoglobin (Hb)(64–76)/I-Ek, and is antagonized by A72/I-Ek. A clonotypic Ab (CAB) was used to follow 3.L2tg-specific responses. High levels of the endogenous antagonist were achieved by expressing the antagonist as a membrane protein in all MHC class II-positive cells. The data demonstrate that the predominate effect in vivo is peripheral TCR antagonism. We do not see enhanced positive selection under the conditions of these experiments.

Materials and Methods

Transgenic mice

We have generated a transgenic mouse line (A72tg) that expresses a membrane form of hen egg-white lysozyme (HEL) containing Hb(64–76) N72A as a 13-amino acid epitope tag (mHEL/A72). The peptide Hb(64–76) N72A (henceforth called A72) is an APL of the self-Ag Hb(64–76), and has been shown to be a strong antagonist of 3.L2 T cell responses (25). The N72A substitution occurs in the P5 position as determined by crystallography (26), which is the TCR contact residue located in the center of the peptide/MHC ligand (27). The construction of a mHEL/Hb(64–76) chimeric gene has been described elsewhere (24). The asparagine to alanine substitution at position 72 of Hb(64–76) was created by PCR mutagenesis with the following nonoverlapping oligonucleotides: 5′-GAAG GCC-TGAAAACCTGTACACCC-3′ (coding) and 5′-CGCAAAGGCAG TTTACACCTTTT-GC-3′ (noncoding). The mutation is contained in the 5′ end of the noncoding oligonucleotide, and the two oligonucleotides are directly adjacent to one another. Sequencing in both directions confirmed the substitution.

We sought to maximize presentation of the antagonist ligand by expressing mHEL/A72 in all APCs. The mHEL/A72 construct was subcloned into the EcoRI site of pDOI-5 (Fig. 1, panel A), which contains the MHC
Eae promoter upstream of a fragment of the rabbit β globin gene. The Eae promoter has been effective in expressing transgenes only in class II-positive cells (28, 29). From this plasmid, a 5.2-kb BglI fragment was isolated and used to inject the male pronuclei of fertilized B6.AKR oocytes. Founders were obtained and bred to the 3.L2tg mouse, which is specific for Hb(64–76)/I-Ek, (G. J. Kersh et al., manuscript in preparation), or to the 3A9 TCR-transgenic mouse, which is specific for HEL(46–61)/I-Ak (30). Progeny were screened by PCR analysis of purified tail digest DNA, and all progeny analyzed were heterozygous for the relevant transgenes, including those for the TCR.

Peptides

The peptides used in this study were synthesized, purified, and analyzed as previously described (25). The peptide sequences (in single letter amino acid code) are: GKKVITAFNEGLK (Hbβd (64–76)); NTDGSTDYGILQINSR (HEL(46–61)); and ADLIAYLKQATK (MCC(92–103)).

T cell hybridomas and stimulation assay

The generation and characterization of the 3.L2–12 (Hb(64–76)/I-Ek-specific), 3A9 (HEL(46–61)/I-Ak-specific), and 2B4 (MCC(92–103)/I-Ek-specific) hybridomas have been described (27, 31, 32). Hybridomas were cultured in RPMI containing 10% heat-inactivated FCS, 2 mM Glutamax (Gibco-BRL, Gaithersburg, MD), 2 × 10–3 M 2-ME, 50 µg/ml gentamicin. The 3.L2–12 and 3A9 hybridomas were used to assay for the presence of stimulatory ligands on splenocytes from A72-transgenic mice. Increasing numbers of splenocytes (10–106 cells/well) in 100 µl were added in triplicate to flat-bottom 96-well microtiter plates containing 1 × 105 3A9 or 3.L2–12 hybridoma cells in 100 µl of media. The cells were incubated for 24 h, after which 100 µl of supernatant was removed from each well and assayed for IL-2 production by CTLL-2 assay. Each point is the mean of triplicate wells (with background subtracted), and the SDs are all less than 10% of the plotted value. This experiment was performed twice with similar results.
column was rinsed with RPMI and then incubated with enough R10 FCS to cover the nylon wool for 1 h. A suspension of splenocytes from two spleens was prepared (300 \times 10^6 cells) and added to the column in a volume of 8 ml. Enough media was drained to lower the level to the top of the nylon wool. The loaded columns were incubated for 1 h at 37°C, and 50 ml of R10 FCS was added. The media was drained over 1 h at a rate of approximately one drop per second. The CD8+ T cells were collected by centrifugation and analyzed by FACScan for class II expression with the 14-4-4S Ab. No class II expression was detected, implying no contamination with APCs (not shown). These purified T cells were then used in T cell proliferation assays at a concentration of 1 \times 10^5 cells/well with 5 \times 10^5 splenocytes/well as APCs, plus Hb(64–76) peptide at concentrations ranging from 0.001 to 100 μM. These assays were incubated for 48 h, pulsed with 0.4 μCi/well of \[^{3}H\]TdR for 18 h, and then harvested. Proliferation was measured as cpm incorporated, and each point represents the mean of triplicate wells.

**Primary T cell proliferation**

Proliferation assays of primary T cells were performed in complete media at 37°C with 5% CO2 as described (24). Briefly, 5 \times 10^5 splenocytes/well were incubated with peptide for 48 h. The cells were then pulsed with 0.4 μCi/well of \[^{3}H\]TdR for 18 h and then harvested. Proliferation was measured as cpm incorporated (mean of triplicate wells).

**Antibodies**

Cells were stained for flow cytometry with the following Abs: phycoerythrin (PE) anti-mouse CD4 (PharMingen, San Diego, CA); FITC anti-mouse CD8α (PharMingen); biotin 3.2L clonotypic Ab (G. J. Kersh et al., manuscript in preparation); biotin 3A9 clonotypic Ab (D. A. Peterson, unpublished observations); biotin F10.6.6 (33); biotin 14-4-4S (34); TRICOLOR (TC) streptavidin (Caltag, San Francisco, CA); and PE streptavidin (Caltag).

**Flow cytometry**

Single cell suspensions of thymocytes or splenocytes were stained in PBS supplemented with 0.5% BSA and 0.1% sodium azide. Cells (1 \times 10^6 sample in 100 μl) were incubated on ice for 1 h with the biotinylated or directly labeled Abs, washed, and incubated for 30 min on ice with the streptavidin-fluorochrome conjugate when appropriate. Cells were then washed again, fixed in 1% paraformaldehyde, and analyzed on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer using CELLQuest (Becton Dickinson) software. Samples were gated on live cells, and 100,000 events collected per sample.

**Results**

**The mHEL/A72 chimeric transgene is expressed by APCs in both the thymus and the spleen**

To achieve high levels of antagonist A72/I-E\(^b\) complexes on the surface of all APCs, we utilized the established expression vector pDOI-5, which contains the MHC Ea promoter, to drive transcription of our chimeric mHEL/A72 gene in transgenic mice (Fig. 1A). This Ea promoter has been quite effective in expressing transgenes at physiologic levels in all class II-positive cells in both the thymus and the spleen (28, 29). Two founders were obtained and characterized. The A72.45 mouse line (A72tg) expressed the mHEL/A72 protein in both the medulla and cortex of the thymus, as detected by immunohistochemistry with the anti-HEL Ab F10.6.6 (data not shown). A single cell suspension of splenocytes from the A72.45 line was also stained with F10.6.6 and with 14-4-4S (anti-I-E\(^b\)) Abs. FACS analysis showed expression of mHEL/A72 on all class II cells (Fig. 1B). The A72.48 mouse line expressed mHEL/A72 on only a subset of APCs (not shown) and was not examined further.

Membrane proteins are efficiently processed and presented in the class II pathway (35, 36). We have previously shown that mHEL/Hb(64–76) can be stably expressed in CH27 cells and that mHEL/Hb(64–76)-transfected CH27 cells strongly stimulated both Hb(64–76) and HEL(46–61)-specific hybridomas, demonstrating efficient processing and presentation of the relevant determinants (24). In the present experiments, by design, we could not directly examine the presentation of the A72 ligand because it is an antagonist with no agonist activity (Transgenic mice expressing mHEL/NT2 (wild-type Hb) and mHEL/TT2 (weak agonist) conclusively demonstrate processing and presentation of Hb epitopes from this chimeric construct in vivo (data not shown). We could, however, utilize the HEL(46–61) determinant. Based on the above, we expected that splenocytes from the A72tg mouse would stimulate the 3A9 T cell hybridoma, which is specific for HEL(46–61)/I-A\(^k\). Conversely, the same splenocytes should fail to stimulate the 3L2.12 T cell hybridoma, which is specific for Hb(64–76)/I-E\(^b\) but antagonized by the A72 substitution (25). The results of these experiments are shown in Figure 1C and conform to our expectations. As few as 1 \times 10^5 splenocytes will stimulate 3A9, while 1 \times 10^6 splenocytes fail to stimulate 3L2.12. These results demonstrate that mHEL/A72 is highly expressed in peripheral APCs and is processed and presented.

**Expression of mHEL/A72 in the thymus results in a small decrease in cells bearing the clonotypic receptor**

To test the effect of the endogenous antagonist on the development of 3L2tg thymocytes, we bred the A72tg mouse to the 3L2tg mouse and performed three-color FACS analysis on thymocytes from mice that were 3 to 12 wk of age. Cells were stained with mAbs against CD4, CD8, and the 3L2 clonotypic receptor (Fig. 2). The data show that 3L2tg and 3L2tg × A72tg mice are the same in terms of the percentage of total cells and the overall number of cells in each compartment (Fig. 2A). However, when we gated on the CD4-singlet positive (SP) cells or the CD4/CD8 double positive (DP) cells, a reduction in thymocytes bearing the highest levels of the clonotypic receptor was seen (Fig. 2B). This loss represents approximately 15 to 20% of the CD4-SP cells with the 3L2 TCR (right panel). Loss of cells with the clonotypic specificity clearly occurs as early in thymocyte development as the DP stage, suggesting that overall, the A72 ligand provides either weak negative selection or antagonism of positive selection. Although we cannot formally distinguish between the two possibilities, the consequence is the same, namely loss of cells with the highest levels of the clonotypic receptor. This experiment was repeated four times (Table I).

Down-regulation of the CD8 coreceptor as a mechanism of tolerance has been demonstrated when transgenic thymocytes were selected on an antagonist peptide in FTOC (37). Importantly, in 3L2tg × A72tg mice, CD4 levels on clonotypic Ab (CAB)\(^\text{high}\) cells were the same as in 3L2tg mice, arguing against down-regulation of the coreceptor as a mechanism of tolerance in this system (data not shown). We conclude from these studies that the endogenous antagonist A72 expressed in the thymus of 3L2tg mice results in a small decrease in cells expressing the highest level of the clonotypic receptor. Many cells expressing the clonotypic receptor mature to become CD4-SP, and these cells express normal levels of CD4.

**The number of clonotypic-positive splenocytes in 3L2tg × A72tg mice is also slightly reduced**

Given the 15 to 20% reduction in CD4-SP thymocytes bearing the clonotypic receptor described above, we expected to observe a similar reduction in the number of CD4+ CAB+ splenocytes and in the overall level of the clonotypic receptor in the periphery. On average, the number of CD4+ CAB+ splenocytes was reduced by 15 to 20% (Table I). However, in two experiments, both the 3L2tg and 3L2tg × A72tg mice were virtually identical in terms of both the number of CD4+ CAB+ cells and the level of clonotypic receptor on those cells (Fig. 3, A and B, taken from Expt. 3). Furthermore, in those experiments where the 3L2tg × A72tg mice had fewer CAB+ splenocytes than their 3L2tg littermates, the
reduction in CAB$^+$ cells was not selective for cells with the highest levels of TCR, as it was in the thymus (not shown).

This variability in numbers of CAB$^+$ splenocytes could be the result of proliferation in the periphery as a consequence of A72 ligand engagement. This is unlikely since 3.L2tg × A72tg T cells have a naive phenotype and are CD25 low, CD69 low, and CD62L high, as shown in Figure 4. Also, 3.L2tg splenocytes do not proliferate when stimulated with as much as 100 μM of A72 peptide (G. J. Kersh et al., manuscript in preparation). Instead, it seems plausible that inefficient negative selection in the thymus of 3.L2tg × A72tg mice allows escape of a few cells with high levels of the clonotypic receptor, which gradually accumulate in the periphery and may reach the levels seen in the 3.L2tg mouse by 10 to 12 wk of age.

The proliferative response of 3.L2tg × A72tg splenocytes is significantly reduced when compared with 3.L2tg splenocytes.

We wanted to examine the proliferative response of 3.L2tg T cells to Hb(64–76) in 3.L2tg × A72tg mice. It follows from the preceding data that the proliferative response of splenocytes from

![Image](https://example.com/image.png)
3.L2tg × A72tg mice should be the same as 3.L2tg controls, particularly in those 3.L2tg × A72tg mice with equal numbers of CD4⁺ CAB⁺ splenocytes and equal levels of the clonotypic receptor. In these mice, a direct comparison is not complicated by reduced numbers of CAB⁺ T cells (Table I, Expts. 3 and 4). When 3.L2tg × A72tg splenocytes from these mice are stimulated with Hb(64–76) peptide, their proliferative response is significantly reduced relative to 3.L2tg splenocytes. A representative experiment showing a sevenfold reduction in the proliferative response at 0.1 μM Hb(64–76) is shown in Figure 5A. This decrease in proliferation in the presence of the antagonist A72 ligand was a consistent feature of all experiments (Table I) and on average ranged from three- to eightfold, depending on the concentration of agonist ligand. We conclude that the proliferative response of 3.L2tg T cells in the presence of the endogenous antagonist A72 is significantly suppressed.

Splenocytes from 3.L2tg × A72tg mice proliferate normally in the absence of the A72 ligand

Three possible mechanisms could account for this marked decrease in proliferative response to Hb(64–76) seen in 3.L2tg × A72tg mice: anergy (38, 39), peripheral antagonism (5, 40), or MHC competition. Regarding the third possibility, studies have demonstrated that continuous delivery of an endogenously expressed membrane protein results in only a fraction of class II molecules (6–15%) loaded with a specific epitope (41, 42). Thus, it is highly unlikely that the endogenous antagonist A72 could occupy enough I-Ek binding sites to inhibit the binding of the exogenously loaded agonist Hb(64–76) and give the appearance of a shift in the dose-response curve. Furthermore, we demonstrated that there was no competition for MHC binding by loading 3.L2tg and 3.L2tg × A72tg splenocytes with MCC(92–103), another well-characterized I-Ek epitope (43), and then using these splenocytes to stimulate the 2B4 hybridoma (I-Ek/MCC(92–103) specific). If the endogenous antagonist A72 was inhibiting the binding of exogenous Hb(64–76) peptide, then it should also inhibit the binding of MCC(92–103). The stimulation of 2B4 by MCC(92–103), from 0.01 μM to 10 μM, was identical on both populations of APCs, thus ruling out competition for MHC binding as an explanation for the observed shift (data not shown).

To distinguish between anergy and antagonism, we examined the inherent reactivity of the T cells. T cells were purified from 3.L2tg × A72tg mice and cultured with B6.AKR splenocytes plus peptide, or with A72tg splenocytes plus peptide. For each distribution, the CD4⁺ and CD8⁺ populations were gated as described in Materials and Methods. These populations were then stained with mAbs specific for the clonotypic receptor (CAB-biotin) and analyzed on a FACScan. A, Three-color FACS analysis was performed on splenocytes from 3- to 12-wk-old mice. Splenocytes were stained with Abs to CD4 (PE), CD8 (FITC), and the clonotypic receptor (CAB-biotin/TC-streptavidin). The data were collected on a Becton Dickinson FACScan and analyzed with CELLQuest software. Shown are dot plots for cells staining with anti-CD4 and anti-CD8 (log₁₀ fluorescence). The boxed region selects CD4⁺ splenocytes. Percentages of cells appearing in each quadrant are indicated. Each plot represents 100,000 live cell events. B, Histograms for all cells in the CD4⁺ region, showing the level of staining (log₁₀ fluorescence) for the clonotypic receptor: A72tg × 3.L2tg (solid line), 3.L2tg (dots), A72tg (dashes). The results are from experiment 3 and are representative of five experiments.
Furthermore, the difference between 3.L2tg T cells and 3.A9tg T cells in the thymus, namely a small decrease in CAB+ CD4-SP thymocytes, mature and are found in the periphery, 133 Clearly then, the two purified populations of T cells have quite similar responses under these two conditions. In the absence of the A72 ligand, both populations respond identically to stimulation with Ag, ruling out anergy. In the presence of the A72 ligand, both populations of T cells have an identical decrease in their proliferative response. These data demonstrate that the decreased proliferative response in 3.L2tg × A72tg mice is an example of peripheral antagonism with an endogenous APL.

Expression of mHEL/A72 in the thymus is sufficient to negatively select transgenic T cells specific for HEL(46–61)/I-A^d

One possible explanation for our unexpected observation that the A72 ligand had a minimal effect in the thymus but was still able to antagonize 3.L2tg T cell responses in the periphery was that expression of the mHEL/A72 protein in the periphery was far greater than in the thymus. Two lines of evidence argue against this explanation. First, immunohistochemical staining of the thymus and spleen with an anti-HEL Ab indicated that expression of mHEL/A72 was equivalent in the thymus and the spleen (not shown).

Another proof that this was not the explanation was obtained by demonstrating processing and presentation of the HEL(46–61) epitope by thymic APCs. The A72tg mouse was bred to the 3A9 TCR-transgenic mouse, which expresses a TCR specific for HEL(46–61)/I-A^d (30). In progeny expressing both transgenes, thymocytes with the 3A9 TCR should be negatively selected by APCs expressing the strong agonist ligand HEL(46–61)/I-A^d. The data from this experiment are shown in Figure 6. Virtually all DP thymocytes are deleted (Fig. 6A, right panel). There was a 75% reduction in CD4-SP cells when comparing this mouse to a 3A9 mouse (center panel). Furthermore, gating on the CD4-SP populations and then analyzing for the presence of the 3A9 TCR reveals that none of these CD4-SP cells in 3A9 × A72tg mice carry the clonotypic receptor (Fig. 6B). Similar results were obtained by Akkaraju et al. (44) when they bred the 3A9 TCR-transgenic mouse to a mouse expressing mHEL under the control of a MHC class I promoter. In double transgenic mice, there were virtually no DP thymocytes and only 9% CD4-SP thymocytes. They concluded that this is the result of negative selection on the strong agonist ligand HEL(46–61).

Our results also demonstrate that all T cells with the 3A9 TCR are efficiently deleted, proving the presence of a properly processed and presented HEL(46–61) epitope in the cortex and the medulla. They also strongly imply that the A72 epitope is expressed in sufficient quantity and in the appropriate thymic APCs for negative selection to occur. Taken together, these data demonstrate expression, processing, and presentation of the mHEL/A72 protein in both the thymus and spleen. Importantly, they demonstrate that the level and location of expression in the thymus is sufficient for negative selection.

Discussion

In this study, we have identified a novel and unexpected effect of the endogenous TCR antagonist A72 expressed in the thymus and in the periphery. We find only a minimal effect on specific T cells in the thymus, namely a small decrease in CAB+ T cells attributable to either negative selection or antagonism of positive selection. After interacting with this ligand in the thymus, most CAB+ thymocytes mature and are found in the periphery, where they antagonize specific T cell responses. This antagonism can be overcome by higher concentrations of Ag, when the stimulating peptide is a strong agonist. We conclude that the
A72 ligand has a minimal effect on thymic selection, and a much more dramatic effect on the peripheral immune response. These findings demonstrate that not all self-peptide/MHC ligands capable of interacting with a given TCR result in positive selection or tolerance by activation-induced cell death. T cells interacting with the endogenous self-antagonist A72 clearly mature and encounter the A72 ligand in the periphery, with the consequence being inhibition of specific immune responses. This suggests that the avidity threshold for negative selection of the 3.1L2tg TCR is approximately that achieved in the A72tg mouse. On balance, it is better for reactive T cells with this avidity to mature and generate a more diverse T cell repertoire than to be deleted in the thymus. The potential tradeoff for this increase in diversity is the inhibition of primary immune responses, an effect that can be overcome by increasing the concentration of stimulating Ag. This phenomenon has not been described in other systems, and may be more relevant to class II-restricted responses. In at least one class I system, T cells that react with a TCR antagonist early in development become tolerant by down-regulating CD8 (37). Down-regulation of CD4 was not observed in our experiments. Lastly, our results suggest that peripheral antagonism could play a role in the maintenance of self-tolerance. For example, the autoimmune potential of self-reactive cells could be held in check by the presence in the periphery of tissue-specific endogenous antagonists (17).

Several studies have implicated a role for APLs in thymic selection. The first reports demonstrated that peptide antagonists of mature T cells could induce positive selection of transgenic T cells with the same receptor in FTOC (12). Subsequent studies demonstrated that T cells positively selected on an APL down-regulated their CD8 coreceptor, again implicating APLs in positive selection and coreceptor down-regulation as a mechanism of self-tolerance in the periphery (37). Page et al. used the deletion of DP thymocytes in culture after stimulation with Ag as a measure of negative selection, and found that negative selection could be induced by ligands of lower affinity than those required for full T cell activation (22). Spain et al. have also shown in FTOC that an antagonist peptide can decrease positive selection, either through negative selection or by antagonizing positive selection (21). Sebzda et al. have shown that positive and negative selection could be induced by different concentrations of the stimulatory peptide (14), and that an APL with moderate agonist properties could mediate positive selection in FTOC (45). However, in another study, T cells selected on low concentrations of the agonist peptide were shown to be nonfunctional (46). Taken together, these data demonstrate that APLs can exert some influence on developing thymocytes in vitro. Importantly, this effect seems to correlate with the affinity of TCR/
ligand interaction (47), implying that for any given peptide, the balance between positive and negative selection will depend on the avidity of the interaction of the specific T cell with thymic APCs (13, 18, 48). Factors influencing this overall avidity include the binding affinity of the TCR and MHC/peptide pair, the concentration of the coreceptor, and the concentration of the relevant ligand on that APC. These studies clearly predict that APLs will have an effect on thymic selection in vivo.

In our in vivo studies with the endogenous antagonist A72, we do not see enhanced positive selection. One explanation for this observation is that positive selection of the 3.L2tg TCR may already be maximized on the B6.AKR background. Should this be the case, adding an additional positively selecting ligand would have no effect. It is important to point out that our experiments only test the effect of adding an antagonist to the endogenous pool of ligands, and not the independent function of the antagonist. These studies clearly predict that APLs will have an effect on thymic selection in vivo.

In our in vivo studies with the endogenous antagonist A72, we do not see enhanced positive selection. One explanation for this observation is that positive selection of the 3.L2tg TCR may already be maximized on the B6.AKR background. Should this be the case, adding an additional positively selecting ligand would have no effect. It is important to point out that our experiments only test the effect of adding an antagonist to the endogenous pool of ligands, and not the independent function of the antagonist. Given this caveat, our results are therefore most consistent with the in vitro results of Spain et al. (21) and Page et al. (22), with a number of important differences that are highlighted above. We see a small but consistent reduction in the number of thymocytes with high levels of the clonotypic receptor in 3.L2tg × A72tg mice. We favor weak negative selection, rather than antagonism of positive selection, as the explanation for a number of reasons. Loss of CAB+ cells in the presence of the endogenous antagonist occurs at the CD4+CD8+ stage of development, and negative selection occurs at this stage in other TCR-transgenic systems (21, 49). Furthermore, the A72 peptide induces deletion of CD4+CD8+ thymocytes in a suspension culture deletion assay, albeit at very high concentrations of peptide (G. J. Kersh et al., manuscript in preparation). The A72 peptide does not induce either the 3.L2–12 hybridoma, the 3.L2 clone, or 3.L2tg T cells to produce IL-2 at any concentration. Given the above, it seems plausible that negative selection is responsible for the loss CAB+ CD4+CD8+ cells, although as mentioned we cannot rule out antagonism of positive selection.

It is instructive to consider the findings presented herein together with our previous studies describing the effects of endogenous antagonists. Our earlier studies utilized a TCR β-chain-transgenic mouse in which Hb(64–76) is an antagonist, and Hb(64–76)T69S (Ser69) is the agonist. Expression of the antagonist Hb(64–76) in vivo resulted in elimination of high avidity Ser69-reactive cells, demonstrating negative selection by an endogenous antagonist (23). In the periphery, low avidity Ser69-reactive cells appeared, but the level of endogenous Hb(64–76)/I-Ek complexes was too low to antagonize the response of
these Ser69-reactive cells without the addition of exogenous peptide (24). In the present study, expression of the endogenous antagonist resulted in a profound lack of central tolerance in which high avidity Ag-specific T cells mature, and are antagonized by endogenously A72/I-E\(^b\) complexes in the periphery. Taken together, these results illustrate the broad range of biologic activity attributable to endogenous antagonists. The differential-avidity model of thymic selection predicts that those antagonists that negatively select will have a higher affinity for their cognate TCR than will those antagonists that are neutral or that positively select (16). While we cannot examine this directly in the Ser69 system due to the oligoclonal T cell response to Ser69, we can determine the kinetics of TCR/ligand interactions in the 3.2L system. Based on the above, the prediction is that thymic expression of 3.2 L2 TCR antagonist ligands with greater biologic activity (and presumed greater affinity) will result in increased negative selection. We conclude that these in vivo data are consistent with the view that there is a correlation between the affinity of antagonist/TCR interactions and the magnitude of their central effect (47).

In summary, we have presented data examining the in vivo effects of expressing one particular 3.2 L2 TCR antagonist at one fixed level in MHC class II-positive cells in the thymus and spleen. Surprisingly, the predominant effect is tolerance by peripheral antigen, rather than central tolerance by negative selection. We see no evidence for positive selection of Ag-specific T cells in addition to that already achieved on the B6.AKR background. Since only a single transgenic founder was examined, our results may be affected to some degree by quantitative effects, which are determined by the level of transgene expression, the site of integration, and the details of tissue-specific expression. Such quantitative effects will need to be addressed before the role of APLs in vivo approach, which we are extending to a spectrum of APLs of the 3.2 L2 system. Based on the above, the prediction is that thymic expression of 3.2 L2 TCR antagonist ligands with greater biologic activity (and presumed greater affinity) will result in increased negative selection. We conclude that these in vivo data are consistent with the view that there is a correlation between the affinity of antagonist/TCR interactions and the magnitude of their central effect (47).

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