Naturally Occurring Low Affinity Peptide/MHC Class I Ligands Can Mediate Negative Selection and T Cell Activation

Bruce Motyka and Hung-Sia Teh

*J Immunol* 1998; 160:77-86; 
http://www.jimmunol.org/content/160/1/77

**References**
This article cites 59 articles, 28 of which you can access for free at: http://www.jimmunol.org/content/160/1/77.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 1998 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Naturally Occurring Low Affinity Peptide/MHC Class I Ligands Can Mediate Negative Selection and T Cell Activation

Bruce Motyka and Hung-Sia Teh

The affinity/avidity model for T cell development postulates that ligands with high affinity for the TCR are efficient in negative selection, whereas those with lower affinity/avidity favor positive selection. Using the 2C TCR transgenic model, we evaluated the efficacy of ligands with widely differing affinity for the TCR (3 × 10^3 to 2 × 10^6 M^-1) in mediating thymocyte deletion. The relative affinities of the 2C TCR for the p2Ca/Ld, dEV-8/Kb, p2Ca-A3/Ld, and p2Ca/Kb ligands are approximately 1000:50:10:1, respectively. Here we show, using an in vitro assay, that the deletion of 2C CD4^+CD8^- thymocytes is mediated not only by p2Ca/Ld, but also by the lower affinity ligands dEV-8/Kb, p2Ca-A3/Ld, and p2Ca/Kb, albeit at relatively higher peptide concentrations. Deletion mediated by low affinity ligands required CD8, whereas high affinity ligand-mediated deletion was CD8 independent. The p2Ca/Kb and dEV-8/Kb ligands are naturally occurring in H-2^b mice, and others have shown that p2Ca/Kb can induce the maturation of CD4^-CD8^-2C-TCR(high) thymocytes in fetal thymic organ culture. In this study we showed that in addition to deletion, the p2Ca/Kb and dEV-8/Kb ligands, in the presence of exogenous IL-2, induced mature 2C T cell proliferation, albeit at a lower level than that induced by the high affinity p2Ca/Ld ligand. Thus, the same low affinity ligands that can effect negative selection and, in the case of p2Ca/Kb, the maturation of CD8 single-positive thymocytes can also induce the activation of mature CD8^+ T cells. The Journal of Immunology, 1998, 160: 77–86.

Although the role of peptides in positive and negative selection is now well accepted (1–5), the types of peptides that may mediate selection are still debatable. For instance, antigenic (6), agonist (7, 8), or antagonist (9, 10) peptides or unrelated peptides that serve an MHC-stabilizing function (6, 11) have been shown to effect positive selection. Studies using \( \beta_2\text{-m}^-/-^{2/3} \) or TAP-1/-/ fetal thymic organ culture (FTOC)\(^3\) have identified peptides that can positively or negatively select a defined peptide/class I ligand-specific TCR (7–9). In one series of studies, high concentrations of the antigenic peptide resulted in the deletion of CD4^+CD8^- (double positive (DP)) thymocytes, whereas low peptide concentrations induced the maturation of TCR transgenic CD4^-CD8^- thymocytes (7, 8). However, the CD4^-CD8^- thymocytes expressed low levels of CD8, and their functional potential was not addressed (7, 8). A more recent study identified a variant of an antigenic peptide (agonist) that was able to activate mature T cells but was unable to negatively select, even at high peptide concentrations (12). Interestingly this agonist could also positively select, with the positively selected cells able to respond to the antigenic peptide but not to the agonist peptide to which they were positively selected (12).

In contrast, other studies using a different TCR specificity found that even low concentrations of the antigenic peptide or its strong agonist variants could not positively select for the class I-specific transgenic TCR (9, 13). One strong agonist was described that did allow maturation to the CD4^-CD8^-TCR^+ stages; however, these cells expressed low levels of CD8 and were nonresponsive to Ag stimulation (13). In this series of studies, peptides that could positively select were all antigenic variants classified as antagonists based on their ability to inhibit mature T cell responses mediated by the antigenic peptide/class I ligand (9, 13). Antagonist peptides that promoted positive selection were inefficient at T cell activation and thymocyte deletion (14). A more recent study indicated that positive selection was mediated in a narrow affinity range, with the antagonist peptides that mediated positive selection possessing a lower affinity than the agonist or antigenic peptides that mediated negative selection (15).

In addition to agonist and antagonist peptides, peptides that are unrelated to the antigenic peptide have also been reported to be able to positively select (6, 11). In one study using \( \beta_2\text{-m}^-/-^{2/3} \) FTOC that were transgenic for the 2C TCR, it was found that various unrelated Kb binding peptides, including some that have no detectable affinity for the TCR (16), can positively select for the transgenic TCR (11). In another study, an adenovirus-mediated delivery of invariant chain and a given peptide was used to express a desired peptide/class II ligand in the thymus of invariant chain^−/− mice. By evaluating for a functional peripheral immune response it was concluded that various unrelated nonagonist/non-antagonist peptides could positively select (6). Furthermore, evidence is presented, in contrast with other findings (9, 12), suggesting that T cells can be activated by the same ligand that induced their positively selection in the thymus (6).

---

1 Address correspondence and reprint requests to Dr. Hung-Sia Teh, Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3. E-mail address: teh@unixg.ubc.ca
2 Abbreviations used in this paper: FTOC, fetal thymic organ culture; DP, double positive; LN, lymph nodes; DC, dendritic cell; I medium, Iscove's Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U of penicillin G/ml, 100 \( \mu \)g of streptomycin/ml, and 5 \( \times \) 10^-5 M 2-mercaptoethanol; FSC, forward scatter; SSC, side scatter; MFI, mean fluorescence intensity; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin; low, low level; high, high level.
The affinity/avidity (17) and the agonist/antagonist (18) models of thymocyte development are two different models to explain how recognition by the TCR of self peptide presented by self MHC molecules can lead to the dramatically different fates of positive or negative selection. The affinity/avidity model postulates that high and low affinity/avidity TCR interactions with peptide/MHC mediate negative or positive selection, respectively (7, 17, 19). From this model it may be predicted that very low concentrations of a deleting peptide/MHC ligand could facilitate positive selection, whereas high concentrations of a positively selecting peptide/MHC ligand could signal deletion. The alternative agonist/antagonist model postulates that the type of peptide/MHC ligand (agonist or antagonist) encountered is critical in determining how a thymocyte is selected (9, 10, 13, 14, 18, 20). Antagonist ligands favor positive selection, whereas agonist ligands favor deletion (9, 13, 15). This model predicts that the affinity of the TCR for peptide/MHC is of lesser importance in the determination of a thymocyte’s fate (20), although antagonism usually correlates with lower affinity (15, 21, 22). For the agonist/antagonist model, emphasis is usually placed on the thymocyte receiving a qualitatively different signal, rather than quantitative changes in a signal (18, 23, 24).

Here, we used the 2C transgenic mouse model, in which the affinities of this TCR to various peptides bound to MHC class I molecules have been determined (16, 25–29), to test the prediction of the affinity/avidity model that low affinity ligands at a relatively high concentration are able to mediate thymocyte deletion. Using this model it is also possible to access whether a naturally occurring ligand with a very low TCR affinity can mediate both the negative and the positive selection of the same TCR, and whether this ligand can also induce the activation of mature T cells. The 2C TCR recognizes the naturally occurring octapeptide p2Ca (LSPFPFDD) bound to both Ld and Kb, although the affinity of the 2C TCR to p2Ca/Ld (2 × 10^6 M^-1) is about 1000-fold greater than that to p2Ca/Kb (1 × 10^3 M^-1) (16, 28). Positive and negative selections of the 2C TCR are mediated on the Kb- and Ld-expressing backgrounds, respectively, although the peptides involved in vivo are not known (3, 30). Of interest, the maturation of 2C thymocytes in FTOC has recently been described to be mediated by the p2Ca/Kb ligand (11). To investigate the deletion of 2C DP thymocytes, we used peptide transporter mutant cells expressing either Ld or Kb as APCs, and different concentrations of peptides. Besides p2Ca, two other peptides were also tested: p2Ca-A3 (16), a variant of p2Ca with a P→A substitution at position 3, and another naturally occurring peptide, deV8 (31, 32), which is structurally distinct from p2Ca. The data support the affinity/avidity model of thymocyte selection and define some of the parameters that are characteristic of the negative selection process mediated by naturally occurring low affinity ligands.

### Materials and Methods

**Mice**

2Cββ(-m/-) mice were produced by backcrossing H-2^b 2C TCR transgenic mice (30) to H-2^d ββ(-m/-) mice (33) (gifts from Dr. Dennis Loh, Nippon Roche Research Center (Kamakura, Japan), and Dr. Oliver Smithies, University of North Carolina (Chapel Hill, NC), respectively). TAP-1/- (34) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained and bred in the animal facility of the Department of Microbiology and Immunology, University of British Columbia.

**Cells and cell culture conditions**

Thymocytes were isolated from 6- to 16-wk-old 2Cββ(-m/-) mice. 2C CD8^+ cells were isolated from the lymph nodes (LN) of 6- to 10-wk-old 2C H-2^d mice and purified by incubating the cells with biotinylated anti-CD8β mAb 53.58 followed by positive selection using a MACS MS^7 Separation Column and MiniMACS magnet following the procedure provided with the product (Miltenyi Biotech, Auburn, CA). This procedure yielded a population of cells of which >95% were CD4^-CD8^-1B2^+ as determined by FACS. In this case, CD8 expression was detected by the anti-CD8α mAb 53.67. The TAP-deficient cell lines T2, T2-Ld^, and T2-Kb (35) (derived from a human T X B hybridoma transfected with murine Ld or Kb), and splenic dendritic cells (DC) isolated from TAP-1/- (H-2^b) mice were used as APCs. The DCs were isolated as previously described (36). In addition, for some studies the fibroblast cell line L-M (tk^) was used. A derivative of L929 (obtained from American Type Culture Collection (ATCC), Rockville, MD), or the Kb^ and Ld^ (37) transfectants of this line (gifts from Dr. W. A. Jefferies, University of British Columbia (Vancouver, Canada), and Dr. I. Stroynowski, University of Texas Southwestern Medical Center (Dallas, TX), respectively) were used as APCs. L-Kb^ and Ld^-transfected thymocytes were used as APCs. L-Kb^ and Ld^-transfected thymocytes in vitro are not known (3, 30). Of interest, the maturation of 2C thymocytes in FTOC has recently been described to be mediated by the p2Ca/Kb ligand (11). To investigate the deletion of 2C DP thymocytes, we used peptide transporter mutant cells expressing either Ld or Kb as APCs, and different concentrations of peptides. Besides p2Ca, two other peptides were also tested: p2Ca-A3 (16), a variant of p2Ca with a P→A substitution at position 3, and another naturally occurring peptide, deV8 (31, 32), which is structurally distinct from p2Ca. The data support the affinity/avidity model of thymocyte selection and define some of the parameters that are characteristic of the negative selection process mediated by naturally occurring low affinity ligands.

**Antibodies and specificities were as follows:** 1B2-biotin, 2C TCR Id (38); F23.1-biotin, Vb8 (39); 53.67 or 53.67-FITC, CD8α (ATCC); 53.58-biotin, CD8β (ATCC); GK1.5-PE, CD4 (Becton Dickinson, Mountain View, CA); 28-14-8S, αδ domain of Ld^ (ATCC); 30-5-7S, αδ domain of Ld^- (ATCC) (40); and Y3, Kb^ (ATCC). All FITC and biotin conjugations of mAb, with the exception of FITC-goat anti-mouse Ig (obtained from Southern Biotechnology Associates, Birmingham, AL), were performed in our laboratory. Streptavidin-Tricolor (PharMingen, San Diego, CA) was used to detect biotinylated mAb. Cell staining and flow cytometry analysis were performed according to standard procedures. A FACScan equipped with LYSIS II software (Becton Dickinson) was used to acquire and analyze data.

**Peptides**

All peptides were synthesized at the University of British Columbia. Peptides p2Ca and p2Ca-A3 were kindly provided by Dr. I. Clark-Lewis. Summary data for the peptides relevant to this study, including peptide affinity to class I, and peptide affinity to the TCR in the context of class I are shown in Table I.

### Flow cytometric analysis of MHC class I expression

The extent of peptide binding to T2-Ld^ or T2-Kb^ cells was measured using a class I induction/stabilization assay as previously described (28). Briefly, 1 × 10^6 T2-Ld^ or T2-Kb^ cells that had been preincubated at 2°C for 8 h were incubated with or without different concentrations of peptide for 8 to 12 h at 37°C in 200 μl of medium. Subsequently, the cells were washed and incubated for 20 min at 4°C with mAb 28-14-8S, 30-5-7S, or Y3. After washing, cells were incubated with FITC-conjugated goat anti-mouse Ig for 20 min at 4°C, washed again, and analyzed by FACS. The degree of labeling of viable cells (based on forward (FSC) and side (SSC) light scatter analysis) for class I was observed as a single peak, with data expressed as the mean fluorescence intensity (MFI).

### Table I. Summary data for peptides used in this study

<table>
<thead>
<tr>
<th>Peptide-Class I affinity^a</th>
<th>2C TCR-Peptide-Class I affinity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peptide</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>p2Ca</td>
<td>LSFPFPFDL</td>
</tr>
<tr>
<td>p2Ca-A3</td>
<td>LSFPFPFDL</td>
</tr>
<tr>
<td>deV8</td>
<td>EQKYFYSV</td>
</tr>
<tr>
<td>pMCMV</td>
<td>YPHFMPTNL</td>
</tr>
<tr>
<td>pOVA</td>
<td>SINEFRL</td>
</tr>
</tbody>
</table>

^a Reference 16 for table, unless otherwise noted.
^b Reference 28.
^c NA, not applicable.
^d Reference 29.
^e Reference 41.
^f ND, not determined.
In vitro deletion assay

Thymocytes (2.5 \times 10^7 cells/ml) were cultured in 96-well microtiter plates in the presence of 1 \times 10^5 T2-L^d, T2-K^b, or T2 cells; 3 \times 10^5 DC; or confluent monolayers of L cells. Splenic DC (42) and L cells (43, 44) have previously been shown to act as efficient APC for the deletion of thymocytes in suspension culture. The APC were first pulsed for 0.5 to 1 h with peptide before thymocyte addition, and deletion was quantitated by FACS using a three-color assay that included the vital dye 7-aminoactinomycin D (7-AAD; Calbiochem, La Jolla, CA) (45). Following culture, cells were washed and incubated for 20 min at 4°C with anti-CD4-FITC, anti-CD4-PE, and 7-AAD. Subsequently, following the procedure of Philpott et al. (46), the cells were washed, fixed with paraformaldehyde, and within 2 h analyzed by FACS. Very small debris and cells larger than the thymocyte population were excluded from collection by setting a gate based on FSC/SSC. Analysis of T2 or L cell APC cultures without thymocytes indicated that usually 4% of cells within this gate were APC. Between 10,000 and 20,000 events were collected per sample. Cells staining 7-AAD- were considered nonapoptotic and viable (45, 46), and it was this population that was subsequently analyzed for CD4 and CD8 expression. The percent recovery of DP thymocytes was calculated using the formula 100 \times (% of viable cells that were DP)/(% of viable input cells that were DP). The percent specific deletion of DP thymocytes was determined as 100 \times (1 - (% of viable cells that were DP in culture with APC and peptide))/(% of viable cells that were DP cultured with APC in the absence of peptide).

The dependency of deletion on CD8 was investigated by adding saturating doses of mAb 53.67 (2 \mu g/ml) at the beginning of culture. The addition of anti-CD8 mAb prevented the detection of CD8 by 53.67-FITC mAb, which was instead detected by a goat anti-mouse Ig FITC-conjugated Ab.

Cell proliferation assay

To determine the proliferative potential of 2C T cells for the p2Ca/K^b, dEV-8/K^b, or p2Ca/L^d ligand, 1 \times 10^5 purified CD8^-T2-L^d or T2-K^b stimulator cells and 10^4 mitomycin C-treated T2-L^d or T2-K^b stimulator cells and 10^4 purified CD8^-2C cells from 2C LN were cultured in 96-well round-bottom plates in 200 \mu l of 1 medium with 5 \times 10^5 mitomycin C-treated T2-L^d or T2-K^b stimulator cells and various concentrations of p2Ca, dEV-8, pMCMV, or OVAp with or without 20 U/ml mouse IL-2. The sequences of these peptides are indicated in Table I. For the final 14 h of a 72- to 96-h culture, cells were incubated with 1 \muCi of [3H]thymidine (DuPont, Boston, MA)/well.

Results

Peptide binding to the TAP-deficient cells T2-L^d and T2-K^b

Although the affinities of p2Ca to L^d and K^b and of p2Ca-A3 to L^d have been established (16, 28) (Table I), it was important to determine the extent of class I expression on T2-L^d or T2-K^b cells after peptide addition under the culture conditions used for the thymocyte deletion assay. MHC class I expression on the T2 cells after peptide addition under the culture conditions used for the thymocyte deletion assay. MHC class I expression on the T2 cells was determined approximately 10-fold greater shift in the expression of L^d induction/stabilization with p2Ca-A3 compared with p2Ca and p2Ca-A3 compared with the 28-14-8S mAb (data not shown), which recognizes the \( \alpha \)3 domain of H-2L^d (48, 49).

Low affinity peptide/MHC class I ligands are deleting at relatively high peptide concentrations

The affinity/avidity model of thymocyte selection stipulates that thymocytes possessing a TCR with high affinity for its cognate peptide/MHC ligand will be deleted. It is not clear, however, whether ligands with low affinity for the TCR can also effect negative selection and, if they do, whether there are qualitative differences associated with the deletion process that are effected by these ligands. As the affinity of the 2C TCR to p2Ca/L^d, p2Ca/K^b, p2Ca-A3/L^d, and dEV-8/K^b has been established (16, 29), it was possible to characterize the degree to which TCR affinity for its ligand plays a role in thymocyte deletion. Deletion was accessed using an in vitro assay (42) with T2-L^d or T2-K^b cells as APC so that the effect of a single type of peptide on thymic deletion could be examined. Analysis using 7-AAD allowed the differentiation of live (7-AAD-) from apoptotic (7-AAD+) and late apoptotic/dead (7-AAD+/4') cells (46), concurrently with the CD4 and CD8 phenotype (45).

For this assay, thymocytes were obtained from 2C/\( \beta_2 m^- \) mice, as these mice lack MHC class I expression and therefore possess a nonselecting environment for the development of mature CD4^-CD8^-1B2^- T cells. 2C/\( \beta_2 m^- \) thymocytes are primarily
CD4\textsuperscript{high}CD8\textsuperscript{high} (~90%) and express a relatively low level of the transgenic 2C TCR that increases dramatically upon culture (Fig. 2). The recovery of DP thymocytes after 20 h of culture without APC with or without peptide was about 85% of the input DP thymocyte population (Table II) and was slightly lower (~70%) for thymocytes cultured with T2-L\textsuperscript{d} or T2-K\textsuperscript{b} cells without peptide (Table II and Fig. 3) or with the control peptides pMCMV and OVAp at concentrations sufficient for maximal L\textsuperscript{d} or K\textsuperscript{b} class I expression, respectively (Table II). As a specificity control for the TCR, thymocytes from 2C TCR nontransgenic b2m\textsuperscript{2/2} mice were incubated with T2-L\textsuperscript{d} cells and various concentrations of p2Ca (Fig. 4) or p2Ca-A3 (data not shown) for up to 40 h. In terms of percent DP thymocyte recovery, no difference was evident with these peptides even at a concentration of 100 \textmu g/ml (Fig. 4 and data not shown).

Incubation of thymocytes with the high affinity p2Ca/L\textsuperscript{d} ligand resulted in marked thymocyte deletion at a p2Ca concentration of \textgreek{0.01} \textmu g/ml (Figs. 3 and 5). The affinity of the 2C TCR for the p2Ca-A3/L\textsuperscript{d} ligand is about 100-fold lower than that for p2Ca/L\textsuperscript{d} (16). As shown in Figure 5, deletion of thymocytes by p2Ca-A3 required about 500- to a 1000-fold more peptide than p2Ca for half-maximal deletion at 18 h. However, p2Ca was only about 30-fold more efficient than p2Ca-A3 in inducing the expression of L\textsuperscript{d} on T2-L\textsuperscript{d} cells (Fig. 1). Thymocytes undergoing deletion down-regulated their TCR (Fig. 2). That some of the DP thymocyte population did not express the 2C TCR may explain why DP thymocyte deletion was not complete at high ligand concentrations (Fig. 2).

Table II. The deletion of DP 2C thymocytes is peptide specific\textsuperscript{a}

<table>
<thead>
<tr>
<th>APC</th>
<th>Peptide</th>
<th>% Recovered DP Cells\textsuperscript{b}</th>
<th>Relative MFI\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>85</td>
<td>NA</td>
</tr>
<tr>
<td>–</td>
<td>100 \textmu g/ml p2Ca</td>
<td>85</td>
<td>NA</td>
</tr>
<tr>
<td>T2-L\textsuperscript{d}</td>
<td>10 \textmu g/ml p2Ca-A3</td>
<td>86</td>
<td>NA</td>
</tr>
<tr>
<td>T2-L\textsuperscript{d}</td>
<td>1 \textmu g/ml p2Ca</td>
<td>69</td>
<td>27</td>
</tr>
<tr>
<td>T2-L\textsuperscript{d}</td>
<td>10 \textmu g/ml pMCMV</td>
<td>73</td>
<td>52</td>
</tr>
<tr>
<td>T2-K\textsuperscript{b}</td>
<td>–</td>
<td>75</td>
<td>24</td>
</tr>
<tr>
<td>T2-K\textsuperscript{b}</td>
<td>100 \textmu g/ml p2Ca</td>
<td>43</td>
<td>67</td>
</tr>
<tr>
<td>T2-K\textsuperscript{b}</td>
<td>10 \textmu g/ml OVAp</td>
<td>68</td>
<td>84</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 2C/b2m\textsuperscript{2/2} thymocytes were cultured with or without T2-L\textsuperscript{d} or T2-K\textsuperscript{b} cells in the presence or absence of peptide for 20 h.

\textsuperscript{b} % Recovered DP cells = % of total viable CD4\textsuperscript{+}CD8\textsuperscript{+} cells recovered from the CD4\textsuperscript{+}CD8\textsuperscript{+} input population. This population included cells which were CD4\textsuperscript{dull}CD8\textsuperscript{dull} but were 7-AAD\textsuperscript{2}.

\textsuperscript{c} Relative MFI of L\textsuperscript{d} or K\textsuperscript{b} on T2-L\textsuperscript{d} or T2-K\textsuperscript{b} cells, respectively, after 10 h of incubation with or without peptide.

Notably, the very low affinity p2Ca/K\textsuperscript{b} ligand was also deleting (Figs. 3 and 5). However, about 1000-fold more p2Ca was needed to obtain half-maximal deletion with T2-K\textsuperscript{b} APC despite the fact that p2Ca was slightly more efficient in inducing MHC class I expression in T2-K\textsuperscript{b} than in T2-L\textsuperscript{d} cells (Figs. 1 and 5). Thus, when comparing the p2Ca/L\textsuperscript{d} and p2Ca/K\textsuperscript{b} ligands, differences in the affinity of the TCR to its ligand are more important in determining the efficiency of deletion than the density of the ligand.
That the differences in deletion of thymocytes by p2Ca on Ld and Kb were not due to differences in the T2-Ld and T2-Kb mutant cells used was suggested by the observation that p2Ca-mediated deletion of thymocytes was very efficient at low peptide doses with Ld-transfected L cells, whereas 1000-fold more peptide (30 μg/ml) was needed for half-maximal deletion with Kb-transfected L cells after 18 h of culture (data not shown). No peptide-specific 2C thymocyte deletion was observed with the parental L cell line (Kk) (data not shown). Deletion of thymocytes in the presence of p2Ca was also evident with H-2b TAP-1−/− DC, where the dose and time dependence of thymocyte deletion was very similar to that of T2-Kb- plus p2Ca-mediated deletion (data not shown).

As shown in Figure 5, the dEV-8/Kb ligand was also deleting in a dose-dependent manner, similar to the p2Ca/Kb ligand. However, the slope of the dEV-8-induced deletion curve did not parallel those observed with the other three ligands (Fig. 5). Intuitively, one would have expected the dEV-8/Kb ligand to

**FIGURE 3.** The very low affinity ligand p2Ca/Kb at high concentrations relative to those of p2Ca/Ld is deleting for 2C DP thymocytes. For determination of thymocyte deletion, 2C/β2m−/− thymocytes were stained in a one-step procedure with 7-AAD, anti-CD4-FITC, and anti-CD8-PE and analyzed by FACS. Shown is the cell viability and the CD4/CD8 expression of viable thymocytes (R1 gate) after 6, 18, or 40 h of culture with T2-Ld or T2-Kb cells without or with 0.1 or 100 μg/ml p2Ca, respectively. The FACS profiles from one representative experiment of six are shown.

**FIGURE 4.** The p2Ca/Ld ligand is nondeleting for β2m−/− thymocytes that do not express the 2C TCR. For determination of thymocyte deletion, 2C/β2m−/− thymocytes were stained in a one-step procedure with 7-AAD, anti-CD4-FITC, and anti-CD8-PE and analyzed by FACS. Shown is the cell viability and the CD4/CD8 expression of viable thymocytes (R1 gate) after 18 or 40 h of culture with T2-Ld cells with or without 100 μg/ml p2Ca.
generate a dose-response curve intermediate among those generated by the p2Ca/Ld, p2Ca/Kb, and dEV-8/Ld ligands. Such a result is expected, since dEV-8 is more efficient than p2Ca in inducing Kb expression (Fig. 1), and the dEV-8/Kb ligand has a higher affinity for the 2C TCR than the p2Ca/Kb ligand (Table I). However, it was found that although low concentrations of dEV-8 were more effective than p2Ca in inducing deletion, high concentrations of dEV-8 were not. Thus, for the dEV-8/Kb ligand, there was a lack of correlation between the affinity of the TCR and the efficiency of deletion.

The kinetics of deletion mediated by varying concentrations of the p2Ca/Ld, p2Ca/Kb, and dEV-8/Kb ligands are shown in Figure 6. It is clear from these data that the kinetics of deletion effected by these ligands are fairly similar. High concentrations of the control peptides pMCMV or OVAp were nondeleting (Fig. 6). Furthermore, thymocyte deletion was not evident with p2Ca or dEV-8 and nontransfected T2 cells as APC, indicating that the deletion seen with the Ld- or Kb-transfected T2 cells is mediated by these transfected class I molecules and not by the endogenous human class I HLA molecules (Fig. 6).

**Proliferation of mature 2C cells induced by relatively high concentrations of low affinity ligands**

The p2Ca/Kb ligand has been suggested to be capable of serving as a positively selecting ligand for the 2C TCR, inasmuch as expression of this ligand in FTOC was capable of inducing the expression of mature CD8 T cells that expressed the 2C TCR (11). The dEV-8/Kb ligand, based on its naturally occurring expression in the H-2b thymus, has also been suggested to positively select the 2C TCR (31, 32). The lower affinity recognition by the 2C TCR of this ligand vs p2Ca/Ld (29) also would support a role for dEV-8/Kb to positively select the 2C TCR. Thus, given that p2Ca/Kb and dEV-8/Kb ligands may both be capable of positively selecting 2C TCR (11, 31, 32) and could also mediate deletion (Figs. 5 and 6), it was of interest to determine whether these ligands could activate mature 2C T cells.

The results presented in Figure 7 indicate that, as expected, the high affinity p2Ca/Ld ligand induced a strong proliferative response by mature CD8 T cells expressing the 2C TCR. This activation did not require the addition of exogenous IL-2, although exogenous IL-2 did improve the response, particularly at low p2Ca concentrations. In contrast, the low affinity ligands, p2Ca/Kb and dEV-8/Kb, at a peptide concentration of 30 μg/ml did not stimulate a proliferative response by CD8 T cells expressing the 2C TCR in the absence of IL-2, although with 100 μg/ml of peptide there was a small IL-2-independent response evident with p2Ca but not with dEV-8 (Fig. 7). In the presence of exogenous IL-2 and at peptide concentrations of 10 μg/ml or more, the p2Ca or dEV-8 peptide, when presented to T2-Kb cells, induced a proliferative response in 2C T cells (Fig. 7). Thus, the low affinity p2Ca/Kb and dEV-8/Kb ligands could elicit a proliferative response by naive 2C T cells, but, unlike the higher affinity p2Ca/Ld ligand, low affinity ligand-mediated proliferation required both a high ligand concentration and exogenous IL-2.

Interestingly, with or without IL-2, the proliferative response to p2Ca/Kb was stronger than that mediated by dEV-8/Kb (Fig. 7). However, based on ligand affinity the opposite finding may have been expected. This lack of correlation of the dEV-8/Kb ligand to 2C TCR affinity compared with the p2Ca/Kb ligand was also observed for the thymocyte deletion response.

**Thymocyte deletion mediated by low avidity TCR-ligand interactions is CD8 dependent**

Deletion of 2C thymocytes on the H-2d background proceeds in the absence of CD8, and this contrasts with the CD8 dependency of thymocyte deletion in the H-Y or the lymphocytic choriomeningitis virus class I-specific TCR transgenic mouse models (50). One likely explanation for this difference was that the 2C TCR might have a higher affinity for its ligand compared with the affinities of the H-Y or the lymphocytic choriomeningitis virus TCR for their ligands (50). Thus it might be predicted that p2Ca/Ld-mediated deletion would occur independently of CD8 in vivo deletion assay, and that the deletion mediated by ligands with lower affinity for the 2C TCR may require CD8. As shown in Figure 8, when anti-CD8 mAb were added at saturating concentrations, deletion of DP thymocytes by p2Ca and T2-Ld cells was unaffected. However, addition of anti-CD8 mAb affected deletion mediated by the other ligands to varying degrees. Deletion effected by the p2Ca-Kb/Ld ligand was partially inhibited by anti-CD8 mAb, whereas deletion effected by the dEV-8-Kb ligand was completely inhibited by the anti-CD8 mAb. The anti-CD8 mAb also caused almost complete inhibition of deletion mediated by the p2Ca/Kb ligand (Fig. 8). These results are consistent with a requirement for CD8 in thymocyte deletion that is mediated by low avidity, but not high avidity, TCR-ligand interactions. Clearly, however, other factors must also impinge on deletion besides ligand avidity, as the dEV-8/Kb ligand has a higher affinity for the
2C TCR than the p2Ca-A3/Ld and p2Ca/Kb ligands, yet the dEV-8/Kb ligand is more sensitive to inhibition by anti-CD8 mAb.

Discussion

Using an in vitro deletion assay with APC that present only one type of peptide, we have shown that very low affinity interactions between peptide/MHC ligand and TCR can mediate thymocyte deletion. However, deletion by low affinity ligands was only evident at relatively high ligand concentrations. Thus, a 1000-fold greater concentration of p2Ca was required to effect 2C DP thymocyte deletion mediated by the p2Ca/Kb ligand compared with the p2Ca/Ld ligand, and this difference correlates with the 1000-fold lower affinity of the 2C TCR for the p2Ca/Kb vs the p2Ca/Ld ligand (16). These results are consistent with the hypothesis that for deletion to occur, a threshold avidity level must be attained (17), and we now show that this is possible with a TCR-ligand affinity as low as $3 \times 10^{-3}$ M$^{-1}$. In mature T cells, activation is based on the number of triggered TCRs and is less dependent on the affinity of the triggering ligand for the TCR (51). According to this model, a lower density of a high affinity ligand is required to trigger the critical number of TCRs to induce T cell activation, whereas a much higher density of low affinity ligand is required to trigger the same critical number of TCRs. Our deletion data are also consistent with this model, since a much higher ligand density is required by low affinity ligands for deletion of DP thymocytes.

The low affinity p2Ca/Kb and dEV-8/Kb ligands function as deleting ligands for the 2C TCR in the assay system used in this study. While it is known that p2Ca/Ld is a naturally occurring ligand (52–54), it is not known whether p2Ca is also naturally present on Kb-expressing tissue (discussed in Ref. 28). It is interesting to note that the p2Ca/Kb ligand has previously been shown to induce the maturation of CD4$^+$ CD8$^+$ 2C-TCR$^{high}$ thymocytes in FTOC (11). The data in Figure 7 showed that 2C T cells can undergo proliferation in response to the p2Ca/Kb and dEV-8/Kb ligands, albeit at high peptide concentrations and in the presence of exogenous IL-2. It is therefore clear that a particular ligand can induce positive selection, negative selection, and activation of mature T cells. However, it is not clear whether thymocytes that are positively selected by the p2Ca/Kb ligand can respond to this ligand in the periphery. The ability of a low affinity self peptide to activate mature T cells under certain circumstances has potential implications for the maintenance of T cell tolerance and memory. That the p2Ca/Kb or dEV-8/Kb ligands can prime naïve 2C cells suggests that an autoimmune response could develop in H-2b 2C
mice if a high enough concentration of ligand was encountered at a site where a sufficient amount of IL-2 was also being produced. However, it seems unlikely that such conditions would be met under physiologic situations, and thus self tolerance would be maintained. Indeed, an increased incidence of autoimmunity has been described in H-2b 2C mice. It has been hypothesized that very low affinity interactions with self-Ags may be important in maintaining the life span of naive T cells and/or memory cells (55, 56). In this regard, the p2Ca/Kb and dEV-8/Kb ligands may represent self Ags that can provide a low grade stimulation signal. Future studies may be able to address whether such low level stimulation signals exist under physiologic conditions in the 2C mouse and, if they do, may suggest the importance of such signals for the maintenance of naive and/or memory T cells.

The finding that the same weak p2Ca/Kb agonist ligand that can negatively select may also be able to positively select agrees with the findings of Fukui et al. (57), who found that a single peptide/MHC class II complex could positively or negatively select depending on the level of expression of the transgenic ligand. However, Szebda et al. (12) found that a moderate agonist peptide was able to positively select over a wide range of concentrations, but was unable even at high concentrations to negatively select. Thus, the strength of an agonist ligand does not necessarily correspond to the efficiency of negative selection. We also did not obtain complete correspondence of increased efficiency in negative selection with TCR/ligand affinity. Since the dEV-8 peptide was more efficient than the p2Ca peptide in inducing Kb expression on T2-Kb cells, and the dEV-8/Kb ligand has a higher affinity than the p2Ca/Kb ligand for the 2C TCR, one would have predicted that the dEV-8/Kb ligand would be more efficient than the p2Ca/Kb ligand in inducing negative selection and T cell activation. However, we observed that the dEV-8/Kb ligand was not more efficient than the p2Ca/Kb ligand in mediating negative selection, particularly at high ligand density. Furthermore, the dEV-8/Kb ligand was more dependent on the CD8 coreceptor than was the p2Ca/Kb ligand in mediating negative selection. It was also anomalous in that it was less effective than the p2Ca/Kb ligand in inducing the proliferation of 2C CD8+ LN cells. A similar lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR has been reported in another study (27). This lack of strict correlation between TCR-ligand affinity and the efficiency of thymocyte deletion and T cell activation may lead one to question the general validity of the affinity/avidity model. These observations suggest that molecular events besides TCR binding of the MHC/peptide complex may be required for negative selection and T cell activation. In this regard it is interesting to note that the ability of various anti-TCR Abs to act as agonists or antagonists appear to be dependent on their capacities to induce the recruitment of CD4 and CD45 molecules into the TCR signaling complex (58). These observations suggest that molecular events besides TCR binding of the MHC/peptide complex may be required for negative selection and T cell activation. In this regard it is interesting to note that the ability of various anti-TCR Abs to act as agonists or antagonists appear to be dependent on their capacities to induce the recruitment of CD4 and CD45 molecules into the TCR signaling complex (58). One speculation is that the greater sensitivity of dEV-8/Kb-mediated deletion to inhibition by anti-CD8 mAb may correlate with the less efficient recruitment of the CD8 coreceptor to the TCR signaling complex by this ligand. Alternatively, a more trivial explanation for these discordant observations is that some of the purified MHC and TCR molecules used for affinity measurements may lack structures that are found on natural cell surface molecules, and these structures are critical for effective TCR-ligand interactions. Further studies are required to distinguish among these possibilities.

That deletion of thymocytes mediated by the high affinity p2Ca/Ld ligand was not blocked by anti-CD8 mAb is in agreement with the observation that negative selection of immature 2C thymocytes occurs efficiently in the CD8α–/– background (50). Our results indicated that unlike high affinity ligand-mediated depletion, deletion mediated by low affinity ligands was dependent on CD8 interactions. An avidity threshold may explain these differences in the requirement for CD8. Thus, although increasing the density of the low affinity ligand increases the avidity of its interaction with the TCR, the overall avidity may still be low enough that a CD8 interaction is necessary. In contrast, for high affinity ligands, the avidity threshold required for efficient negative selection may be exceeded even in the absence of CD8. Indeed, recent findings suggest that the CD8/MHC interaction enhances the stability of the TCR/ligand complex by reducing its dissociation rate (59).
FIGURE 8. Differential requirement for the CD8 coreceptor for high and low affinity ligands in thymocyte deletion. Following a 20-h culture with T2-L 1d or T2-K b cells and peptide p2Ca, p2Ca-A3, or dEV-8 with or without anti-CD8, 2C/β2m−/− thymocytes were incubated with 7-AAD and mAb specific for CD4 and CD8 and analyzed by flow cytometry, except that CD8 expression on anti-CD8-treated thymocytes was detected using an anti-mouse Ig-FITC Ab. The extent of thymocyte deletion was expressed as the percent specific deletion. The data from one representative experiment of three are shown.

References


