Strong Agonist Ligands for the T Cell Receptor Do Not Mediate Positive Selection of Functional CD8+ T Cells

Kristin A. Hogquist,* Stephen C. Jameson,*
and Michael J. Bevan
Howard Hughes Medical Institute
Department of Immunology
University of Washington
Seattle, Washington 98195

Summary

Positive selection of functional CD8+ T cells expressing an MHC class I–restricted T cell receptor can be induced in fetal thymus organ culture by class I–binding peptides related to the antigenic peptide ligand. Peptides that act as antagonist or weak agonist/antagonist ligands for mature T cells work efficiently in this regard. In the present study, we have investigated whether low concentrations of the original agonist peptide, or variants that still have a strong agonist activity can also mediate positive selection. The antigenic peptide did not induce positive selection at any concentration tested. A strong agonist variant was capable of stimulating the differentiation of TCR^{hi} CD8+ cells, giving the appearance of phenotypic positive selection. However, these cells lacked biological function, since they could not proliferate in response to antigen. The most efficient positive selection resulted with ligands that did not activate mature T cells or stimulate negative selection.

Introduction

Developing thymocytes are spared from programmed cell death if they express a T cell receptor (TCR) capable of engaging specific self-peptide–major histocompatibility complex (MHC) ligands in the thymus. This step is known as positive selection. The induction of activation-induced death (negative selection), however, also results from the engagement of the TCR with self-peptide–MHC ligands. Immunologists have long sought to understand what distinguishes ligands that induce positive versus negative selection, and how these different ligands are perceived biochemically.

Recently, we and others have developed an approach to identifying peptides that will induce positive selection when presented by the appropriate MHC (Hogquist et al., 1994; Ashton-Rickardt et al., 1994; Sebzda et al., 1994). This approach utilized class I–restricted TCR transgenic mice for which the antigenic peptide ligand is known. The TCR transgenic mice were bred to mice that cannot present endogenous class I antigens via the normal pathway, but that are capable of binding exogenous peptides provided in the media. The deficient mice (β2-microglobulin

*Present address: Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455.

−/− [β2m$^{-/-}$] or TAP$^{-/-}$) did not positively select cells with the transgenic receptor either in vivo or in fetal thymic organ culture. Addition of class I–binding peptides to organ culture restored positive selection of thymocytes with the transgenic receptor.

Interestingly, among the many peptides capable of binding the appropriate class I molecule, only peptides related to the antigenic peptide were found to induce positive selection. In the case of the ovalbumin-specific H-2K^b-restricted receptor, the positively selecting peptides were single amino acid variants of the antigenic peptide, and acted as antagonists for mature T cells with this receptor (Hogquist et al., 1994). Many other peptides that bound K^b, but were not related to the antigenic peptide, had no effect. The antigenic peptide induced negative selection efficiently, but did not induce positive selection. On the other hand, two groups using an lymphocytic choriomeningitis virus (LCMV)-specific H-2D^b-restricted receptor found that the antigenic peptide (actually a very conservative single amino acid variant of it) did induce positive selection at low concentrations, while inducing negative selection only at higher concentrations (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). In this report, we have tried to reconcile these two results, and discuss the implications of this data on how a T cell chooses life or death in the thymus, based on recognition of self-peptide–MHC ligands.

Results

The predominant ovalbumin peptide epitope that is naturally processed and presented by K^b is an octamer composed of residues 257–264 (Rotzschke et al., 1991). T cells recognizing this epitope were produced and the genes were cloned and used to generate TCR transgenic mice (OVA-tcr-1) (Hogquist et al., 1994). To study the effect of peptides on T cell development, we have used fetal thymic organ culture, wherein immature lobes are cultured ex vivo for 1 week. During this time, T cells divide and differentiate in a manner which mimics that seen in vivo. Figure 1 shows that day 16 fetal thymic lobes from OVA-tcr-1 mice contain predominantly double-negative cells, and that these cells differentiate over time, generating a high percentage of CD8 single-positive cells (CD8SP) in β2m$^{+/-}$ lobes, or a distinct absence of CD8SP in β2m$^{-/-}$ lobes. This absence reflects the lack of positive selection due to the β2m deficiency. In the β2m$^{+/-}$ lobes, the CD8SP that accumulate resemble mature adult thymic CD8SP. They have a high level of the transgenic TCR (Figure 1), are HSA^{lo}, and express Bcl-2 (Hogquist et al., 1994). The system described here allows us to look at the effect of class I–binding peptides on deletion, positive selection, or both. For these experiments, we used three peptides (Figure 2). OVAp is the naturally processed and presented fragment of the ovalbumin protein for which this TCR is specific. A2 is a single amino acid variant (alanine for isoleucine at position 2), which retains stimulatory or agonist
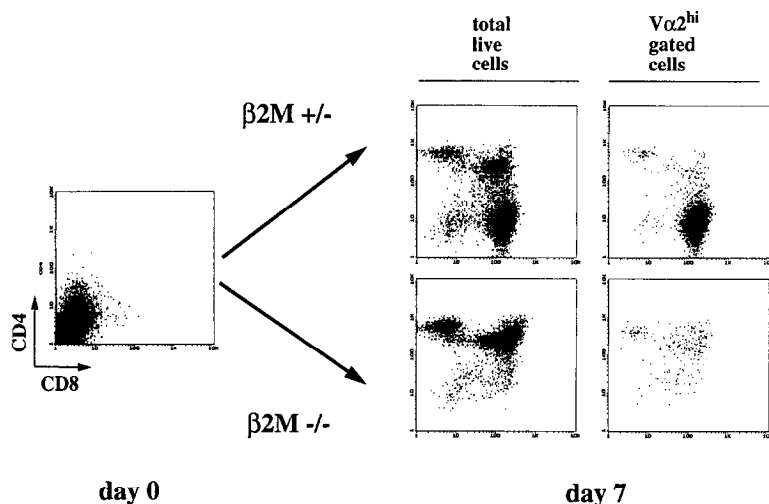
Figure 1. Thymocyte Development in TCR Transgenic $\beta_2 m^{+/-}$ and $\beta_2 m^{-/-}$ Fetal Thymus Organ Culture

(Left) Thymi from fetal mice at a gestational age of day 16 were stained for CD4, CD8, and TCR Vα2. CD4 and CD8 staining are shown. Vα2 staining was negative.

(Right) Lobes were cultured for 7 days and stained. CD4 and CD8 staining on total viable thymocytes or on viable thymocytes after gating on TCR Vα2 high-expressing cells is shown as indicated.

activity (Figure 2A) but is roughly 10-fold weaker than OVAp. E1 is a variant (glutamic acid for serine at position 1), which can antagonize the stimulatory activity of OVAp (Jameson et al., 1993). On its own, E1 stimulates a feeble response, and only at very high concentrations (Figure 2A). All of the peptides bind K^b to a similar degree (Figure 2B).

The Antigenic Peptide Causes Profound Deletion and Results in Cells with Lower CD8 Levels

As has been seen with many TCR transgenics, exposure of developing thymocytes to antigenic peptide results in clonal deletion by activation-induced cell death. This was seen in vivo (Murphy et al., 1990; Vasquez et al., 1992), in suspensions cultures in vitro (Swat et al., 1991; Pircher et al., 1993; Iwabuchi et al., 1992), and in fetal thymic organ cultures (Spain and Berg, 1992; Hogquist et al., 1994; Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Figure 3A shows the results of adding the ovalbumin antigenic peptide (OVAp) to fetal thymic organ cultures of OVA-tcr-1 mice. At high concentrations (100 nM) complete deletion prevails; the cellularity of the lobes is low (about 10% of untreated), and the remaining cells are CD4⁻, CD8⁻. At intermediate concentrations of peptide (10 pM to 1 nM), an unusual phenotype is seen. As peptide is increased, cellularity decreases and cells with a distinct CD8^{lo} phenotype remain. The more peptide present, the lower the level of CD8 on the remaining cells. It is unclear at this point whether cells that had a preexisting low level of CD8 selectively survive the exposure to peptide, or whether cells that had a high level of CD8 down-regulate it in response to peptide. That cells with lower levels of CD8 are less responsive to antigen than CD8^{hi} cells has been demonstrated in several systems (Auphan et al., 1992; Blok et al., 1992; Jameson et al., 1994). Interestingly, an analogous down-regulation of CD4 was not seen when antigenic peptide was added to fetal thymic organ cultures of a CD4-dependent class II-restricted TCR transgenic (Spain and Berg, 1992; K. A. H. and J. Goverman, unpublished data). This may reflect differences in the way the cell surface expression of these two proteins are regulated. Alternatively, since more Lck is known to

associate with CD4 than CD8 (Wiest et al., 1993), it is possible that cells with an intermediate level of CD4 do not exhibit a survival advantage (i.e., are equally responsive to the antigenic peptide as cells with a high level of CD4).

Positive Selection Is Not Seen with Any Concentration of the Antigenic Peptide

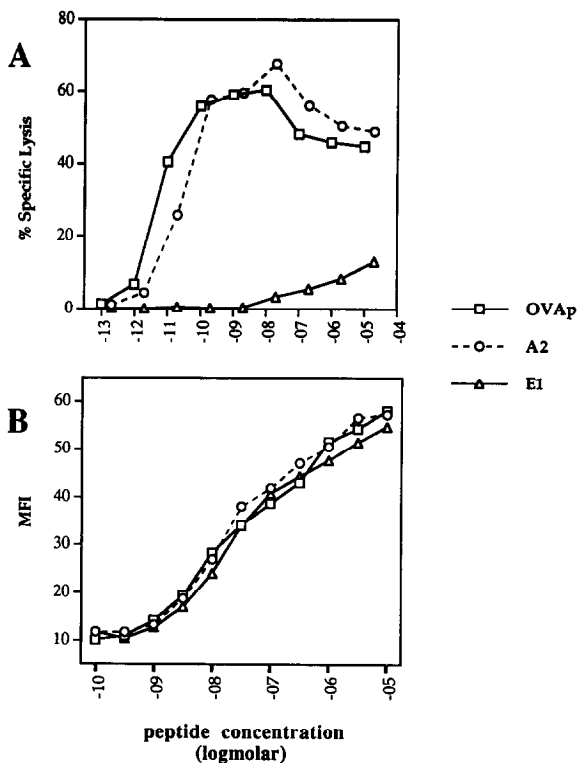TCR engagement of MHC-antigenic peptide complexes

Figure 2. Characteristics of OVAp and Two Variant Peptides

(A) Compares three peptides: the antigenic peptide OVAp (SIINFEKL), and two single amino acid variants, A2 (SAINFEKL), and E1 (EIINFEKL), for their ability to target lysis on EL4 by a mature transgenic CTL clone.

(B) Compares the ability of these three peptides to bind and stabilize K^b on the cell line RMA-S. The mean fluorescence intensity of staining for surface K^b is shown.

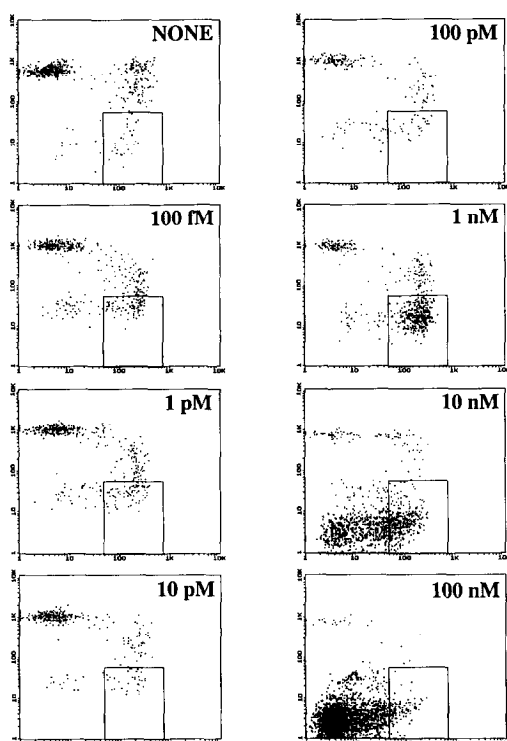## A OVAp titration (on β2M+/-)
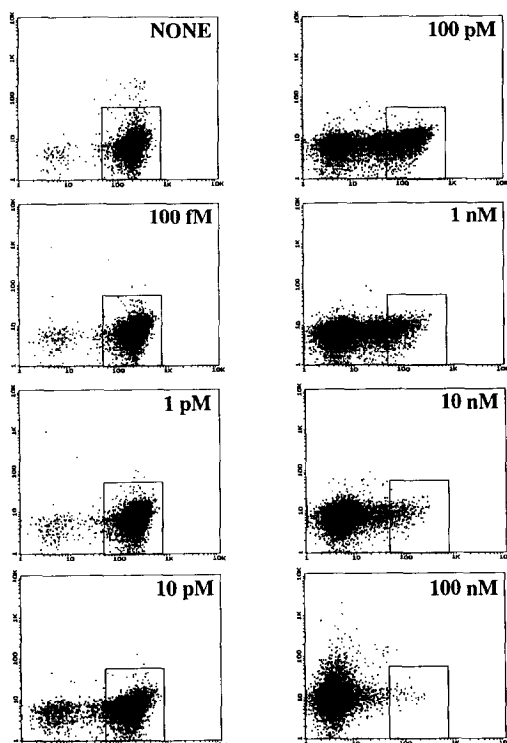
B OVAp titration (on β2M-/-)

Figure 3. The Antigenic Peptide (OVAp) Induces Negative but Not Positive Selection: CD8[b] Cells Accumulate at Intermediate Concentrations

Human β2m and various concentrations of the octamer peptide SIIN-

results in negative selection, but positive selection also requires TCR engagement of MHC–peptide ligands. One hypothesis (Lo et al., 1986) predicted that MHC–peptide complexes directing negative selection would have a high affinity for the TCR, while those directing positive selection would have a low affinity. A modification of this, which takes into account the overall avidity of the TCR–MHC interaction between two cells, predicts that high affinity ligands would cause positive selection when present at very low concentrations (low avidity) and negative selection only at high concentrations (high avidity). To test this, we looked for antigenic peptide–induced positive selection in the β2m^{-/-} FTOC lobes. As previously noted in the OVA system, the antigenic peptide did not induce positive selection (Hogquist et al., 1994). In Figure 3B, we further tested this antigenic peptide over a 7 log concentration range. At no concentration did we see development of CD8SP cells similar to that seen in β2m^{+/-} OVA-tcr-1 FTOC. The peptide did, however, induce complete negative selection (Figure 3B, 100 nM). OVAp appeared to have no effect on thymic development when used at less than 1 nM. This is in contrast with the β2m^{+/-} OVA-tcr-1 lobes, where negative selection was induced by picomolar concentrations of the peptide. This is presumably due to the markedly less efficient presentation of exogenous peptide by β2m^{-/-} cells compared with β2m^{+/-} cells (Hogquist et al., 1993).

At the 1 and 10 nM points in Figure 3B, a small increase in the percentage of CD8SP cells is seen. Does this reflect positive selection? True positive selection should result in an increased number of thymocytes in the CD8SP subset per thymic lobe. In Figure 4 (left), we show the number of CD8SP thymocytes recovered per thymic lobe. Because cellularity of the lobes is decreased with OVAp, the actual number of CD8SP is not increased, despite the increase in the percentage of CD8SPs seen in Figure 3B. The absence of positive selection at the 1 or 10 nM concentration is confirmed by the lack of biological activity developing in these lobes (see below).

A Slightly Weaker Agonist Peptide Induces Positive Selection at Low Concentration

These data are in contrast with that seen by two other groups using an LCMV-specific D[b]-restricted TCR transgenic, P14 (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). In those experiments, a very close analog of the antigenic peptide was used. This peptide differs from the virally encoded antigenic epitope by 1 aa; methionine for cysteine at the carboxyl terminus. This modification was made to prevent dimer formation of the peptide. Although the carboxyl terminus position is an anchor residue, other natural D[b] epitopes contain methionine here, so it is unlikely that this modification would result in decreased bind-

FEKL (OVAp) were added to fetal thymic organ cultures from mice that express β2m (A) and those that do not (B). At the end of the culture, thymocytes were harvested and stained for CD4, CD8, and the transgenic TCR Vα2 chain. This figure shows the expression of CD4 and CD8 on TCRα^{hi} cells. As peptide concentration increased, the total cellularity of the lobes decreased, for both β2m^{+/-} and β2m^{-/-} lobes.
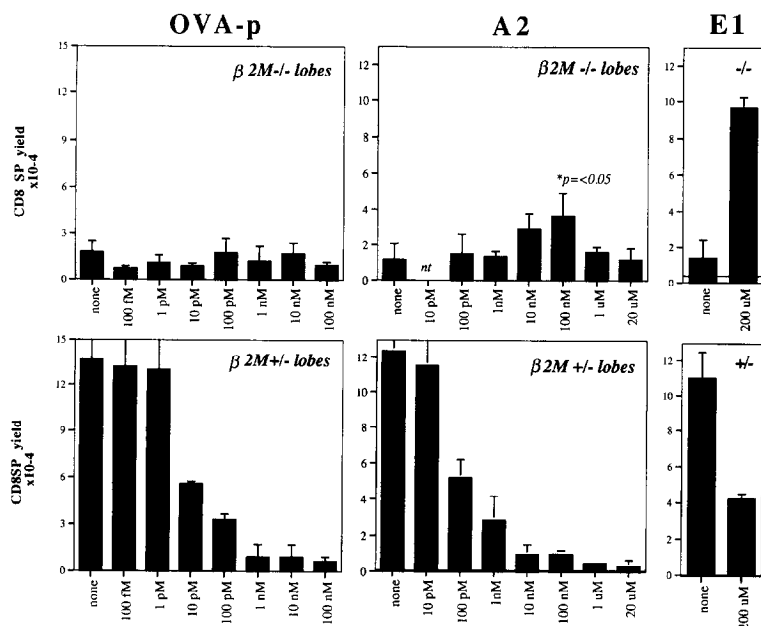
Figure 4. The Effect of Peptides on Total CD8SP Recovered from Organ Cultures

Fetal thymic organ culture was performed and cells were stained as in Figures 1 and 3. Total viable thymocyte yields from the lobes were determined. This figure shows the absolute number of CD8 single-positive TCR Vα2 high cells recovered from lobes after culture in the presence of human β_2m and various peptides. The data are the average yields of 3–17 lobes per condition. Error bars show the standard error of the mean. The difference between β_2m$^{-/-}$ lobes treated with no peptide or 100 nM A2 was statistically significant (p < 0.05).

ing to D^b. In fact, this peptide is biologically active in the low picomolar range, indicating its MHC interaction must be fairly stable, and that its ability to engage the TCR is not compromised. When present in fetal thymic organ culture, this LCMV antigenic peptide caused deletion at high concentrations, but positive selection at low concentrations. In the experiments of Ashton-Rickardt et al. (1994), the deleting dose was 300 μM, while the selecting dose was 30 μm. Sebza et al. (1994) saw deletion at 1 μM, and selection at 1 pM. This difference could be attributed to the use of TAP1$^{-/-}$ by one group and β_2m$^{-/-}$ by the other; although it is difficult to imagine that the TAP1$^{-/-}$ mice would not, in fact, be more efficient at presenting peptide antigen.

Nonetheless, it remains that the antigenic peptide induced positive selection, as judged by an increase in the actual numbers of CD8SP cells, in contrast with what was seen in the OVA-tcr-1 system. How can these results be reconciled? One possibility is that the inherent affinity of the TCR for MHC–antigenic peptide is different for the OVA-tcr-1 transgenic compared with the P14 transgenic. Specifically, if this interaction is stronger in the OVA-tcr-1 mice, it may be more difficult to identify a "window" where positive selection could be seen. To test this, we tried to induce positive selection with A2, the slightly less stimulatory variant of the antigenic peptide. This peptide was only 10-fold less efficient at stimulating peripheral cytotoxic T lymphocyte (CTL) when compared with the antigenic peptide OVAp (see Figure 2A). However, its binding to K^b was as strong as OVAp or E1 (see Figure 2B). Not surprisingly, then, it was efficient at inducing deletion in β_2m$^{+/-}$ OVA-tcr-1 thymic lobes. Figure 5A shows that deletion of thymocytes was well underway in the low nanomolar range, being complete at 200 nM. This peptide also caused deletion in the β_2m$^{-/-}$ lobes (Figure 5B), but here again deletion was not complete unless higher concentrations of the peptide

were used. In the β_2m$^{-/-}$ lobes, it was also apparent that the variant was capable of producing a more significant positive selection than the antigenic peptide. This is true both in terms of an increase in the percentage of CD8SP cells as well as the yield (see Figure 4, middle). Although the statistical significance of this difference was not profound, it should be stressed that these are the data from many individual animals. The variation seen in total cellular yield was as great in the untreated lobes as it is in the peptide-treated lobes. Where sister lobes from the same animal were compared, 100 nM A2 consistently resulted in at least a 2-fold increase in CD8SP cells over untreated cells. These data suggest that only for weaker antigen–TCR interactions, the positively selecting and negatively selecting ligands could be one and the same: positive selection being induced at low concentrations, negative selection prevailing if the antigen was expressed at high concentration.

Nonstimulatory Variants Induce Substantial Positive Selection

That efficient positive selection can be restored in this system was demonstrated previously (Hogquist et al., 1994; Jameson et al., 1994) and is shown again for comparison in Figure 6. Lobes treated with the peptide E1 demonstrate a reconstitution of the CD8SP population approaching the level seen with wild-type cells. This is seen both in the percentage (Figure 6) as well as number (see Figure 4) of transgene-bearing CD8 cells. We have identified many peptides, such as E1, that can induce positive selection. In fact, the most efficient peptides are, without exception, TCR antagonists for mature CTL with this receptor.

Antagonists are defined as ligands that, in the presence of a suboptimal concentration of the antigenic peptide, will inhibit mature T cell responses (Sette et al., 1994).
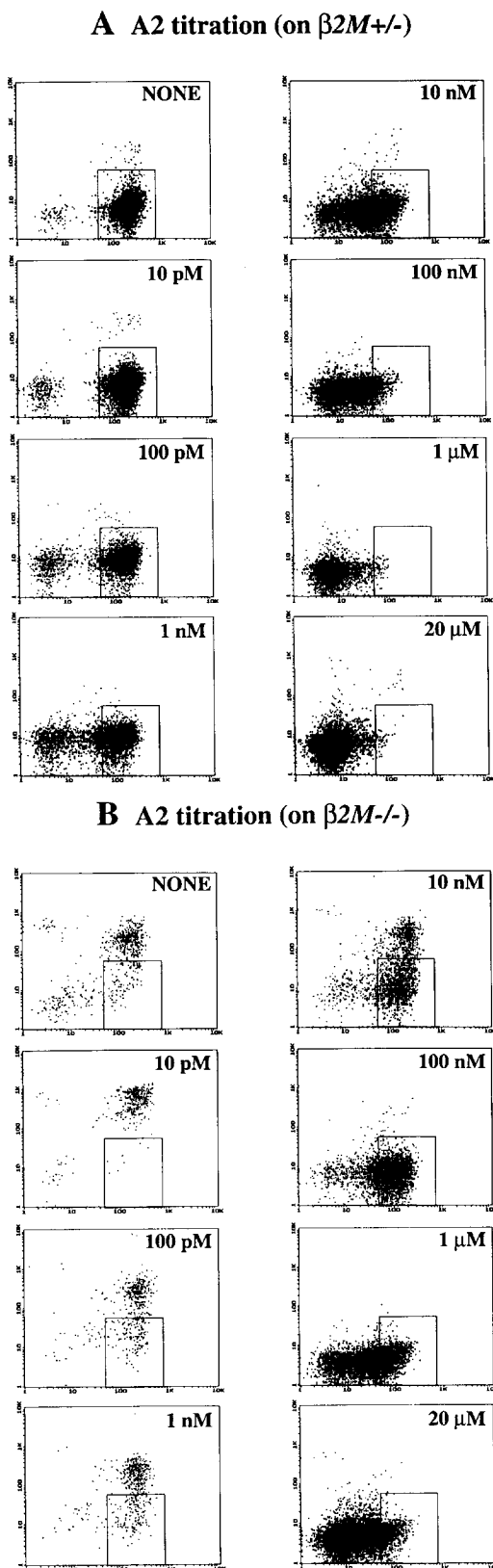
A A2 titration (on β2M+/-)

NONE | 10 nM
10 pM | 100 nM
100 pM | 1 μM
1 nM | 20 μM

B A2 titration (on β2M-/-)

NONE | 10 nM
10 pM | 100 nM
100 pM | 1 μM
1 nM | 20 μM

Figure 5. The A2 Peptide Induces Negative and Positive Selection at Different Concentrations

Human β2m and various concentrations of the octamer peptide SAIN-FEKL (A2) were added to fetal thymic organ cultures from mice that

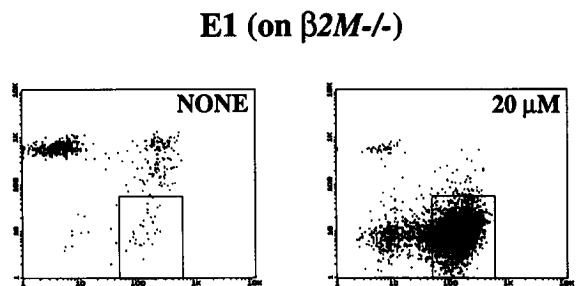## E1 (on β2M-/-)

NONE | 20 μM

Figure 6. The E1 Peptide Induces Substantial Positive Selection

Human β2m and none or 20 μM of the octamer peptide EIINFEKL (E1) were added to fetal thymic organ cultures from mice that do not express β2m. At the end of the culture, thymocytes were harvested and stained for CD4, CD8, and the transgenic TCR Vα2 chain. This figure shows the expression of CD4 and CD8 on TCRα^{hi} cells.

Although E1 does have some stimulatory activity at high concentrations, we feel this is not essential to its ability to induce positive selection, since another variant (V-OVA), which has no agonist activity, induced positive selection of CD8 T cells and these T cells were functional (Hogquist et al., 1994).

Agonist-Induced Thymocytes Are Nonfunctional

As mentioned above, the positive selection mediated by A2 was modest. The yield of CD8SP T cells was less than half that achieved by E1 (Figure 4, right) and less than a third compared with the natural ligand (see Figure 4, β2m^{+/-}, NONE). To investigate this further, we asked whether selection on A2 enhanced the biological function of thymocytes from the lobes. Figure 7 shows the combined results of four proliferation assays, where unselected thymocytes were compared with thymocytes selected on peptides or the natural ligand, for their ability to respond to the antigenic peptide. Thymocytes selected on the natural ligand (β2m^{+/-}) mount a vigorous proliferative response to the OVAp peptide plus EL4 antigen-presenting cells (APCs). Unselected thymocytes (β2m^{-/-} NONE) mount a poor response. While E1-selected cells dramatically recovered their ability to respond, A2 or OVAp selected cells did not (Figure 7). In the experiments shown, 100 nM A2 and 10 nM OVAp were included in the FTOC. In other experiments, 10 and 50 nM A2 and 100 nM OVAp were tested and also found to yield cells that did not mount a proliferative response (data not shown).

It is surprising in light of the increased yield of CD8SP cells in A2-treated lobes, that greater proliferation to the OVAp peptide does not occur in the test cultures. The absence of functional capacity may be due to the decreased levels of CD8 seen on A2-selected cells. Alterna-

express β2m (A) and those that do not (B). At the end of the culture, thymocytes were harvested and stained for CD4, CD8, and the transgenic TCR Vα2 chain. This figure shows the expression of CD4 and CD8 on TCRα^{hi} cells.
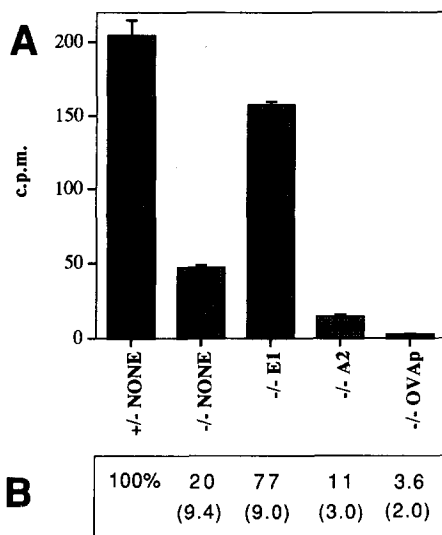
Figure 7. Agonist-Selected Cells Are Not Functional

Organ culture from $\beta_2 m^{+/-}$ or $\beta_2 m^{-/-}$ was performed in the presence of human $\beta_2 m$ and the peptides OVAp, A2, or E1. The concentration of peptide used (10 nM OVAp, 100nM A2, or 20 μM E1) were the optimal concentrations for positive selection seen in the above experiments. Thymocytes were harvested and counted. A fraction of the cells from each lobe was stained as in Figure 3. The remaining cells from an equivalent number of lobes in each group were added to cultures with EL4 APCs with or without 10 nM OVAp. (In previous experiments this was found to be the optimal concentration for a proliferative response from $\beta_2 m^{+/-}$ FTOC cells.) [³H]thymidine incorporation was determined after 48 hr. The data shown in (A) are the mean (and standard error of the mean) of triplicate cultures from one experiment. The data shown in (B) are the average (and standard error of the mean) of four separate experiments. The cpm value for proliferation from $\beta_2 m^{+/-}$ lobes was considered 100%, and the others normalized to it.

tively, the thymocytes may have been rendered unresponsive by some other means.

Discussion

T cells emerging from the thymus have three essential features: they are tolerant to self-protein antigens, they are MHC restricted, and they are capable of recognizing novel foreign protein antigens. These features are achieved during thymic development by the balanced influences of positive and negative selection, both of which are induced by TCR recognition of self-peptide–MHC ligands. Previous studies suggested that the variety of self-peptide ligands on cells that induce positive versus negative selection are nearly identical (Marrack et al., 1993). How does recognition of the same set of ligands by a random pool of receptors achieve an educated peripheral T cell pool?

The affinity hypothesis proposes that cells bearing receptors that have strong interactions with self-peptide–MHC complexes are deleted, those with receptors that have weak interactions are spared, while cells with receptors that have no interaction are left to die. This hypothesis has an inherent explanation of why positive selection should occur more often than negative selection: because

a random receptor will have more low affinity ligands than high. This is why the selective forces in the thymus yield a net positive number of cells. It also offers that receptors with low affinity for self-ligands will fortuitously have high affinity for some foreign ligands, but only in the context of MHC presentation. What has historically been called the affinity hypothesis has really been discussed by immunologists as an avidity hypothesis, where the overall avidity between a T cell and an APC is considered part of the positive/negative selection paradigm. In other words, the influencing factors are not just the affinity of the T cell receptor for MHC–peptide, but the density of the receptor or ligand, as well as contribution of cell surface molecules such as CD8 and CD4, among others (Berg et al., 1990; Lee et al., 1992; Robey et al., 1992).

In terms of the avidity model of positive selection, there is good evidence that ligands of intermediate affinity for the TCR serve as excellent selecting ligands in vitro (Hogquist et al., 1994, Ashton-Rickardt et al., 1994). Thus, some peptides that are sequence-related to the antigenic peptide, yet are not strong agonists select well. At the high affinity and low affinity ends of the spectrum, however, there is much discussion. At the extreme low affinity end of TCR–MHC/peptide interactions, Schumacher and Ploegh (1994) have argued that peptide does not contribute to binding and that the best peptides for positive selection are those that keep out of the way of the TCR–MHC interaction. In this model, empty MHC molecules (if they were stable) would be excellent ligands for positive selection, and multiple MHC–peptide "faces" would not be better than a single MHC face, i.e., there is no role for ligand heterogeneity in positive selection. The model also predicts that the relevant ligands for positive selection are a mixture of peptides unrelated to the agonist peptide that cumulatively make a high ligand density. We have not addressed this question in the current work.

The other affinity end of the avidity model for positive selection is that high affinity (agonist) ligands for the TCR expressed at extremely low ligand density can also mediate positive selection. Our current work argues against this notion. We have shown here and previously (Hogquist et al., 1994), that the antigenic peptide OVAp does not stimulate positive selection at any dose. A variant peptide, A2, which is able to stimulate mature T cell activation or immature thymocyte deletion at about a 10-fold lower level than OVAp, was able to increase the maturation of TCR^{hi} CD8$^+$ T cells in organ culture, but these cell were not functional. It seems possible that agonist induction of the appearance of CD8$^+$ T cells, as seen in FTOC experiments, does not happen in the physiological situation. One feature of agonist-selected cells is their low level of CD8 (Ashton-Rickardt et al., 1994; Jameson et al., 1994; Sebzda et al., 1994). Cells with a low level of CD8 are not found at a significant frequency in normal animals. Perhaps agonist ligands can induce positive selection in fetal thymic organ culture because the representation of APCs is limited to what was in the thymus at day 16. In a normal animal, efficient APCs from the blood would circulate through the thymus and might mediate more vigorous deletion than

seen in organ culture. In addition to the data here, the observations that low concentrations of bacterial superantigens (J. Kappler, personal communication) or endogenous superantigens (C. Janeway, personal communication) do not induce positive selection, also argue against a strict interpretation of the avidity hypothesis.

Experimental Procedures

Mice
H-2^b (129 × C57BL/6 F2) β_2m$^{-/-}$ mice, and C57BL/6, OVA-tcr-1 mice were maintained in our colony at the University of Washington (Seattle, Washington). Timed matings were set up by adding one male to a cage of three females for 16 hr. The day the males were removed was considered day 1, as opposed to day 0.

Fetal Thymic Organ Culture
Thymic lobes were excised from fetal mice at day 16 and placed on cellulose ester filters (Millipore, Bedford, Massachusetts), which sat upon gelfoam sponges (Upjohn, Kalamazoo, Michigan) in media with or without peptide (used at the concentrations indicated in the figures). At the beginning of each experiment, a 10× stock of peptide was made and frozen in aliquots. Media, inclusive of freshly thawed peptide, was replenished each 24 hr. The media consisted of RPMI plus 10% fetal calf serum, penicillin, streptomycin, L-glutamine, 2-ME, and 5 µg/ml human β_2m (Calbiochem, La Jolla, California). The peptides used in these experiments did not undergo more than 10-fold degradation during 24 hr in media containing serum, as judged by ability to stimulate CTL. After 7 days, thymocytes were released from the lobes by pressing through a steel mesh.

Peptide Synthesis
Peptides were synthesized at the Howard Hughes Chemical Synthesis Facility (University of Washington, Seattle, Washington) or using an Applied Biosystems (Foster City, California) Synergy peptide synthesizer.

Flow Cytometry
Biotinylated anti Vα2 antibody B20.1.1 was used for detection of the transgenic TCR. Tricolor streptavidin (Caltag, San Francisco, California) was used as a secondary reagent. Anti-CD4–phycoerythrin and anti CD8–fluorescein isothiocyanate (Becton Dickinson, Mountainview, California) were used directly. Cells were analyzed on a FAC-Scan (Becton Dickinson, Mountainview, California), using ReproMan software (True Facts Software, Seattle, Washington).

CTL Lysis Assay
A CTL line was established from C57BL/6 OVA-tcr-I mice. In brief, splenocytes were stimulated with 100 pM OVAp for 14 days and subsequently restimulated weekly with E.G7 (EL4 transfected with the ovalbumin gene; Moore et al., 1988) in the presence of rat concanavalin A supernatant.

For lysis assays, EL4 cells were labeled with ^{51}Cr-sodium chromate in RP10 for 1 hr at 37°C. After washing, test peptides were added at various concentrations together with 3 × 10^4 CTL per well. After 4 hr, the plate was centrifuged and 100 µl of the supernatant removed and counted.

Proliferation Assays
Cells from thymic lobes after 7 days of in vitro culture were washed three times and added to a 96-well plate at .5 lobe equivalents per well with 3 × 10^4 irradiated EL4 APCs, and with or without 10 nM OVAp peptide. After 48 hr at 37°C, 0.5 µCi per well of [^{3}H]thymidine (New England Nuclear Research Products, Boston, Massachusetts) was added. The cells were harvested 6 hr later and the incorporated radioactivity measured.

RMA-S Stabilization Assay
To detect binding of peptides to K^b, we used the RMA-S stabilization assay (Schumacher et al., 1990). In brief, RMA-S cells were incubated at 31°C overnight to provide maximum MHC expression at the cell surface. Peptide was added at various concentrations for 30 min at 31°C. Cultures were shifted to 37°C for 4 hr. Cells were then washed and stained for K^b expression using the Y3 antibody (American Type Culture Collection, Rockville, Maryland), followed by fluorescein isothiocyanate goat anti-mouse immunoglobulin G (Cappel, Malvern, Pennsylvania).

References

Ashton-Rickardt, P. G., Bandeira, A., Delaney, J. R., Van Kaer, L., Pircher, H.-P., Zinkernagel, R. M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell 76, 651–663.

Auphan, N., J'ezo Br'emond, A., Schonrich, G., Hammerling, G., Arnold, B., Malissen, B., and Schmitt Verhulst, A. M. (1992). Threshold tolerance in H-2K^b-specific TCR transgenic mice expressing mutant H-2K^b: conversion of helper-independent to helper-dependent CTL. Int. Immunol. 4, 1419–1428.

Berg, L. J., Frank, G. D., and Davis, M. M. (1990) The effects of MHC gene dosage and allelic variation on T cell receptor selection. Cell 60, 1043–1053.

Blok, R., Margulies, D. H., Pease, L., Ribaudo, R. K., Schneck, J., and McCluskey, J. (1992). CD8 expression alters the fine specificity of an alloreactive MHC class I-specific T hybridoma. Int. Immunol. 4, 455–466.

Hogquist, K. A., Gavin, M. A., and Bevan, M. J. (1993). Positive selection of CD8$^+$ T cells induced by major histocompatibility complex binding peptides in fetal thymic organ culture. J. Exp. Med. 177, 1469–1473.

Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J., and Carbone, F. R. (1994). T cell receptor antagonist peptides induce positive selection. Cell 76, 17–27.

Iwabuchi, K., Nakayama, K., McCoy, R. L., Wang, F., Nishimura, T., Habu, S., Murphy, K. M., and Loh, D. Y. (1992). Cellular and peptide requirements for in vitro clonal deletion of immature thymocytes. Proc. Natl. Acad. Sci. USA 89, 9000–9004.

Jameson, S. C., Carbone, F. R., and Bevan, M. J. (1993). Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. J. Exp. Med. 177, 1541–1550.

Jameson, S. C., Hogquist, K. A., and Bevan, M. J. (1994). Specificity and flexibility in thymic selection. Nature 369, 750–752.

Janeway, C. A. (1993). High fives or hand clasps? Curr. Biol. 2, 591–593.

Lee, N. A., Loh, D. Y., and Lacy, E. (1992). CD8 surface levels alter the fate of alpha/beta T cell receptor–expressing thymocytes in transgenic mice. J. Exp. Med. 175, 1013–1025.

Lo, D., Ron, Y., and Sprent, J. (1986). Induction of MHC-restricted specificity and tolerance in the thymus. Immunol. Res. 5, 221–232.

Marrack, P., Ignatowicz, L., Kappler, J. W., Boymel, J., and Freed, J. H. (1993). Comparison of peptides bound to spleen and thymus class II. J. Exp. Med. 178, 2173–2183.

Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988). Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell 54, 777–785.

Murphy, K. M., Heimberger, A. B., and Loh, D. Y. (1990). Induction by antigen of intrathymic apoptosis of CD4$^+$CD8$^+$TCR^{lo} thymocytes in vivo. Science 250, 1720–1723.

Pircher, H., Brduscha, K., Steinhoff, U., Kasai, M., Mizuochi, T., Zinkernagel, R. M., Hengartner, H., Kyewski, B., and Muller, K. P. (1993). Tolerance induction by clonal deletion of CD4$^+$8$^+$ thymocytes in vitro

does not require dedicated antigen-presenting cells. Eur. J. Immunol. 23, 669–674.

Robey, E. A., Ramsdell, F., Kioussis, D., Sha, W., Loh, D., Axel, R., and Fowlkes, B. J. (1992). The level of CD8 expression can determine the outcome of thymic selection. Cell 69, 1089–1096.

Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., and Rammensee, H. G. (1991). Exact prediction of a natural T cell epitope. Eur. J. Immunol. 21, 2891–2894.

Schumacher, T. N. M., and Ploegh, H. L. (1994). Are MHC-bound peptides a nuisance for positive selection? Immunity 1, 721–723.

Schumacher, T. N., Heemels, M. T., Neefjes, J. J., Kast, W. M., Melief, C. J., and Ploegh, H. L. (1990). Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. Cell 62, 563–567.

Sebzda, E., Wallace, V. A., Mayer, J., Yeung, R. S. M., Mak, T. W., and Ohashi, P. S. (1994). Positive and negative thymocyte selection induced by different concentration of a single peptide. Science 263, 1615–1618.

Sette, A., Alexander, J., Ruppert, J., Snoke, K., Franco, A., Ishioka, G., and Grey, H. M. (1994). Antigen analogs/MHC complexes as specific T cell receptor antagonists. Annu. Rev. Immunol. 12, 413–432.

Spain, L. M., and Berg, L. J. (1992). Developmental regulation of thymocyte susceptibility to deletion by "self"-peptide. J. Exp. Med. 176, 213–223.

Swat, W., Ignatowicz, L., von Boehmer, H., and Kisielow, P. (1991). Clonal deletion of immature CD4+8+ thymocytes in suspension culture by extrathymic antigen-presenting cells. Nature 351, 150–153.

Vasquez, N. J., Kaye, J., and Hedrick, S. M. (1992). In vivo and in vitro clonal deletion of double-positive thymocytes. J. Exp. Med. 175, 1307–1316.

Wiest, D. L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R. D., Glimcher, L. H., Samelson, L. E., and Singer, A. (1993). Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56[lck] tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J. Exp. Med. 178, 1701–1712.