

Evidence for a Differential Avidity Model of T Cell Selection in the Thymus

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Summary

Positive and negative selection of a lymphocytic choriomeningitis virus (LCMV) peptide-specific, H-2D^b-restricted T cell clone (P14) was studied using TAP1⁻ and TAP1⁺ mice transgenic for P14 T cell receptor (TCR) α and β genes. Positive selection of transgenic CD8⁺ P14 cells was impaired in TAP1⁻ mice. Addition of the LCMV peptide to TAP1⁻ fetal thymic organ cultures (FTOCs) at low and high concentrations induced positive and negative selection of CD8⁺ P14 cells, respectively, while addition of the same peptide to TAP1⁺ FTOCs induced negative selection even at low concentrations. Both types of selection were peptide specific. Thus, a critical parameter that controls the fate of a thymocyte seems to be the number of TCRs engaged with complexes of peptide and major histocompatibility complex. When this number is low, positive selection occurs, and when it is high, negative selection takes place. These findings support a differential avidity model of T cell selection.

Introduction

The repertoire of antigen specificities of T cells of an individual animal is shaped by two types of cellular selection that take place in the thymus. Positive selection (Bevan, 1977; Zinkernagel et al., 1978) ensures that T cells leaving the thymus are capable of major histocompatibility complex (MHC)-restricted recognition of antigen; that is, they possess the capacity to recognize antigen presented by products of self-MHC (Zinkernagel and Doherty, 1974; Fink and Bevan, 1978). Positive selection is thought to involve engagement of T cell receptors (TCRs) on immature CD3⁺ low CD4⁺CD8⁺ thymocytes with self-MHC products expressed on the surface of thymic epithelial cells (Lo and Sprent, 1986): only those thymocytes expressing

TCRs with sufficient affinity for self-MHC are thought to be given the signal to avoid programmed cell death and to mature (Kisielow et al., 1988; Sha et al., 1988). Engagement of TCRs with MHC class I molecules leads to the differentiation into CD4⁺CD8⁺ (hereafter referred to as CD8⁺) cytotoxic T cells, whereas engagement of TCRs with MHC class II molecules leads to differentiation into CD4⁺CD8⁻ (hereafter referred to as CD4⁺) T helper cells (Teh et al., 1988). Negative selection eliminates through clonal deletion those T cells that are potentially autoreactive (for reviews see Schwartz, 1989; Hugo et al., 1993). Negative selection is also thought to involve engagement of TCRs on immature thymocytes; however, in this process self-peptide-MHC complexes expressed on the surface of bone marrow-derived thymic stromal cells are thought to be recognized (macrophages and dendritic cells). Those thymocytes expressing TCRs with relatively high affinity for the self-peptide-MHC complexes are thought to be given the signal to undergo programmed cell death. One of the central questions that remains to be solved is how the similar interactions that take place between TCRs and self-MHC or self-peptide-MHC complexes trigger two very different cell fates during the two types of selection.

It is well established that in MHC-restricted recognition of antigen, a TCR interacts not only with an MHC product but also with an antigen-derived peptide entrenched in the groove of the MHC product (Ziegler and Unanue, 1981; Townsend et al., 1986; Bjorkman et al., 1987). Since a peptide is essential for the stable expression of an MHC class I molecule on the surface of cells (Townsend et al., 1989, 1990; Schumacher et al., 1990; Ljunggren et al., 1990), it is expected that the self-MHC class I molecules expressed on thymic epithelial cells and recognized by TCRs during positive selection also contain peptides. Indeed, the analyses of CD8⁺ T cell development in mice expressing mutant class I molecules with altered peptide-binding grooves suggested a role of peptides in determining the specificity of TCR-MHC interaction during the positive selection of CD8⁺ T cell precursors (Nikolić-Zugčić and Bevan, 1990; Sha et al., 1990; Jacobs et al., 1990; Ohashi et al., 1993).

To address this issue more directly, we exploited TAP1 (for transporter associated with antigen processing 1) mutant mice (Ashton-Rickardt et al., 1993) that were produced using embryonic stem cell technology (Cappechi, 1989; Van Kaer et al., 1992). The TAP1 gene encodes an ATP-dependent peptide pump (Shepherd et al., 1993), which translocates peptides from the cytosol into the lumen of the endoplasmic reticulum. Peptides then associate with newly synthesized class I heavy chain and β 2-microglobulin to form stable class I molecules that are then transported to the cell surface (Townsend et al., 1989, 1990; Nuchtern et al., 1989; Schumacher et al., 1990). In TAP1 mutant mice, this major route of peptide loading of class I molecules is blocked. Consequently, surface

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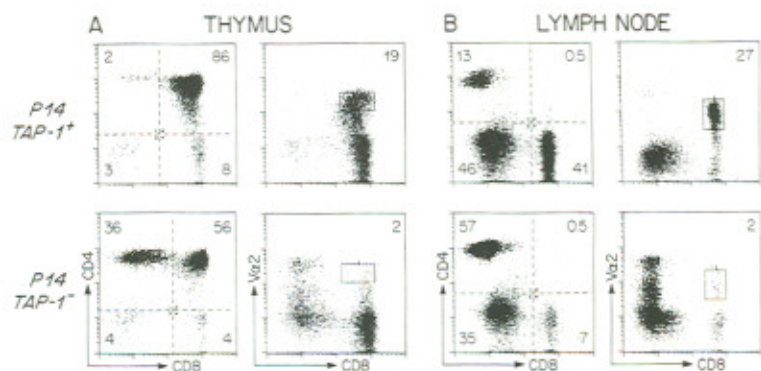


Figure 1. Positive Selection of CD8⁺ Transgenic P14 Cells Is Impaired in TAP1⁻ Mice

Thymocytes and mesenteric lymph node cells (1×10^5) were prepared from P14-positive TAP1⁺ or TAP1⁻ mice and stained (1.5×10^4 cells) with anti-CD8-FITC and anti-CD4-PE MAbs or with anti-Va2-PE and anti-CD8-FITC MAbs and then analyzed by FACS. The percentages of cells in each quadrant or window are indicated.

expression of MHC class I molecules is reduced, and positive selection of CD8⁺ T cells is severely hampered. Only a few "empty," ill-folded, and heat-sensitive class I molecules appear on the surface of cells in TAP1 mutant mice (Van Kaer et al., 1992). These empty class I molecules could be loaded and stabilized with peptides that were provided extracellularly in the presence of β 2-microglobulin. Thus, addition of peptides to cultured fetal thymi from TAP1 mutant mice rescued class I expression on thymic stromal cells, but only some of these peptides promoted the positive selection of CD8⁺ T cell precursors (Ashton-Rickardt et al., 1993). Furthermore, as observed in a fetal thymus organ culture (FTOC) system (Hogquist et al., 1993) from β 2-microglobulin-deficient mice (Zijlstra et al., 1990), complex mixtures of class I-binding peptides (synthetic or thymus derived) were more efficient than single peptides in promoting the positive selection of CD8⁺ T cells (Ashton-Rickardt et al., 1993). These data confirmed that peptides contribute to the specificity of positive selection of CD8⁺ T cells in the thymus.

In the present study, we crossed TAP1 mutant mice (Van Kaer et al., 1992) with a TCR α and β transgenic mouse (P14) to study the role of peptides in thymic selection in greater detail. The P14 TCR recognizes a peptide from the lymphocytic choriomeningitis virus (LCMV) glycoprotein (amino acids 33–41) presented by H-2D^b molecules (Pircher et al., 1989, 1990). As expected, positive selection of CD8⁺ T cells expressing the P14 TCR was hampered in mice deficient in TAP1. Analyses of cultured fetal thymi from P14 TAP1⁻ transgenic and P14 TAP1⁺ transgenic mice that were supplemented by various H-2D^b-binding peptides allowed us to draw two major conclusions. First, peptides determine the specificity of TCR-MHC interaction during positive selection, most probably by being directly recognized by TCRs. Second, the critical parameter that controls the fate of thymocytes is the number of TCRs engaged with peptide-MHC complexes that is determined by the avidity between these two cell surface components; regardless of the intrinsic affinity of the TCR-peptide/MHC interaction, when the number of engaged TCRs is moderate, positive selection takes place, and when the number is high, negative selection occurs.

Results

Positive Selection of P14 Transgenic T Cells Is Impaired in TAP1-Deficient Mice

We have shown previously that positive selection of CD8⁺ T cells, but not CD4⁺ T cells, is severely hampered in the thymus of TAP1 mutant mice (Van Kaer et al., 1992; Ashton-Rickardt et al., 1993). Transgenic mice (P14) expressing a TCR (Va2, V β 8.1) specific for the LCMV glycoprotein peptide (33–41) (hereafter referred to as LCMV peptide or nominal antigen peptide) presented by H-2D^b (Pircher et al., 1989, 1990) were crossed to TAP1-deficient mice. We analyzed thymocytes and lymph node cells derived from the P14 TAP1⁻ mice as well as the cells from the control P14 TAP1⁺ mice by flow cytometry. Typical results are shown in Figure 1. The thymi of P14 TAP1⁻ mice contained as many thymocytes as those of P14 TAP1⁺ mice. However, the percentage of CD8⁺ cells was reduced in P14 TAP1⁻ mice ($n = 4$; range, 3%–4%) compared with P14 TAP1⁺ mice (range, 7%–10%). In contrast, the percentage of CD4⁺ cells was augmented in P14 TAP1⁻ mice (range, 30%–40%) compared with P14 TAP1⁺ (range, 2%–4%). Most of the few remaining CD8⁺ thymocytes in the P14 TAP1⁻ mice did not express the P14 TCR as indicated by the low percentage of Va2⁺ CD8⁺ thymocytes for P14 TAP1⁻ mice (range, 2%–4%) compared with P14 TAP1⁺ mice (range, 19%–23%). Similar results were obtained with peripheral T cells. The mesenteric lymph nodes of P14 TAP1⁻ mice also contained as many cells as those of P14 TAP1⁺ mice. However, a substantial reduction in the percentage of CD8⁺ cells was observed in P14 TAP1⁻ mice (range, 5%–8%) compared with P14 TAP1⁺ control mice (range, 40%–45%). Here again, the percentage of CD4⁺ cells was increased in P14 TAP1⁻ mice (range, 55%–46%) compared with P14 TAP1⁺ mice (range, 11%–14%). Most of the remaining CD8⁺ cells in the P14 TAP1⁻ mice did not express the P14 TCR, as indicated by the relatively low percentage of Va2⁺ CD8⁺ cells (range, 2%–3%) compared with P14 TAP1⁺ control mice (range, 26%–29%). These data indicate that the development of CD8⁺ transgenic P14 cells is severely impaired in the TAP1⁻ background. Most of the CD4⁺ thymocytes or lymph node T cells ex-

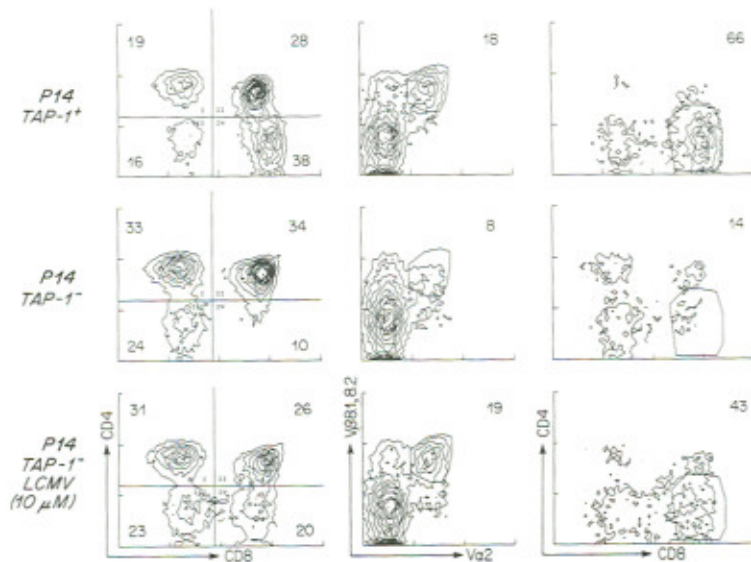


Figure 2. Positive Selection of CD8⁺ Transgenic P14 Cells Is Impaired in TAP1⁻ FTOC and Is Restored by the Nominal Antigen Peptide

P14-positive TAP1⁺ or TAP1⁻ thymic lobes were cultured with or without peptide as indicated for 9 days in RPMI, 10% fetal calf serum, and then thymocyte suspensions were prepared by mechanical disaggregation (typically 2×10^5 cells per lobe). Cells (4×10^4 , unless otherwise indicated) were stained with anti-CD8-FITC, anti-CD4-allophycocyanin (APC), anti-TCR V α 2-PE, and anti-TCR V β 8.1,8.2-biotin-streptavidin-Texas red MAbs and then analyzed by FACS. The percentages of cells falling into the quadrants resulting from the analysis of CD4 and CD8 staining are indicated. Stained cells were gated for high expression of V α 2 and V β 8.1,8.2 (P14) (percentage shown next to window) and then displayed for log fluorescence intensity resulting from staining for CD4 and CD8. The percentage of this gated population positive for CD8 and negative for CD4 is shown next to the window. The overall percentage of cells determined as V α 2⁺ high V β 8.1,8.2⁺ high (CD8⁺ P14) was calculated by multiplying the percentage of cells V α 2⁺ high V β 8.1,8.2⁺ high by the percentage of CD8⁺ cells of the gated population.

press non-P14 (i.e., nontransgenic) TCR (data not shown). The proportional increase of CD4⁺ cells in P14 TAP1⁻ mice compared with P14 TAP1⁺ mice can be explained by preferential selection of these cells by class II MHC molecules whose cell surface expression is not affected by the lack of TAP1.

When we cultured day 16 fetal thymic lobes from P14 TAP1⁻ mice (hereafter referred to as TAP1⁻ FTOC) or P14 TAP1⁺ mice (TAP1⁺ FTOC) for 9 days, we also observed a reduction in numbers of CD8⁺ P14 thymocytes recovered from TAP1⁻ thymi compared with similarly cultured TAP1⁺ thymi. Figure 2 shows a representative result of multiple ($n = 8$) experiments. We determined the proportion of CD8⁺ P14 cells by multiplying the percentage of V α 2⁺ high V β 8.1,8.2⁺ high cells (which are virtually equivalent to P14 cells) among the total recovered thymocytes with the percentage of CD8⁺ cells among the P14 cells. Thus, for the experiment shown in Figure 2, in TAP1⁺ FTOC the percentage of CD8⁺ P14 cells was 12% (V α 2⁺ high V β 8.1,8.2⁺ high [18%] \times CD8⁺ [66%]), and in TAP1⁻ FTOC, the percentage was 1% (V α 2⁺ high V β 8.1,8.2⁺ high [8%] \times CD8⁺ [14%]). In all experiments performed, the percentage of CD8⁺ P14 cells ranged from 1%–2% in TAP1⁻ FTOC to 12%–19% in TAP1⁺ FTOC. Using these percentages and the total numbers of thymocytes recovered from FTOCs, we calculated the absolute numbers of CD8⁺ P14 cells per thymic lobe. Thus, in the representative experiment shown in Figure 2, we observed a deficit in positive selection of these cells in TAP1⁻ FTOC (5×10^3 CD8⁺ P14 cells per thymic lobe) compared with TAP1⁺ FTOC (37×10^3 CD8⁺ P14 cells per thymic lobe). In all experiments performed, the number of CD8⁺ P14 cells per thymic lobe ranged from

5×10^3 to 7×10^3 in TAP1⁻ FTOC and 35×10^3 to 45×10^3 in TAP1⁺ FTOC ($n = 8$).

Positive Selection of CD8⁺ Transgenic P14 T Cells Is Induced by Addition of Nominal Antigen Peptide

To understand the role of peptides in T cell development in greater detail, we examined the selection of CD8⁺ transgenic P14 cells in TAP1⁻ FTOC in the presence of various peptides. To this end we first examined the ability of the peptides to stabilize H-2D^b expression on the surface of TAP2-deficient RMA-S cells (Ljunggren and Karre, 1985; Attaya et al., 1992). The data in Table 1 indicate that LCMV peptide stabilized H-2D^b on the surface these cells about 300 times less efficiently than the H-2D^b-binding (Townsend et al., 1986) influenza virus 1934 nucleoprotein peptide (366–374) (hereafter referred to as IF peptide). To estimate relative affinity of the two peptides for the P14 TCR, we examined the proliferation of P14 spleen cells in vitro in response to the LCMV and IF peptides. The IF peptide was at least 10^3 times less efficient than the LCMV peptide in inducing the proliferation of P14 spleen cells in vitro (Table 1). A third peptide, DB-S, retains asparagine and methionine at positions 5 and 9, respectively, which are thought to be required for binding with H-2D^b (Falk et al., 1991), but contains serine at all other positions. This peptide stabilized H-2D^b as efficiently as the LCMV peptide but failed to induce the proliferation of P14 spleen cells in vitro (Table 1).

The LCMV peptide supported the development of CD8⁺ P14 precursor cells when added to TAP1⁻ FTOC at concentrations as low as $10 \mu\text{M}$, whereas at similar concentrations neither the IF nor the DB-S peptide was effective

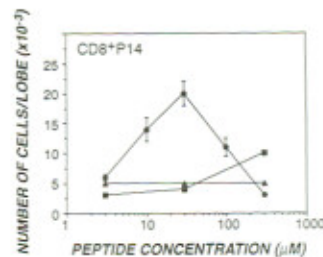
Table 1. Relative Abilities of Peptides to Stabilize Surface H-2D^b and to Stimulate P14 Spleen Cells In Vitro

Peptide	Sequence	50 _{max} H-2D ^b (M)	50 _{max} P14 (M)
LCMV	KAVYNFATM	2×10^{-4}	6×10^{-10}
LCMV-8.1	KAMYNFATM	3×10^{-4}	2×10^{-7}
LCMV-8.7	KALYNFATM	6×10^{-5}	-1×10^{-4}
LCMV-M	KAVYNFATM	2×10^{-6}	2×10^{-7}
	<u>RGIF YGS</u>		
LCMV'	RGIFNYGSM	2×10^{-4}	$>1 \times 10^{-4}$
IF	ASNNMETM	6×10^{-7}	$>1 \times 10^{-4}$
DB-S	SSSSNSSSM	6×10^{-5}	$>1 \times 10^{-4}$

Compared with the LCMV peptide (Pircher et al., 1991), the LCMV-8.1 and LCMV-8.7 variants have single amino acid changes at position 3 (underlined). The LCMV' peptide has every amino acid substituted with the most chemically related residue except for the H-2D^b anchor residues at positions 5 and 9 (Falk et al., 1991). The LCMV-M mix of peptides (128 peptides) was synthesized by incorporating the relevant amino acid from either the LCMV or LCMV' peptide at each position. The resulting mixture thus includes a spectrum of peptides differing in relatedness to LCMV or LCMV'. Both the IF (Towsend et al., 1986) and DB-S peptides differ from the LCMV peptide at every position except the H-2D^b anchor residues. The relative abilities of peptides to stabilize H-2D^b molecules is expressed as the 50_{max} H-2D^b value. This is defined as the concentration of peptide required to rescue the surface expression of H-2D^b on RMA-S to a level that is 50% of maximum (maximum being about 35% of RMA) in an overnight in vitro experiment. Surface expression of H-2D^b was detected by staining with an anti-H-2D^b-biotin MAb (B22.249.R1, $\alpha 1$ specific) and then with streptavidin-PE and by FACS analysis. The relative ability of peptides to stimulate P14 spleen cells in vitro is expressed as the 50_{max} P14 value. This is defined as the concentration of peptide required to induce the proliferation of P14 TAP1⁺ spleen cells to a level of 50% of the maximum value observed using the nominal antigen peptide. Proliferation was assessed by culturing P14 spleen cells (2×10^5) with peptide for 36 hr and then measuring the rate of cell division by pulsing with [³H]thymidine for 12 hr and determining the amount of cell-associated label by counting the rate of β particle emission.

(Figures 2 and 3). The LCMV peptide-mediated selection increased when we increased the concentration of the peptide, reaching a maximum level of selection at 30 μ M. However, above this concentration, selection decreased with increasing peptide concentrations. When added at a high concentration (300 μ M), the IF peptide also induced a moderate level of positive selection of CD8⁺ P14 cells, whereas the DB-S peptide did not (Figure 3).

We attempted to determine the relative level of H-2D^b expression induced by peptides on the surface of thymic stromal cells in P14 TAP1⁺ FTOC (see Experimental Procedures). The IF peptide, which is a relatively potent H-2D^b stabilizer (Table 1), induced H-2D^b expression on the surface of I-A^b-positive thymic stromal cells at levels that were 5% (30 μ M added IF peptide) and 35% (300 μ M added IF peptide) of the level observed on the same type of cells from P14 TAP1⁺ thymi. However, the LCMV peptide is a less potent H-2D^b stabilizer (Table 1) and at 30 or 300 μ M did not induce the H-2D^b expression above the background level on the surface of thymic stromal cells. These data indicate that the positive selection by the LCMV peptide of CD8⁺ P14 cells in TAP1⁺ FTOC requires a very low density of peptide-H-2D^b complexes on the surface of thymic stromal cells, whereas the selection by the IF

Figure 3. CD8⁺ Transgenic P14 Cells Generated in TAP1⁺ FTOC in the Presence of Various H-2D^b-Binding Peptides

P14 TAP1⁺ or TAP1⁻ thymic lobes were cultured as described in the legend to Figure 2 with or without peptide at the concentrations shown. Thymocyte suspensions were prepared from FTOC treated with LCMV (closed circles), IF (closed squares), or DB-S (closed triangles) and analyzed for surface markers. The percentage of CD8⁺ P14 cells was determined as described in the legend to Figure 2. The levels for the number of CD8⁺ P14 cells per lobe is the mean (\pm SEM) of values determined from the analysis of between three and seven independently cultured thymic lobes (points without error bars had an SEM of $<0.25 \times 10^3$). TAP1⁺ FTOC gave $38 \pm 5 \times 10^3$ CD8⁺ P14 cells per lobe and TAP1⁻ FTOC without peptide gave $5 \pm 1 \times 10^3$. Similar data was obtained from two other experiments performed at different times.

peptide requires a very high density of peptide-H-2D^b complexes.

We wanted to examine the possibility that the majority of CD8⁺ P14 thymocytes that appeared in TAP1⁻ FTOC supplemented with the LCMV peptide arose by proliferation of a small number of preexisting CD8⁺ P14 cells rather than by differentiation of CD4⁺CD8⁺ cells. To this end, we sorted CD8⁺ P14 cells from TAP1⁻ FTOC treated with 10 μ M LCMV peptide and, as a control, from TAP1⁺ FTOC. We then estimated the size of the sorted cells by determining the mean forward light scattering value of cells (after fluorescence-activated cell sorting [FACS] analysis) as well as the percentage of cells in S phase. For comparison we also analyzed the cell cycle status and size of CD8⁺ P14 spleen cells that had been stimulated in vitro with the LCMV peptide. The data shown in Table 2 (experiment 1) indicate that the CD8⁺ P14 cell population recovered from TAP1⁻ FTOC supplemented by 10 μ M LCMV peptide is similar to that recovered from TAP1⁺ FTOC without added peptide in both cell size and percentage of dividing cells. In contrast, CD8⁺ P14 cells recovered from a proliferating spleen cell culture exhibited a larger average cell size as well as a higher percentage of dividing cells. The data from Table 2 (experiment 2) show that at day 7 and day 8 in P14 TAP1⁻ FTOC, the proportion of CD8⁺ P14 cells in the S phase of the cell cycle is 20% and 11%, respectively. On the other hand, 51% of CD8⁺ P14 spleen cells proliferating in response to the LCMV antigen are in S phase. Assuming that the proliferating cells in FTOC are equivalent to those in the spleen cell culture, we calculate that about 40% and 22% of the CD8⁺ P14 cells are in the cell cycle at day 7 and day 8, respectively, in P14 TAP1⁻ FTOC. We therefore conclude that the majority of the CD8⁺ P14 cells that appeared in TAP1⁻ FTOC upon an addition of the LCMV peptide arose by

Table 2. CD8⁺ P14 T Cells Selected by LCMV Peptide in TAP1⁻ FTOC Are Not Proliferating

Experiment	Concentration of LCMV Peptide (μM)	Duration of FTOC (Days)	Mean Forward Scatter	S Phase (%)
Experiment 1				
P14 TAP1 ⁻ FTOC	—	9	373 ± 24	16 ± 6
P14 TAP1 ⁻ FTOC	10	9	377 ± 24	13 ± 6
P14 TAP1 ⁻ spleen cell culture	10	NA	525 ± 17	37 ± 5
Experiment 2				
P14 TAP1 ⁻ FTOC	30	7	ND	20 ± 4
P14 TAP1 ⁻ FTOC	30	8	ND	11 ± 4
P14 TAP1 ⁻ spleen cell culture	10	NA	ND	51 ± 4

In experiment 1, P14 TAP1⁻ (n = 56) or TAP1⁻ (n = 49) thymic lobes were cultured with 10 μM LCMV peptide for 9 days as described previously (Ashton-Rickardt et al., 1993), and then thymocyte suspensions were prepared. The suspensions were pooled before staining for cell surface markers and analyzed by FACS. Cells determined as Vα2⁺ high Vβ8.1,8.2⁺ high CD8⁺ (CD8⁺ P14) were sterilely sorted by FACS (TAP1⁻ FTOC, 9 × 10⁶ cells; TAP1⁻ FTOC with LCMV 10 μM, 1.8 × 10⁶ cells). We then estimated the size of the sorted cells by determining the mean value (± variance of histogram) of the forward light scatter from a histogram of forward scatter versus cell number. These cells were then permeabilized and chromosomal DNA stained overnight with PI. Quantitation of PI staining intensity was performed by FACS analysis. Statistical analysis (Modfit program) of the histogram of PI staining intensity versus cell number then allowed the determination of the percentage of cells in G0/G1 (n PI staining intensity), G2/M (2n PI staining intensity), and S phases of the cell cycle (intermediate staining intensity). P14-positive TAP1⁻ spleen cell suspensions were prepared and incubated for 36 hr (1 × 10⁶/ml) with the LCMV peptide at 10 μM. The sorted CD8⁺ P14 cells (4 × 10³) were then stained with PI, and the percentage of cells in the S phase of the cell cycle and the mean forward light scatter of the sorted population were determined as above. In experiment 2, P14 TAP1⁻ thymi were cultured for 7 or 8 days with or without 30 μM LCMV peptide. As in experiment 1, at the end of culture, thymocyte suspensions were prepared and CD8⁺ P14 cells purified by appropriate staining with MAbs and FACS sorting. In this experiment the number of CD8⁺ P14 cells per P14 TAP1⁻ lobe treated with 30 μM LCMV peptide after 7 days of FTOC was 7 ± 2 × 10³ and after 8 days was 30 ± 4 × 10³. P14 TAP1⁻ lobes incubated without peptide gave 4 ± 0.7 × 10³ after 7 days and 7 ± 2 × 10³ after 8 days of FTOC. The level of CD8⁺ P14 cells per thymic lobes is the mean (± SEM) of values determined from the analysis of between four and eight separately cultured lobes. Sorted CD8⁺ P14 cells (P14 TAP1⁻ FTOC with 30 μM LCMV peptide on day 7, 1 × 10⁴ cells; on day 8, 8 × 10⁴ cells) were stained with PI, and the percentage of cells in the S phase of the cell cycle was determined as in experiment 1. Proliferating P14 TAP1⁻ spleen cells were prepared, and CD8⁺ P14 cells were sorted (1 × 10⁴ cells) and stained with PI as described in experiment 1. NA, data not applicable; ND, data not determined.

differentiation of CD4⁺CD8⁺ cells rather than by the proliferation of a small population of preexisting CD8⁺ P14 cells.

Analogues of the Nominal Antigen Peptide Induce the Positive Selection of CD8⁺ Transgenic P14 T Cells in TAP1⁻ FTOC

From the data described in the previous section, it would appear that positive selection of CD8⁺ P14 cells is peptide specific in that at input concentrations of between 10 and 100 μM, LCMV peptide is effective in inducing the positive selection while neither the IF nor the DB-S peptide is. To examine the peptide specificity of CD8⁺ P14 cell selection in greater detail, we tested the ability of several LCMV-related peptides to drive the positive selection of CD8⁺ P14 cells in TAP1⁻ FTOC. Two naturally occurring LCMV peptide variants, LCMV-8.7 and LCMV-8.1 (Pircher et al., 1991), both stabilized H-2D^b on the surface of RMA-S cells to similar extents (more precisely, LCMV-8.1 is 1.5-fold better than LCMV, and LCMV-8.7 is 3-fold better than LCMV), but only LCMV-8.1 could induce the proliferation of CD8⁺ P14 spleen cells in vitro to a detectable (albeit low) level. These peptides differ from the LCMV peptide by a single amino acid replacement at position 3 (see Table 1). We also tested a synthetic peptide, LCMV', which still has the anchor residues necessary to bind to H-2D^b (Falk et al., 1991) but is replaced at every other position with the residue that is most chemically related to the corresponding residue of the LCMV peptide. The LCMV' peptide stabilized H-2D^b as well as the LCMV peptide but failed to induce the proliferation of CD8⁺ P14 spleen cells in

vitro (see Table 1). We also synthesized a mixture of 128 peptides, called LCMV-M, by incorporating during the synthesis either the LCMV or LCMV' amino acid residue at each position. This resulted in a mixture including a spectrum of peptides differing in relatedness to LCMV or LCMV'. The LCMV-M peptides stabilized surface H-2D^b on RMA-S cells 100-fold more efficiently than the LCMV peptide and could induce the proliferation of CD8⁺ P14 spleen cells in vitro to a level distinctly lower than the level attained by the LCMV peptide but similar to the level attained by the LCMV-8.1 peptide. When added at 30 μM, the LCMV-8.1 peptide and LCMV-M peptides induced the positive selection of CD8⁺ P14 cells, albeit at levels distinctly lower than those attained by the LCMV peptide (Figure 4). In contrast, neither LCMV-8.7 nor LCMV' at 30 μM induced the positive selection of CD8⁺ P14 cells to a detectable level (Figure 4). At 300 μM the LCMV-8.1 peptide induced the positive selection of CD8⁺ P14 cells to a level that is about 15% greater than the level attained at 30 μM (data not shown).

Negative Selection of Transgenic P14 Cells by the Nominal Antigen Peptide

As described above, when we increased the concentration of the LCMV peptide in TAP1⁻ FTOC beyond 30 μM up to 300 μM, we observed a decrease in the number of CD8⁺ P14 cells compared with the cultures supplemented with 30 μM of the same peptide (see Figure 3). We wanted to determine whether the decline in the number of CD8⁺ P14 cells was due to negative selection. Negative selection of

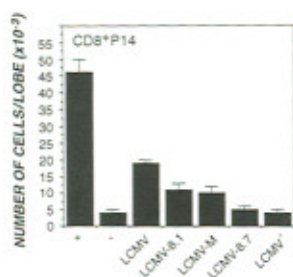


Figure 4. Effects of LCMV Peptide Analogs Added to TAP1⁺ FTOC on the Positive Selection of CD8⁺ Transgenic P14 Cells

P14 TAP1⁺ or TAP1⁻ thymic lobes were incubated with or without peptide as described in the legend to Figure 2. The plus sign designates the data obtained with TAP1⁺ FTOC with no added peptide, and the minus sign designates the data obtained with TAP1⁻ FTOC with no added peptide. All other data were obtained with TAP1⁺ FTOC supplemented with 30 μM of the indicated peptide. The numbers of selected CD8⁺ P14 cells were determined by staining for the appropriate cell surface markers and by subsequent FACS analysis as described in the legend to Figure 2. The levels for the number of CD8⁺ P14 cells per lobe is the mean (± SEM) of values determined from the analysis of between five and seven independently cultured lobes. Similar data was obtained from two other experiments performed at different times.

CD8⁺ transgenic P14 cells by nominal antigen has previously been described both in vivo (Pircher et al., 1989, 1990) and in vitro (Pircher et al., 1993). In these studies, negative selection led to a drastic reduction in the number of total thymocyte numbers and in the percentages and absolute numbers of CD8⁺ P14 cells and their CD4⁺CD8⁺ precursor cells.

We first examined whether we could induce negative selection of CD8⁺ P14 cells by addition of the nominal antigen peptide to TAP1⁺ FTOC. In one experiment the total numbers of thymocytes recovered from 9-day-old TAP1⁺ FTOC supplemented with the LCMV peptide at 3, 30, and 300 μM were 100%, 42%, and 37%, respectively,

of the control FTOC (no peptide). In two other experiments, the range of the total number of thymocytes recovered from 9-day-old TAP1⁺ FTOC supplemented with the LCMV peptide at 3 μM was 100%–50%, at 30 μM was 47%–42%, and at 300 μM was 37%–18% of the number of thymocytes generated in FTOC without peptide. In TAP1⁺ FTOC with 30 μM LCMV peptide, the percentages of CD8⁺ P14 cells were clearly reduced compared with the control FTOC. In the representative experiment shown in Figure 5, the CD8⁺ P14 cells were 5% (20% × 25%) with the peptide versus 20% (27% × 73%) without the peptide. The absolute number of CD8⁺ P14 cells was also reduced by the addition of the LCMV peptide (Figure 6). The reduction was by 7- to 8-fold at the three input LCMV peptide concentrations tested (3, 30, and 300 μM) compared with the control FTOC with no added peptide. About a 4-fold reduction of the number per lobe of precursor CD4⁺CD8⁺ cells was also observed in TAP1⁺ FTOC supplemented with 30 or 300 μM LCMV peptide, but no reduction was seen in the cultures supplemented with 3 μM LCMV peptide (Figure 6). In another experiment, similar results were obtained. However, in a third experiment, the number per lobe of CD4⁺CD8⁺ cells in the presence of 3 μM LCMV peptide was reduced by about 3-fold compared with the control FTOC without added peptide. We also quantitated the CD4⁺ cell subset, most of which does not express the P14 TCR, and found that its number per lobe was reduced by up to 2-fold in TAP1⁺ FTOC supplemented with 30 or 300 μM LCMV peptide, but not in the culture supplemented with 3 μM LCMV peptide (Figure 6). The reduction in the number of the CD4⁺ cells is probably due to the fact that in the absence of the added peptide many of these cells arise from CD4⁺CD8⁺ cells whose TCRs have switched from the transgenic type to nontransgenic types by the lack of allelic exclusion in the TCR α locus. Depletion of CD4⁺CD8⁺ cells with P14 TCR by the added peptide will reduce the number of CD4⁺CD8⁺ cells with additional non-P14 TCRs and hence the number of CD4⁺ cells.

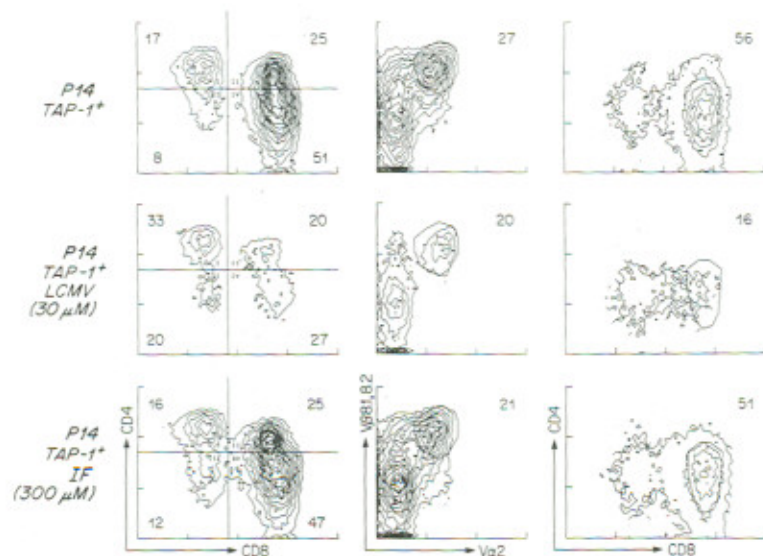


Figure 5. Flow Cytometric Data Showing Negative Selection of CD8⁺ Transgenic P14 Cells by the Nominal Antigen Peptide in TAP1⁺ FTOC

P14 TAP1⁺ thymic lobes were incubated with or without the LCMV (30 μM) peptide or the IF peptide (300 μM) as described in the legend to Figure 2. Cell surface markers were stained and subsequently analyzed by FACS as described in the legend to Figure 2, except that all of the cells recovered from individual lobes were analyzed. Thus, the TAP1⁺ lobe without peptide gave 8 × 10⁴ stained cells, TAP1⁺ lobes with the LCMV peptide (30 μM) gave 2 × 10⁴ stained cells, and TAP1⁺ lobes with the IF peptide (300 μM) gave 1 × 10⁴ stained cells. The percentages of cells in each quadrant or window are indicated.

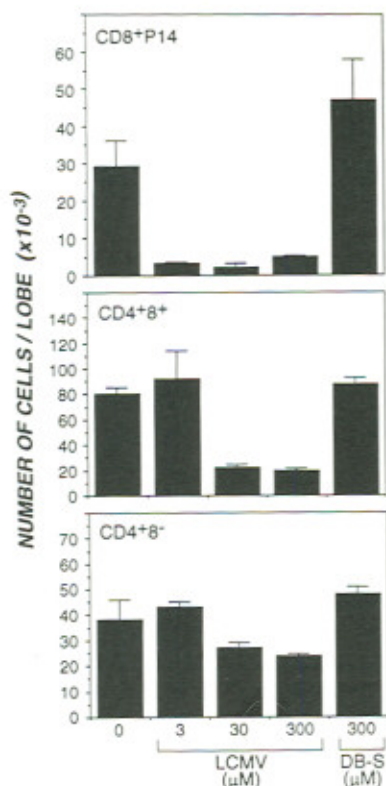


Figure 6. Histograms Showing Negative Selection of CD8⁺ Transgenic P14 Cells by the Nominal Antigen Peptide in TAP1⁺ FTOC

P14 TAP1⁺ thymic lobes were cultured as described in the legend to Figure 2 with or without peptide at the concentrations indicated. Thymocyte suspensions were prepared, and the presence of the appropriate surface markers was detected by staining with the appropriate MAbs and subsequent analysis by FACS. The percentages of CD8⁺ P14, CD4⁺CD8⁺, and CD4⁺ cells were then determined as described in the legend to Figure 2. The level for the absolute number of a given cell type per lobe is the mean (\pm SEM) of values determined from the analysis of between three and five independently cultured thymic lobes. Similar data was obtained from two other experiments performed at different times.

The LCMV peptide-mediated reduction in the total number of thymocytes and in the numbers of cells in CD8⁺ P14, CD4⁺CD8⁺, and CD4⁺ subsets in P14 TAP1⁺ FTOCs seemed to be peptide specific. Thus, neither the IF peptide (see Figure 5) nor the DB-S peptide (Figure 6), even at

300 μ M, caused a reduction in the numbers of the total thymocytes or any of the three thymocyte subsets. The overall results demonstrate that the added LCMV peptide can induce negative selection of CD8⁺ P14 cells in TAP1⁺ FTOC. We suspect that not only the bulk TCR⁺ low CD4⁺CD8⁺ cells but also a subset of cells that are derived from the CD4⁺CD8⁺ subset with somewhat up-regulated levels of TCR are susceptible to negative selection. In the presence of a low concentration (3 μ M) of the LCMV peptide, only the latter subset will undergo negative selection, leading to a reduction in the number of CD8⁺ P14 cells but not in the number of the bulk CD4⁺CD8⁺ subset (Figure 6). It should be noted that the concentrations of the LCMV peptides required for negative selection of CD8⁺ P14 precursor cells in TAP1⁺ FTOC is considerably greater than those required for negative selection of these cells in thymocyte suspension cultures (Pircher et al., 1993). This most probably reflects ineffective diffusion of peptides into thymic lobes during FTOC.

Having characterized the changes in the levels of total thymocytes and thymocyte subsets that accompany negative selection of CD8⁺ P14 cells by the nominal antigen peptide in TAP1⁺ FTOC, we then analyzed thymocytes from TAP1⁻ FTOC supplemented with a relatively high concentration (300 μ M) of the peptide. The total number of thymocytes per cultured lobe was reduced by about 2.5-fold in TAP1⁻ FTOC with the LCMV peptide as compared with the cultures without the peptide. Since the number of CD8⁺ P14 cells is low in TAP1⁻ FTOC without added peptide, we did not use reduction in the number of this thymocyte subset as a reliable measure for negative selection in TAP1⁻ FTOC by added LCMV peptide. Indeed, the numbers of CD8⁺ P14 cells in the peptide-supplemented FTOC fell within the background range (i.e., the numbers for peptide-negative FTOC) (see Figures 3 and 8). However, reduction in the numbers of CD4⁺CD8⁺ and CD4⁺ subsets was clearly observed in TAP1⁻ FTOC supplemented with 300 μ M LCMV peptide (Figures 7 and 8). Such reduction was not observed in TAP1⁻ FTOC supplemented with 3, 30, or 300 μ M LCMV peptide or in the similar cultures supplemented with 3, 30, or 300 μ M DB-S peptide (Figure 8). We conclude that at a high concentration (300 μ M) the LCMV peptide causes negative selection of CD8⁺ P14 thymocytes in TAP1⁻ FTOC. It is interesting to note that the negative selection was induced in TAP1⁺ FTOC with

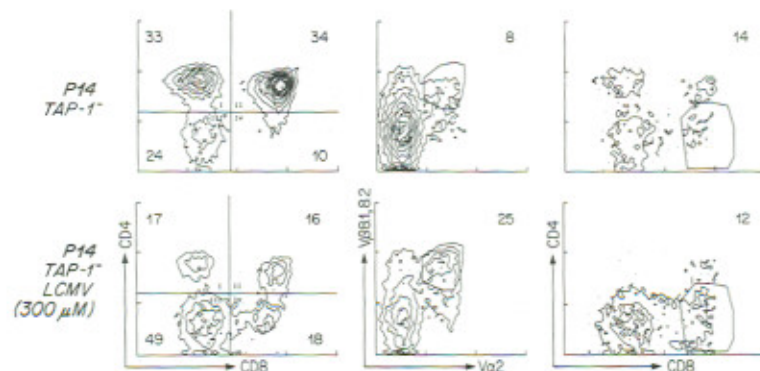


Figure 7. Flow Cytometric Data Showing Negative Selection of CD8⁺ Transgenic P14 Cells by the Nominal Antigen Peptide in TAP1⁻ FTOC

P14 TAP1⁻ thymic lobes were cultured with or without LCMV peptide (300 μ M) as described in the legend to Figure 2. Thymocyte preparations were prepared and analyzed (4×10^4 stained cells for lobes cultured without peptide and 3×10^4 for lobes cultured with 300 μ M LCMV peptide) for the expression of surface markers as described in the legend to Figure 2. The percentages of cells in each quadrant or window are indicated.

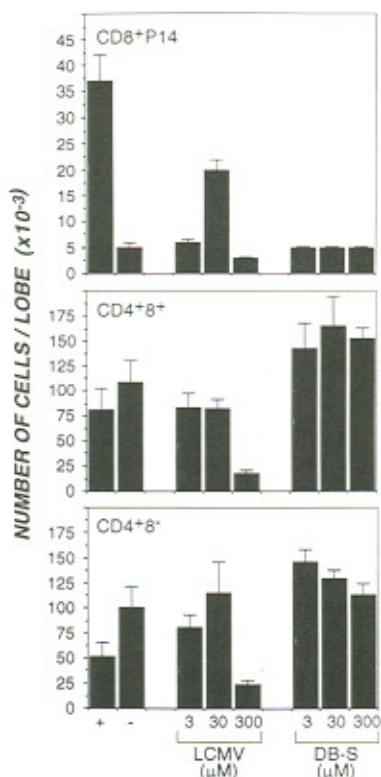


Figure 8. Histograms Showing Negative Selection of CD8⁺ Transgenic P14 Cells by the Nominal Antigen Peptide in TAP1⁻ FTOC

P14 TAP⁺ or TAP1⁻ thymic lobes were cultured as described in the legend to Figure 2 with or without peptide at the concentrations shown. The plus sign designates the data obtained from P14 TAP⁺ FTOC with no added peptide, and the minus sign designates the data obtained from P14 TAP1⁻ FTOC with no added peptide. All other data were obtained from FTOC supplemented with the peptides indicated. Thymocyte suspensions were prepared and the presence of the appropriate surface markers was detected by staining with the appropriate MAbs and by subsequent analysis by FACS as described in the legend to Figure 2. The percentages of CD8⁺ P14, CD4⁺CD8⁺, and CD4⁺ cells were then determined as described in the legend to Figure 2. The level for the absolute number of a given cell type per lobe is the mean (\pm SEM) of values determined from the analysis of between three and seven independently cultured thymic lobes. Similar data was obtained from two other experiments performed at different times.

distinctly lower concentrations of the LCMV peptide than in TAP1⁻ FTOC (see below for a discussion on this point).

Discussion

In an earlier paper, we demonstrated that some, but not all, single H-2D^b- or K^b-binding peptides can induce positive selection of a diverse set of CD8⁺ T cell clones when they are added at a relatively high input concentration (500 μM) to TAP1⁻ (non-TCR transgenic) FTOC (Ashton-Rickardt et al., 1993). That study as well as another study performed using β 2-microglobulin mutant mice (Hogquist et al., 1993) also showed that diverse mixtures of synthetic or natural H-2D^b- or K^b-binding peptides are effective positive selectors. Since the activity of a peptide (or a peptide mixture) to induce the positive selection and its activity to restore

stable cell surface expression of class I H-2 molecules on thymic cells did not correlate, we concluded that the role of peptide in positive selection is not just to stabilize the class I expression (the stability model); peptides also contribute to the specificity of positive selection of CD8⁺ T cells in the thymus (the specificity model).

In the present paper, we extended our study on the role of peptides during thymic selection by examining the development of cells expressing a transgenic TCR. We draw two major conclusions. First, peptides shape the T cell repertoire, most probably by being directly recognized by the TCR during the positive selection. Second, the avidity of the interaction between TCRs and peptide-MHC complexes determines the fate of the immature (CD4⁺CD8⁺) thymocytes by inducing either positive or negative selection.

Peptides Have a Specificity-Determining Role in Positive Selection

The most striking finding made in the present study is that even the nominal antigen peptide, the LCMV glycoprotein (amino acids 33–41), can induce the positive selection of the P14 clone along the CD8⁺ T cell pathway as long as the peptide is provided to the TAP1⁻ FTOC at an appropriate concentration (10–100 μM). The selection is peptide specific in that its extent depends on the sequence of the peptide. The LCMV peptide and its three analogs possess similar H-2D^b-stabilizing activities. However, at 30 μM input concentration, the LCMV peptide induces the positive selection of CD8⁺ P14 cells at a high level (about 40% of the level observed in TAP1⁺ FTOC) and LCMV-8.1 peptide at an intermediate level (about 25%), whereas neither LCMV-8.7 nor LCMV' peptide induces the positive selection to a detectable level. Therefore, the substitution of just one amino acid has the potential either to abolish or to reduce the ability of a peptide to select. We also examined two other peptides, IF nucleoprotein (366–374) and DB-S, which have no sequence similarity to the LCMV peptide except for the two H-2D^b anchor residues. Although these peptides are clearly either more effective (IF peptide) than or about as effective (DB-S peptide) as the LCMV peptide in stabilizing the surface expression of H-2D^b molecules, they could not induce positive selection of CD8⁺ P14 cells at 30 μM.

We have not directly measured the affinity of P14 TCR to any of the peptide-H-2D^b complexes. However, assuming a similar strength of binding to H-2D^b, the ability of a peptide to promote the proliferation of P14 spleen cells in vitro provides an indirect measure of the affinity. We found that among the LCMV peptide and its analogs with similar H-2D^b stabilizing activities, CD8⁺ P14 cell selecting activities roughly correlate with P14 spleen cell stimulating activities. Thus, it is likely that the affinity between a TCR and a peptide-MHC complex is an important parameter in positive selection.

The second important parameter is the density of a peptide-MHC complex on the surface of selecting cells. This is suggested by the dose-response curve of positive selection of CD8⁺ P14 cells by the LCMV peptide. At an input concentration below 3 μM or less, no CD8⁺ P14 cells were

generated in TAP1⁻ FTOC above the background level. Between 3 μ M and 30 μ M, increasing levels of positive selection could be observed. It is reasonable to assume that with increasing concentrations of the LCMV peptide in TAP1⁻ FTOC, the density of peptide-MHC complexes on thymic selecting cells increases since we have previously demonstrated a dose-response relationship for a peptide added to TAP1⁻ FTOC and H-2D^b stabilization on thymic stromal cells (Ashton-Rickardt et al., 1993).

It is interesting that the IF peptide can induce a detectable level of positive selection of CD8⁺ P14 cells at a high input concentration (300 μ M) (Figure 3). This peptide exhibited no detectable P14 spleen cell stimulatory activity but is highly effective in stabilizing the surface expression of H-2D^b molecules not only on RMA-S cells (Table 1) but also on thymic stromal cells in FTOC (see above). The observation that the IF peptide can induce positive selection of CD8⁺ P14 cells if it is given at a high concentration (Figure 3) suggests that even a low affinity peptide (so low that it cannot stimulate mature P14 T cells to a detectable level) has the capacity to promote positive selection of T cells as long as it is expressed at a very high density in association with an appropriate MHC molecule on selecting cells. These data thus illustrate the importance of peptide-MHC density as a parameter for positive selection.

It has been shown that the positive selection of a TCR transgenic clone restricted to class II MHC is enhanced in mice expressing higher than normal levels of the selecting thymic MHC molecules (Berg et al., 1990). This observation also supports the view that peptide-MHC densities are a critical parameter in determining positive selection.

Specificity Models of Positive Selection

It is now quite clear that peptide has a repertoire-shaping role during positive selection. A remaining question is whether peptide is directly recognized by TCR during this process as it is during the activation of mature T cells in the periphery. An alternative notion is that peptide plays a repertoire-shaping role by an indirect means without directly being recognized by TCR. Our finding that the same peptides that are recognized by mature T cells promote the positive selection of their precursor thymocytes argues for a direct role of peptide.

One way in which peptide can indirectly shape the T cell repertoire during positive selection is through steric hindrance of the interaction between TCR and MHC molecules (Ashton-Rickardt et al., 1993). According to this model, a T cell clone is positively selected only when its TCR interacts with MHC molecules without interference from the MHC-bound peptide. However, our data with the DB-S peptide does not support this model. Except for the anchor residues at positions 5 and 9, this peptide is composed of serines that carry less-bulky side chains than the corresponding amino acid residues of the LCMV peptide in 5 out of 7 positions. For each of the remaining two positions, the LCMV residue is alanine, whose side chain is only slightly less bulky than that of serine. Thus, it is very likely that the DB-S peptide when presented by H-2D^b molecules will sterically hinder the interaction between MHC and TCR less than the LCMV peptide. However, we found

that the DB-S peptide did not select CD8⁺ P14 cells, while the LCMV peptide did. These data do not support the steric hindrance model.

Another way in which peptide may shape T cell repertoire indirectly is by inducing conformational changes in MHC molecules, which might be preferentially recognized by TCR during positive selection. Our data do not argue against this possibility, and the occurrence of such conformational changes has been observed (Fremont et al., 1992; Matsumura et al., 1992). However, other studies found that these structural changes are relatively limited (Madden et al., 1993). In contrast, the structure of MHC-bound peptide was found to be a highly complex function of its entire sequence, potentially sensitive to even small sequence differences (Madden et al., 1993). It is difficult to imagine how TCR will ignore the structural variability of bound peptide and simply focus on relatively minor conformational changes induced in MHC molecules, either during positive selection or peripheral activation.

Differential Avidity Model of T Cell Selection

That apparently similar TCR-MHC interactions result either in the positive selection of MHC-restricted thymocytes or in the negative selection of autoreactive thymocytes has long intrigued many immunologists. While many hypotheses have been proposed to explain this thymic paradox (for review see Hugo et al., 1993), the parameters of the TCR-MHC interactions (or subsequent signal transductions) that are crucial for the determination of different cell fate have remained largely unknown. Our present study begins to identify some of these parameters. Our results suggest that a single thymocyte clone can be either positively or negatively selected by the same peptide-MHC complexes depending on the avidity of the interaction between the TCRs and the peptide-MHC complexes. Here, we use the term avidity to indicate the product of the intrinsic affinity between a TCR and a peptide-MHC complex, the density of the TCR on the thymocyte, and the density of the peptide-MHC complex on the selecting cell. Avidity defined in this way is a measure of the number of engaged TCRs on the thymocyte. When the avidity is relatively low and within a certain range, the thymocyte undergoes positive selection. When the avidity exceeds this low range and reaches a certain threshold, negative selection occurs.

Several observations made in this study support this differential avidity model of T cell selection. We have already pointed out that the IF peptide that presumably has a very low affinity to P14 TCR can nevertheless positively select CD8⁺ P14 cells in TAP1⁻ FTOC when it is expressed at a very high density on thymic cells, presumably because a minimal avidity required for positive selection is reached. Conversely, the LCMV peptide that presumably has a high affinity for P14 TCR can also positively select CD8⁺ cells in TAP1⁻ FTOC when its expression on thymic cells is kept at a relatively low density so that the avidity is within the range for appropriate positive selection. However, when the same peptide is expressed at a higher density and hence the avidity exceeds a certain threshold, negative selection occurs. We have identified another peptide,

LCMV-8.1, which can induce both positive (this work) and negative (Pircher et al., 1991) selection. It is likely that the different outcomes observed are due to differences in avidity under the two experimental conditions.

Another observation that supports the differential avidity model of T cell selection is that different fates are taken by the precursors of CD8⁺ P14 in TAP1⁻ FTOC and TAP1⁺ FTOC supplemented by relatively low concentrations (3–30 μ M) of the LCMV peptide. In TAP1⁺ FTOC without the exogenous peptide, an avidity sufficiently high for the positive selection of CD8⁺ P14 cells is already reached by a variety of endogenous peptides. Hence, the system is very sensitive for negative selection when the high affinity LCMV peptide is added to the culture. By contrast, in TAP1⁻ FTOC, the avidity is lower than the minimal level necessary for the positive selection in the absence of the exogenous peptides. When we add increasing concentrations of the LCMV peptide, the avidity necessary for positive selection is first reached, and then, upon a further increase in concentration, the avidity sufficient for negative selection is reached.

When transgenic mice that overexpress the CD8 coreceptor were crossed to class I-restricted TCR transgenic mice, negative selection of the transgenic CD8⁺ T cells was observed (Robey et al., 1992; Lee et al., 1992). These observations are consistent with negative selection being determined by an increase in TCR-MHC avidity over that needed for positive selection. In those cases the increase in avidity was afforded by overexpression of the TCR-MHC class I coreceptor CD8.

In the past, a large amount of work on T cell selection was devoted to the identification of thymic stromal cell subsets that induce positive or negative selection. Initial studies seemed to indicate that radioresistant cortical epithelial cells and radiosensitive bone marrow-derived cells (macrophages and dendritic cells) specialize in positive and negative selection, respectively (for review see Schwartz, 1989). However, recent studies challenge these earlier notions and suggest that wider arrays of cell types, including fibroblasts, have the capacity to induce positive selection, negative selection, or both (Pircher et al., 1993; Pawlowski et al., 1993; also for review see Hugo et al., 1993). Our differential avidity model, in its minimal form, does not depend on distinct thymic stromal cells specializing in each of the two types of selection. If distinct cell types are responsible for positive or negative selection and some adhesion molecules are differentially expressed on the positively and negatively selecting cells, they may modulate the avidity of interactions. However, the observation that a single peptide (the LCMV peptide) can induce either positive or negative selection depending on the concentration (Figure 3) indicates that the role of adhesion molecules in differentially influencing cellular interactions in positive versus negative selection is limited. This observation also argues against the notion that different sets of self-peptides must be produced by different selecting cells that, when recognized by thymocyte TCRs, lead to positive or negative selection (Marrack and Kappler, 1987).

As to the target of selection, thymocytes, the differential avidity model again does not depend on the occurrence of distinct subsets of cells for positive and negative selection. However, at least for the selection of thymocytes involving superantigens, it has been suggested that the thymocytes undergoing negative selection express TCRs at higher density than the thymocytes undergoing positive selection (Guidos et al., 1990). When we supplemented P14 TAP1⁻ FTOC with the LCMV peptide, we found that only a 10-fold difference (30 μ M versus 300 μ M) in the input peptide concentration resulted in distinct cell fates. Even if the TCR density is only slightly higher on thymocytes undergoing negative selection than those undergoing positive selection, that difference would amplify the discriminative power of the avidity difference resulting from differences in affinity and peptide-MHC density.

A differential affinity model was previously formulated to explain the thymic paradox (Sprenst et al., 1988). In this model it is the affinity of TCR for a self-MHC or a peptide-self-MHC complex that is critical in the determination of the fate of thymocytes: low affinity results in positive selection and high affinity in negative selection. The differential avidity model (Janeway et al., 1992) is distinct from the differential affinity model in that it incorporates as the critical parameters not only the intrinsic affinity of the TCR (perhaps including its coreceptor) for a peptide-MHC complex but also the cell surface density of the TCR as well as the density of the peptide-MHC complexes. In the thymus, TCRs of an immature thymocyte encounter MHCs loaded with a variety of peptides that have different binding affinities to the TCR and are expressed at different densities on the surface of selecting cells. In addition, it is possible that thymocytes with different levels of TCR density are involved in positive and negative selection. In the differential avidity model, it is the avidity or the summation of the interactions of the multiple but homogeneous TCRs with multiple and heterogeneous peptide-MHC complexes that determines the fate of an individual thymocyte. Thus, a relatively high affinity TCR-peptide/MHC interaction can lead to positive selection as long as the density of the TCR and that of the peptide-MHC complex are below certain levels. Conversely, a low affinity TCR-peptide/MHC interaction can theoretically lead to negative selection, although in practice the required high density of peptide-MHC is unlikely to be reached under physiological conditions. Of course, there is a minimal affinity below which no signal can be delivered through the TCR. In this case, the density of neither TCR nor peptide-MHC complexes will be able to compensate the lack of sufficient affinity.

The nature of differential signals delivered to thymocytes during positive and negative selection remains unknown. Two possibilities can be considered. Owing to higher numbers of engaged TCRs, cells undergoing negative selection may receive more of the same signals than the cells undergoing positive selection. Alternatively, engaged TCRs at high densities may generate signals that are qualitatively different from the signals generated by engaged TCRs at low densities. For example, engaged TCRs at high densities may dimerize and thereby activate

attached effector molecules (e.g., protein kinase) into a form that differs from the form generated by an engaged monomeric TCR.

After submission of this paper, Hogquist et al. (1994) reported positive selection of another transgenic T cell clone by peptides in FTOC derived from β 2-microglobulin mutant mice. Contrary to our findings, these authors reported that the nominal antigen peptides do not induce positive selection. We suggest that a range of peptide concentrations appropriate for positive selection was not tested in this study. Hogquist et al. (1994) emphasize the correlation between the selecting peptides and so-called TCR antagonist peptides. However, we believe that this correlation is only apparent and is probably attributed to the fact that these peptides have a suitable range of affinity for promoting positive selection at the concentration (20 μ M) the authors employed. Our view is that antagonicity is not a required property of positively selecting peptides. Rather, an appropriate range of the avidity as defined above is the critical parameter.

Experimental Procedures

Mice

Control C57BL/6 mice (obtained from The Jackson Laboratory, Bar Harbor, Maine), TAP1-deficient mice ([129/SvJ \times C57BL/6]F2; Van Kaer et al., 1992), and P14 transgenic mice (C57BL/6; Pircher et al., 1989) were maintained and bred under standard conditions. Mice homozygous for the TAP1 mutated allele and for the P14 transgene were crossed, and the progeny (F1) were then intercrossed to produce F2 animals. TAP1^{-/-}P14^{+/+} F2 animals were typed by screening peripheral blood for the absence of class I MHC expression by monoclonal antibody (MAb) staining and FACS analysis (Van Kaer et al., 1992) to detect TAP1^{-/-} mice and by PCR analysis (*V β 8.1* primer, 5'-CATGGAGG-CATGCAGTCACCC-3'; *C β 2* primer, 5'-GTTTGTTCGAGCTCTG-TTTTGATGGCTC-3') of genomic DNA prepared from tail biopsies to detect P14 transgenes. These mice were then intercrossed to produce TAP1^{-/-}P14^{+/+} F3 mice, which were typed by backcrossing to normal C57BL/6 mice and screening the progeny for the presence of the transgene. F3 mice that gave 20 consecutive P14⁺ progeny on backcrossing were considered to be homozygous for the transgene.

FTOCs

TAP1 P14^{+/+} males were mated with TAP1^{-/-} females to produce P14 TAP1^{-/-} fetal thymi, while TAP1^{+/+} (C57BL/6) P14^{+/+} males were mated with C57BL/6 females to produce TAP1^{+/+} P14 fetal thymi. Fetal thymi (day 16 postcoitum) were cultured according to the method described previously (Ashton-Rickardt et al., 1993) except that RPMI, 10% fetal calf serum (FCS) medium was used.

Flow Cytometric Analyses

The following MAbs were used: anti-CD4 (allophycocyanin [APC] or R-phycoerythrin [PE] labeled), anti-CD8 α (fluorescein isothiocyanate [FITC] labeled), anti-V β 8.1,8.2 (biotin labeled), anti-V α 2 (R-PE labeled), anti-H-2D^b (biotin-labeled B22-249R1, α 1 specific, American Type Culture Collection), and anti-I-A^b (PE labeled). Thymocyte suspensions were prepared from cultured fetal thymi and analyzed as described previously (Ashton-Rickardt et al., 1993). Thymocyte and mesenteric lymph node cell suspensions were prepared and analyzed as previously described (Van Kaer et al., 1992). Chromosomal DNA of FACS-sorted cells was stained with propidium iodide (PI). In brief, cells were washed in phosphate-buffered saline (PBS) and then fixed in 70% ethanol (1 ml at -20°C) for 30 min. After washing in PBS, cells (1 \times 10⁶/ml) were permeabilized by incubation in 3 M HCl, 0.5% Tween 20 for 20 min at 20°C and then neutralized by the addition of 3 ml of 0.1 M sodium borate. Washed cells (2 \times PBS) were incubated with RNase A (150 μ g/ml, 0.5 ml) for 20 min at 37°C, washed, and

resuspended overnight with PI (50 μ g/ml in PBS) at 4°C in the dark. Quantitation of the intensity of PI staining was performed by FACS analysis. The proportion of cells in G0/G1 (*n* PI staining), G2/M (2*n* PI staining), and S phases of the cell cycle (intermediate staining) was determined by statistical analysis (Modfit program) of the histogram of PI staining intensity versus cell number.

For the determination of H-2D^b expression on the surface of thymic stromal cells from FTOC, total thymic stromal cell suspensions from six whole fetal thymi were prepared exactly as described in Ashton-Rickardt et al. (1993). Stromal cells were analyzed for surface expression of I-A^b and H-2D^b molecules. To quantitate the surface expression of H-2D^b on gated I-A^b cells, the log fluorescence intensity resulting from staining with biotin-labeled anti-H-2D^b MAb (B22-249R1, α 1-specific) and streptavidin-FITC was plotted against the log relative cell number. Analysis of the resulting histograms allowed the determination of the median level of fluorescence intensity resulting from anti-H-2D^b staining on I-A^b-positive cells. The relative level of H-2D^b was thus expressed as a percentage of the TAP1⁺ level minus the level observed on cells from TAP1^{-/-} thymi incubated without peptide.

Peptides

Amino acid sequences of synthetic peptides are as follows: LCMV (amino acids 33–41), KAVYNFATM; IF (amino acids 366–374), AS-NENMETM; DB-S, SSSSSSSSM; LCMV-8.1 (amino acids 33–41), KAMYNFATM; LCMV-8.7 (amino acids 33–41), KALYNFATM; LCMV', RGIFNYGSM. For technical reasons (to prevent dimer formation), the original cysteines at anchor position 41 in LCMV, LCMV-8.1, and LCMV-8.7 were replaced by methionines. The LCMV, IF, and DB-S peptides were synthesized by the Biopolymers Laboratory (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts), the LCMV-8.1 and LCMV-8.7 were synthesized by Neosystem Laboratoire (Strasbourg, France), and the LCMV' peptide and LCMV-M peptides were synthesized by Research Genetics (Huntsville, Alabama). All were purified by reverse phase high pressure liquid chromatography (HPLC) and the concentration determined by quantitative Ninhydrin colorimetric assay. All peptides used were over 99% pure as determined by HPLC analysis. Three separate preparations of LCMV (from three different syntheses) were used in FTOC experiments. All gave similar results.

H-2D^b Stabilization Assay for Peptides

The relative abilities of peptides to stabilize H-2D^b surface expression was determined using the procedures described previously (Townsend et al., 1989). After incubation for 16 hr, RMA-S (duplicate cultures with peptide dilutions 10⁻⁴ M to 10⁻⁹ M) cells (1 \times 10⁶/ml, 0.2 ml) were washed, and the surface expression of H-2D^b was determined by staining with B22-249R1-biotin (then streptavidin-PE) and FACS analysis. The relative level of surface H-2D^b expressed in arbitrary fluorescence units (RMA, 3251; RMA-S alone, 60) was determined after FACS analysis. The relative ability of peptide to stabilize surface H-2D^b was expressed as the concentration necessary to give 50% of maximum rescue (maximum rescue was 35% of RMA).

P14 Spleen Cell Proliferation Assay for Peptides

Spleen cell suspensions (1 \times 10⁶ cells/ml) were prepared from P14 transgenic mice (C57BL/6) and incubated with peptide (triplicate wells, 2 \times 10⁶ cells) diluted from 10⁻⁴ M to 10⁻¹¹ M for 36 hr in RPMI, 10% FCS medium. Cultures were pulsed with tritiated thymidine (1 μ Ci per well) for 16 hr, the cells in the wells were harvested, and the amount of radioactivity was determined by detection of β particle emission. The relative abilities of peptides to induce the proliferation of P14 spleen cells were expressed as the concentration of peptide necessary to give 50% of the maximum [³H]thymidine incorporation (1.1 \times 10⁵ cpm) observed for the LCMV peptide. With the exception of the LCMV, LCMV-8.1, LCMV-8.7, and LCMV-M peptides, all other peptides tested failed to induce proliferation of P14 spleen cells at concentrations up to 10⁻⁴ M.

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Note Added in Proof

Recently, it was shown that some antagonist peptides can negatively select a cytochrome c-specific T cell clone (Page, D. M., Alexander, J., Snoke, K., Appella, E., Sette, A., Hedrick, S. M., and Grey, H. M. [1994]. Negative selection of CD4⁺ CD8⁺ thymocytes by T cell receptor peptide antagonists. *Proc. Natl. Acad. Sci. USA*, in press).