Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen

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THE crucial role of the thymus in immunological tolerance[1–5] has been demonstrated by establishing that T cells are positively selected to express a specificity for self major histocompatibility complex (MHC)[6–8], and that those T cells bearing receptors potentially reactive to self antigen fragments, presumably presented by thymic MHC, are selected against[9–11]. The precise mechanism by which tolerance is induced and the stage of T-cell development at which it occurs are not known. We have now studied T-cell tolerance in transgenic mice expressing a T-cell receptor with double specificities for lymphocytic choriomeningitis virus (LCMV)-H–2D^b and for the mixed-lymphocyte stimulatory (Mls^a) antigen. We report that $\alpha\beta$TCR transgenic mice tolerant to LCMV have drastically reduced numbers of CD4$^+$CD8$^+$ thymocytes and of peripheral T cells carrying the CD8 antigen. By contrast, tolerance to Mls^a antigen in the same $\alpha\beta$TCR transgenic Mls^a mice leads to deletion of only mature thymocytes and peripheral T cells and does not affect CD4$^+$CD8$^+$ thymocytes. Thus the same transgenic TCR-expressing T cells may be tolerized at different stages of their maturation and at different locations in the thymus depending on the antigen involved.

Transgenic mice expressing the P14-TCR specific for LCMV-H-2D^b ($V\alpha_2 J\alpha_{TA31}/V\beta_{8.1}D\beta J\beta_{2.4}$) (refs 12, 13) offer the unique possibility to examine T-cell tolerance to two independent antigens with the same transgenic mouse line. First, T-cell tolerance to LCMV has been studied in transgenic mice carrying the non-cytopathic LCM virus after neonatal infection[14]. Second, the transgenic TCR use the β-chain variable gene segment $V\beta_{8.1}$ that reacts preferentially with Mls^a antigen: in Mls^a mice, therefore, such $V\beta_{8.1}^+$ T cells are clonally eliminated in the thymus[10].

In a 3-day *in vitro* stimulation, spleen cells from uninfected transgenic Mls^b and Mls^a mice, but not from transgenic LCMV-carrier mice (Mls^b) or from normal unprimed mice, showed specific primary immune responses by proliferation and cell-mediated lysis of LCMV-infected target cells (Fig. 1). The response of transgenic Mls^a spleen cells to LCMV was three times less than that of transgenic Mls^b spleen cells; this decrease was probably due to the reduced number of CD8$^+$ T cells in the spleen of transgenic Mls^a mice (Table 1), because comparable frequencies of LCMV-specific T-cell precursors among CD8$^+$ T cells were found in transgenic Mls^a and Mls^b mice. The frequency of LCMV-specific cytolytic T-cell precursor cells among CD8$^+$ T cells was determined, by limiting dilution analysis, to be 1/1.8–4.2 in Mls^b $\alpha\beta$-transgenic mice, 1/2.4–3.9 in Mls^a $\alpha\beta$-transgenic mice, and below $1/10^4$ in normal unprimed mice[15]

TABLE 1 Peripheral T-cell subsets in transgenic mice

Mice	T-cell population	Percentage of lymph node cells	Population expressing $V\beta_8$ (%)	Absolute numbers per spleen ($\times 10^6$)
$\alpha\beta$-transgenic H-2^b, Mls^b	CD3	55±5	92±3	14.9±3.0
	CD4	9±3	62±5	3.0±0.5
	CD8	48±11	94±2	10.1±2.3
$\alpha\beta$-transgenic H-2^{bd}, Mls^a	CD3	26±3	62±8	3.3±1.5
	CD4	9±2	25±3	1.2±0.6
	CD8	16±4	83±5	1.5±0.8
$\alpha\beta$-transgenic LCMV-carrier H-2^b, Mls^b	CD3	36±4	67±9	4.9±1.2
	CD4	10±3	23±4	1.4±0.4
	CD8	4±1	33±3	0.5±0.1

Lymph-node cells of the indicated mice were stained with KJ16 monoclonal antibody (MAb)[21] (anti-$V\beta_{8.1+8.2}$) and (FITC)-conjugated goat anti-rat IgG (TAGO, Burlingame, California). The staining was followed either by biotinylated anti-CD8 monoclonal antibody (Becton-Dickinson) and phycoerythrin (PE)-conjugated avidin (TAGO) or by PE-conjugated anti-CD4 monoclonal antibody (Becton-Dickinson). For the CD3/$V\beta_8$ double staining, cells were first stained with F23.1 (anti-$V\beta_8$ monoclonal antibody[22] and FITC-conjugated anti-mouse Ig2a (Southern Biotechnology, Birmingham, Alabama) followed by KT3 mAb[23] (anti-CD3) and PE-conjugated goat anti-rat IgG (Southern Biotechnology). The average percentages (after subtraction with the fluorescien conjugate alone (<2%) of three mice per group are shown. Absolute values per spleen were calculated on the basis of the total number of spleen cells and cytofluorometric analysis of the cells stained with anti-CD3, CD4 and CD8 monoclonal antibodies. Red blood cells were removed before cytofluorometric analysis with FACS lysing solution (Becton-Dickinson). The data represents the analysis of three mice (6–12 weeks of age) per group.

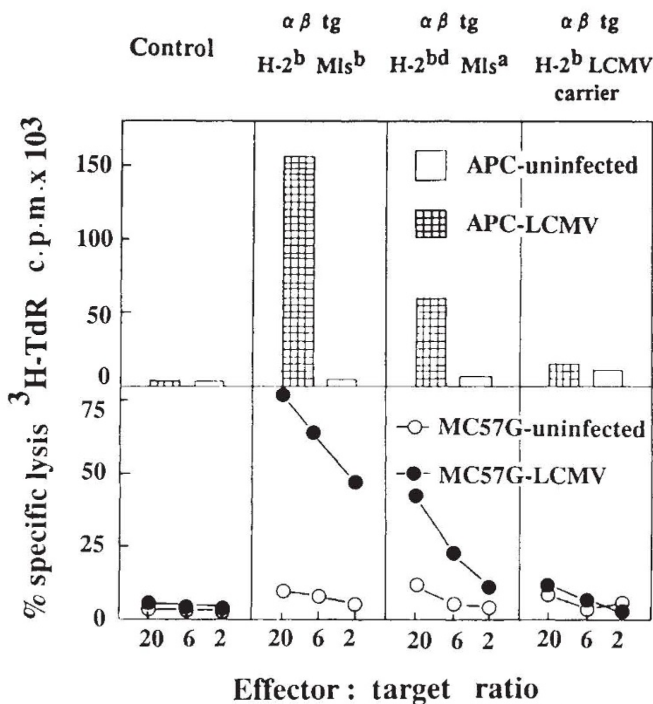

FIG. 1 Spleen cells from $\alpha\beta$ transgenic Mls^b and Mls^a mice generate a primary anti-LCMV cytotoxic T-cell response *in vitro* within three days.
METHODS. The $\alpha\beta$ transgenic mice were generated by co-injection of the P14 TCR α- and β-chain gene constructs[25] into (C57BL/6×DBA/2) F_2-generation fertilized eggs. The male founder 327 bearing 10–20 copies of both α- and β- transgenes integrated at the same chromosome was backcrossed to C57BL/6 (H-2^b, Mls^b) mice. $\alpha\beta$-transgenic Mls^a mice were obtained by mating the H-2^b, Mls^b transgenic line with DBA/2 (H-2^d, Mls^a) mice, and $\alpha\beta$-transgenic LCMV-carrier mice were generated by neonatal LCMV infection (<24 h after birth; 5×10^6 plaque-forming units (PFU) LCMV-WE strain per newborn) of transgenic H-2^b, Mls^b offspring. To examine the T-cell activities of these mice, spleen cells (4×10^6 ml^{-1}) were cultured with irradiated (3,000 rad) LCMV-infected and uninfected peritoneal macrophages (4×10^5 ml^{-1}) from C57BL/6 mice. Cell proliferation was determined after 2 days by ^{3}H-labelled thymidine uptake ^{3}H-TdR (upper panel). The lytic activities (lower panel) of the LCMV-stimulated cultures were determined after 3 days against LCMV-infected and uninfected MC57G (H-2^b) target cells in a 4–5 h ^{51}Cr-release assay, as described previously[12]. The data show a representative experiment for the three types of $\alpha\beta$-transgenic mice.

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(Table 2). These findings indicate that the clonal deletion of transgenic receptor-bearing T cells due to Mls^a was incomplete.

Thymus size and total thymocyte numbers of $\alpha\beta$-transgenic Mls^a and Mls^b mice did not differ significantly from those of their transgene-negative litter-mates. In marked contrast, thymus size and total thymocyte numbers of transgenic LCMV-carrier mice were drastically reduced (1–10% those of negative litter-mates). A strong skewing towards the carrying of CD8 antigen was observed for thymocytes and peripheral T cells of transgenic H-2^b Mls^b mice (Fig. 2*b* and Table 2) but not for those of transgenic H-2^d Mls^b mice (data not shown). This reflects the origin of the transgenic TCR from a H-2^b-restricted CD8$^+$ T-cell clone, and in addition provides evidence for the positive selection of T cells by thymic MHC in the absence of the nominal antigen. These results confirm observations in H-Y antigen-specific and alloreactive TCR transgenic mice[7,8].

Double staining with CD4- and CD8-specific antibodies revealed high numbers of TCRVβ8$^+$ CD4$^+$8$^+$ thymocytes (~80%) in transgenic Mls^a mice (Fig. 2*c*, *g*). The mature single CD8$^+$ thymocyte subset, however, was clearly reduced compared with that of transgenic Mls^b mice (2.3% versus 18.6%). Correspondingly, the number of mature thymocytes expressing a high TCR density were decreased in Mls^a mice. Transgenic TCR V$\beta_{8.1}$$^+$ CD8$^+$ peripheral T cells did not exhibit an Mls^a reactivity *in vitro* (data not shown). Only 31% of the few remaining thymocytes of $\alpha\beta$-transgenic LCMV-carrier mice were double positive for CD4CD8, and the percentage of thymocytes expressing Vβ_8 was drastically reduced (Fig. 2*d*, *h*). Single CD4$^+$ (14.8%) and CD8$^+$ (9.1%) thymocytes carried the CD3 antigen but were mostly Vβ_8^-, indicating that the transgenic β-chain gene was not expressed on these cells (Fig. 2*o*, *p*). The CD4$^-$CD8$^-$ thymocytes of transgenic LCMV-carrier mice expressed high levels of CD3 antigen (Fig. 2*m*) but only low levels of TCR Vβ_8 and $\alpha\beta$ determinants (Fig. 2*o*, *p*). These cells probably belong to the thymic $\gamma\delta$-compartment which was not affected by tolerance induction, and the relative but not the absolute frequency of these cells increased through the massive deletion of $\alpha\beta$ CD4$^+$CD8$^+$ thymocytes.

The drastically reduced number of CD4$^+$CD8$^+$ thymocytes and of transgenic receptor-bearing CD8$^+$ peripheral T cells (Table 2) is direct evidence of tolerance induction by clonal deletion in LCMV-carrier mice. Clonal deletion seems to occur in the thymic cortex, where LCMV has been shown to be present in neonatally infected mice[16]. The fact that ~60% of the Vβ_8^+ T cells in transgenic carrier mice did not express detectable CD4 or CD8 molecules indicates that besides deletion, down-regulation of CD8 could be a different pathway to achieve nonreactivity of transgenic TCR-bearing T cells[17]. In general our results parallel the observations made with H-Y antigen-specific[17] and alloreactive (H-2L^d)[8] transgenic TCR mice. The study using the LCMV model extends these observations by demonstrating that besides self antigens (MHC, H-Y, Mls), foreign antigens (for example, LCMV) are also able to induce deletion of reactive T cells when introduced at an early stage of T-cell development.

Because of the double specificity of the transgenic TCR (LCMV-H-2^b, Mls^a), tolerance to Mls^a could be examined with the same transgenic mouse line. In contrast to the $\alpha\beta$-transgenic LCMV-carrier mouse, thymocyte numbers and CD4$^+$8$^+$ thymocytes were not affected in transgenic Mls^a mice; the single CD8$^+$ thymocyte subset and peripheral CD8$^+$ T cells, however, were clearly reduced compared with those of transgenic Mls^b mice (Table 2).

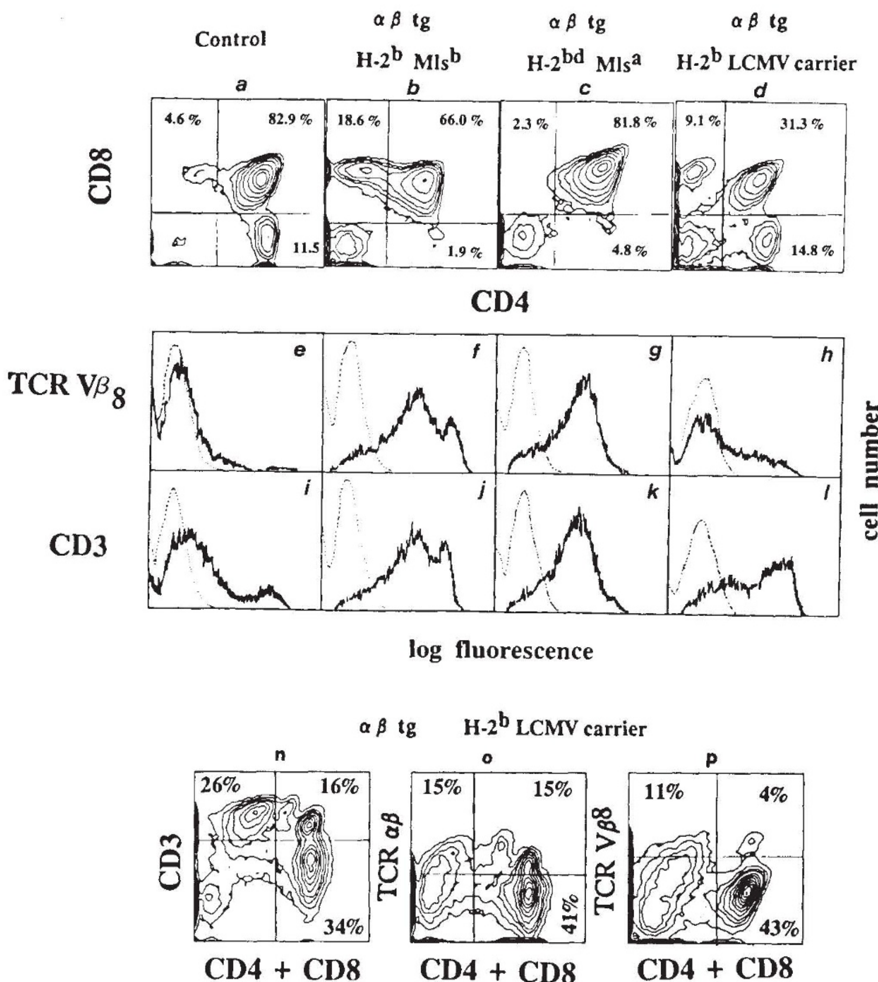

FIG. 2 Expression of CD3, CD4, CD8, $\alpha\beta$TCR and Vβ_8 TCR molecules on thymocytes from nontransgenic control, P14 $\alpha\beta$TCR transgenic Mls^b, Mls^a, and LCMV-carrier (H-2^b, Mls^b) mice. In the first panel (Fig. 2*a–d*) thymocytes were stained with PE-conjugated anti-CD4 and FITC-labelled anti-CD8 monoclonal antibody. The second panel (Fig. 2*e–l*) shows single staining profiles (solid lines) with KJ16 (anti-V$\beta_{8.1+8.2}$) and KT3 (anti-CD3) monoclonal antibodies followed by FITC-conjugated goat anti-rat IgG. Dotted lines indicate staining with the fluorescent conjugate alone. The third panel (Fig. 2*n–p*) shows two-colour flow-cytometric analysis of CD4CD8/TCR expression on thymocytes from $\alpha\beta$-transgenic LCMV-carriers mice The cells were incubated first with KT3, KJ16 and H57-597 (anti-$\alpha\beta$TCR) monoclonal antibodies and goat FITC-conjugated anti-rat/hamster IgG followed by PE-conjugated anti-CD4 and biotinylated anti-CD8 monoclonal antibody and finally PE-streptavidin.

METHODS. Single-cell suspension of thymocytes were stained in PBS with 2% FCS and 0.2% sodium azide with the various antibodies. Incubations were for 30 min at 4 °C (except for KJ16 and H57-597, which was at 37°C). First-step reagents used were monoclonal antibodies anti CD4-PE, FITC-conjugated anti-CD8, biotinylated anti-CD8 (Becton-Dickson); KT3[23], KJ16[21], H57-597[26]. Second-step reagents used were FITC-conjugated goat anti-rat IgG (TAGO), FITC-conjugated goat anti-hamster IgG FITC (Jackson Immunoresearch) and PE-streptavidin (Becton-Dickinson). Viable cells (50,000) per sample were analysed by flow cytometry on a Epics Profile Analyzer with logarithmic scales. Viable cells were gated by a combination of narrow-angle forward light-scatter and perpendicular scatter. Tg, Transgenic.

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TABLE 2 Frequency of LCMV-specific cytotoxic T-cell percursors among $CD8^+$ T cells of $TCR\alpha\beta$ transgenic mice

Mice	Reciprocal frequencies
C57BL/6 Uninfected	$>10^4$ (ref. 15)
C57BL/6 acutely infected with LCMV, day 8	3.0–4.1
$\alpha\beta$-transgenic H-2^b, Mls^b Uninfected	1.8–4.1
$\alpha\beta$-transgenic H-2^{bd}, Mls^a Uninfected	2.4–3.9
$\alpha\beta$-transgenic H-2^b, Mls^b Neonatally infected LCMV-carrier	$>10^4$

Limiting numbers of responder spleen cells were cultured in 96-well round-bottom plates with irradiated (3,000 rad) LCMV-infected peritoneal macrophages (10^3 cells per well) and with irradiated spleen filler cells from LCMV-infected mice (10^5 cells per well) in the presence of 25% (v/v) supernatant from concanavalin A-stimulated rat spleen cells. The precise number of $CD8^+$ T cells was determined by staining of an aliquot of the used responder spleen population with CD8-specific monoclonal antibody and cytofluorometric analysis. After 7 days, the cytolytic activities of the microcultures were tested on LCMV-infected and noninfected MC57G target cells in a ^{51}Cr-release assay[12]. Cultures were scored as positives when LCMV-specific lysis was >15%. Frequencies were calculated according to Taswell[24].

Thus tolerance induction involving the same TCR can vary with the antigen involved. It could be that the time-dependent appearance or the localization of the two antigens (LCMV, Mls^a), or both of these factors, are different in the thymus. In LCMV-carrier mice, LCMV antigens have been detected very early throughout the thymus[16], so thymoctyes could meet LCMV antigen and MHC class I molecules in the thymic cortex and medulla. The distribution of the Mls^a antigen is not known. But because Mls^a antigen is recognized by T cells in the context of MHC class II molecules which are not detected in the outer cortex[18], deletion of most double $CD4^+CD8^+$ cells would not occur in $\alpha\beta$-transgenic Mls^a mice. Alternatively, the affinity of the transgenic receptor for LCMV-H-$2D^b$ could differ from that for Mls^a. Double positive thymocytes having a high-affinity receptor for the appropriate antigen (LCMV-H-$2D^b$ in our model) are therefore deleted at an early state of development. Thymocytes expressing a low-affinity receptor to Mls^a would be deleted at a later stage of development when TCR densities on these cells are increasing[19]. In conclusion, tolerance induction to the two antigens (LCMV, Mls^a) examined with the same transgenic receptor differed drastically. These findings indicate that T-cell tolerance by clonal deletion does not occur at one single discrete stage of T-cell development. □

Note added in proof: Since the submission of this manuscript, Berg *et al.*[20] have reported that $\alpha\beta$TCR ($V\alpha_{11}$, $V_{\beta 3}$) transgenic Mls-2^a and -3^a mice exhibit deletion of $CD4^+CD8^+$ thymocytes, whereas βTCR $V\beta_3$ transgenic Mls-2^a and-3^a mice do not delete $CD4^+CD8^+$ thymocytes. Because the TCR density on thymocytes of $\alpha\beta$-transgenic mice is higher than that in β-transgenic mice, it was argued that deletion of $CD4^+CD8^+$ thymocytes correlates wth TCR density and maturation state. Because we found that the same $CD4^+CD8^+$ thymocytes are deleted in the LCMV-carrier but not in Mls^a mice, TCR density and maturation state alone cannot explain our findings, suggesting that affinity could also play a part. □

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In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine

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CYTOTOXIC T lymphocytes (CTL) constitute an essential part of the immune response against viral infections[1]. Such CTL recognize peptides derived from viral proteins together with major histocompatibility complex (MHC) class I molecules on the surface of infected cells[2–4], and usually require *in vivo* priming with infectious virus[5]. Here we report that synthetic viral peptides covalently linked to tripalmitoyl-*S*-glycerylcysteinyl-seryl-serine (P_3CSS) can efficiently prime influenza-virus-specific CTL *in vivo*. These lipopeptides are able to induce the same high-affinity CTL as does the infectious virus. Our data are not only relevant to vaccine development, but also have a bearing on basic immune processes leading to the transition of virgin T cells to activated effector cells *in vivo*, and to antigen presentation by MHC class I molecules.

The epitopes recognized by MHC class I-restricted, virus-specific CTL can be defined by short synthetic peptides (ref. 3; see also Fig. 1). These peptides are thought to bind to a groove on top of the MHC molecule, as indicated by its crystallographic structure[4]. CTL can recognize target cells incubated in picomolar concentrations of a given peptide (ref. 6; see also Fig. 2). Paradoxically, mice cannot be primed with the same peptide at any concentration tested (H. S. *et al.*, manuscript in preparation). An example for this failure is shown in Fig. 2*a*, *d*, *g*: BALB/c (H-2^d) mice were immunized with influenza nucleoprotein (NP) NP147-158 (R-) epitope (Fig. 1), which is the most efficient peptide to be recognized by H-2^d-restricted CTL specific for influenza nucleoprotein[6]. On stimulation of recipient spleen cells *in vitro*, no killing of peptide-incubated or virus-infected target cells was observed. By contrast, mice immunized with infectious influenza virus (Fig. 2*b*, *e*, *h*) responded well.

The failure of peptide to prime *in vivo* could be related to the non-physiological form of antigen presentation, because

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