Clonal Deletion Induced by Either Radioresistant Thymic Host Cells or Lymphohemopoietic Donor Cells at Different Stages of Class I-restricted T Cell Ontogeny

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Summary

Major histocompatibility complex (MHC) products and self-antigens expressed in the thymus determine the repertoire of mature α/β T cells. While positive selection of self-MHC–restricted T cells is directed by MHC molecules expressed by thymic epithelial cells, negative selection depends to a large extent on self-antigens presented by lymphohemopoietic cells. However, radioresistant components of the thymus also influence negative selection, but it remains controversial whether this is accomplished by clonal deletion, clonal anergy, or other mechanisms. In this study, T cell development in mice expressing a transgenic T cell receptor (TCR) specific for lymphocytic choriomeningitis virus (LCMV) plus H-2D^b was analyzed in the presence or absence of the viral antigen. A novel approach to analyze the thymic tissue requirements for negative selection was possible by comparing thymocyte selection in H-2D^b versus H-2D^{bm13} mice, since the latter allowed positive selection but not LCMV-specific deletion of transgenic TCR-expressing thymocytes. In irradiation bone marrow chimeras expressing the restriction element for negative selection (H-2D^b) on host tissue, we show that radioresistant recipient cells in the thymus deleted developing T cells at an early stage of differentiation. In contrast, chimeras expressing H-2D^b on lymphohemopoietic donor cells showed clonal deletion at a later stage during ontogeny.

The thymus provides a highly efficient environment for differentiation of T lymphocytes (1, 2). Engagement of the α/β TCR in immature T cells plays a critical role both in inducing the development of T cells bearing receptors specific for foreign antigen presented by self-MHC molecules (positive selection), and the deletion of potentially harmful self-reactive T cells (negative selection). The sequence of thymocyte developmental stages and the cellular interactions may provide important clues to understand how T cells "learn" to discriminate self from nonself. The cells involved in thymic selection have been analyzed extensively. It has become clear that positive selection is largely controlled by thymic epithelial cells (3–13).

Negative selection, however, is influenced by both bone marrow–derived cells and thymic epithelia (5–7, 14–27). It has been demonstrated that antigens presented by lymphohemopoietic cells induce tolerance (reviewed in references 5, 6, 14, and 15). For example, clonal deletion of T cells specific for I-E$^+$ (28–30), Mls-1^a (31, 32), Mls-2^a/3^a (33), or staphyloccocal enterotoxin B (SEB) (34) is induced by bone marrow–derived thymic cells (7, 21–23). In contrast, the role of thymic epithelial cells in negative selection is less defined. While early investigations using thymus grafting failed to demonstrate negative selection by the MHC molecules displayed on thymic epithelium (35–37), other studies in chimeras and/or transgenic mice indicate that tolerance may be induced by thymic epithelial cells (16–22, 24–27). Often it was not possible to determine whether nonresponsiveness was due to deletion, anergy, or other mechanisms. Recently, it has been postulated that a nondeletional mechanism such as clonal anergy may occur when lymphocytes encounter their specific self-antigen on thymic epithelia (21, 22, 24, 25). However, while some authors claimed that thymic epithelial cells induced anergy of Mls-1^a-specific T cells (21, 22), our comparative analysis indicated that Mls-1^a-specific anergy was induced by lymphohemopoietic cells (23).

In this study transgenic mice were used carrying the P14 TCR α/β chains (Vα2-JαTA31, Vβ8.1-DJβ2.4) specific for the lymphocytic choriomeningitis virus (LCMV)[1] glycopro-

[1] *Abbreviations used in this paper:* aa, amino acids; LCMV, lymphocytic choriomeningitis virus; tg, transgenic.

tein epitope amino acids (aa) 32–42 presented in the context of H-2D^b (38). Positive selection of transgenic T cells has been shown to depend on the presence of the H-2^b molecule, while clonal deletion occurred in H-2^b LCMV carrier mice (38). The virus carrier state (established by virus infection either neonatally or during immunosuppression) was associated with ubiquitous virus expression, including thymic epithelia and lymphohemopoietic cells (39, 40). In this study the respective roles of the host's thymus versus lymphohemopoietic donor cells in clonal deletion was evaluated by investigating thymocyte maturation in LCMV carrier bone marrow chimeras. The results show that transgenic T cells were clonally deleted at different stages of development depending upon expression of the viral antigen restriction element H-2D^b either on host-derived radioresistant or donor-derived lymphohemopoietic cells.

Materials and Methods

Animals. Inbred BALB/c (H-2^d) and C57BL/6J (H-2^b) mice were purchased from the Institute für Zuchthygiene (Tierspital, University of Zürich, Switzerland). Breeders of the two C57BL/6 H-2D^b mutant inbred mouse strains B6.C-H-2^{bm13} and B6.C-H-2^{bm14} (41, 42) (referred to as H-2^{bm13} and H-2^{bm14} mice) were kindly provided by Dr. Cornelis J. Melief (The Netherlands Cancer Institute, Amsterdam, Holland). H-2^{bm13} mice express a mutated H-2D^b molecule with three amino acid changes at the bottom of the peptide antigen binding groove (β-pleated sheet) at positions 114 (Leu → Gln), 116 (Phe→Tyr), and 119 (Glu→Asp). The mutated H-2D^{bm14} molecule has a single amino acid changed on the α_1 helix of the TCR binding site at position 70 (Gln→His) (42).

The transgenic mouse line 327 expressing the α/β TCR derived from the cytotoxic T cell clone P14 specific H-2D^b plus LCMV glycoprotein peptide aa 32–42 was generated by coinjection of the P14 TcR α and β chain gene constructs into (C57BL/6 × DBA/2)F$_2$-fertilized eggs (38). The male founder 327 bearing 10–20 copies of both α and β transgenes integrated at the same chromosome was backcrossed to C57BL/6 mice. α/β TCR transgenic (tg) H-2^d and H-2^{bm13} mice were obtained by backcrossing to BALB/c and B6.C-H-2^{bm13}, respectively. Characteristics of the strains relevant to the present study are summarized in Table 1.

Chimeras. Bone marrow recipients were lethally irradiated (950 rad, 117 rad/min, ^{137}Cs source) and reconstituted 1 d later with 1–2 × 10^7 T cell–depleted bone marrow cells as described (3). To establish a LCMV carrier state, chimeras were infected intravenously with 5 × 10^6 PFU of LCMV-WE strain 4–14 h after bone marrow transfer. For the following 5 wk, chloramphenicol (0.4 g/liter) was added to the drinking water. Both infected and noninfected chimeras had a survival rate of 85–100%. Analysis was performed between 8 and 12 wk after reconstitution. Chimerism was monitored by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) of lymph node cells with H-2 haplotype–specific mAbs.

Virus, MLR, and Cytotoxicity Assay. The WE strain of LCMV had been originally obtained from Dr. Fritz Lehmann-Grube (Hamburg, FRG) (43). Virus stocks were diluted in MEM supplemented with 2% FCS. To examine the functional response of T cells from activities of chimeric mice, spleen cells (4 × 10^5/ml) were cultured with irradiated (2,000 rad) LCMV-infected peritoneal macrophages (4 × 10^4/ml) from C57BL/6 mice in 96-well plates. Cell proliferation was determined after 3 d by 10-h [^{3}H]thymidine uptake. The lytic activity of spleen cells from chimeras infected 4 d earlier with 5 × 10^6 PFU LCMV-WE intravenously was determined against LCMV-infected and uninfected MC57G (H-2^b) target cells in 5-h ^{51}Cr-release assays as described previously (3).

Cytofluorographic Analysis. Aliquots of untreated thymocytes or lymph node cells were stained at 4°C in balanced salt solution (BSS) containing 2% FCS and 0.2% NaN_3 with rat mAbs B20.1 (Vα2 specific; reference 44) or KJ16-133 (Vβ8.1/Vβ8.2 specific; reference 31) detected with FITC goat anti-rat IgG (Tago Inc., Burlingame, CA). Biotinylated CD8-specific mAb 53–6.7 (Becton Dickinson & Co.) detected with avidin-PE (Tago Inc.) or PE-conjugated CD4-specific mAb GK 1.5 (Becton Dickinson & Co.), respectively, was used for double staining. To assess the chimerism of transplanted mice, haplotype-specific mAbs H141-31 (D^b specific), K7-309 (K^b specific), or 34-2-12 (D^d specific), respectively, were used followed by fluorescent goat anti-mouse IgG or IgG2a reagents (Southern Biotechnology Associates, Inc., Birmingham, AL); samples were similarly double stained as described above. Viable cells (20,000 per sample) were analyzed by flow cytometry on an Epics profile analyzer (Coulter Electronics, Inc., Hialeah, FL) with logarithmic scales.

Results

Early Deletion by Host Radioresistant Cells of Maturing Thymocytes in LCMV Carrier Bone Marrow Chimeras. (C57BL/6 × BALB/c)F$_1$ mice were lethally irradiated and reconstituted with T cell–depleted P14 α/β TCR transgenic bone marrow from either H-2^b or H-2^d mice. To establish a virus carrier state, chimeras were infected with 5 × 10^6 PFU of LCMV strain WE (LCMV-WE) between 4 and 14 h after irradiation and bone marrow reconstitution. Livers and spleens obtained up to 30 wk after infection contained ~10^6 PFU LCMV per gram organ (data not shown), confirming that these mice were virus carriers. Between 8 and 12 wk after bone marrow transplantation, T cell maturation was analyzed by flow cytometry with mAbs specific for the transgenic TCR domains Vα2 (B20.1) and Vβ8.1 (KJ16). A strong reduction of thymocytes expressing the transgenic TCR was observed in F$_1$ chimeric virus carriers reconstituted with either H-2^b or H-2^d transgenic bone marrow (Fig. 1 *c* and *d*). The extent of deletion was comparable with nonchimeric virus carriers published previously (38) and similar to the deletion described in other class I–restricted α/β TCR transgenic mouse models (8, 9). In contrast, thymocytes from uninfected control chimeras expressed transgenic Vα2 (Fig. 1, *a* and *b*) and Vβ8.1 (not shown) at high levels. Lymph node cells in uninfected chimeras were ~40% Vα2$^+$CD8$^+$, whereas in chimeric virus carriers <2% were Vα2$^+$CD8$^+$ (not shown). In summary, the data suggest that radioresistant thymic cells expressing the appropriate restriction element H-2D^b in H-2^d→H-2^{bxd} carrier mice induced clonal deletion of virus-specific CTL precursors.

Deletion by Lymphohemopoietic Cells Occurred at a Later Stage of T Cell Differentiation. Since bone marrow chimeras inevitably contain remaining radioresistant lymphohemopoietic cells of host origin, further experiments were necessary to evaluate the possible involvement of the thymic epithelium for induction of clonal deletion. A conventional protocol would have been to generate fully allogeneic or F$_1$ → parent

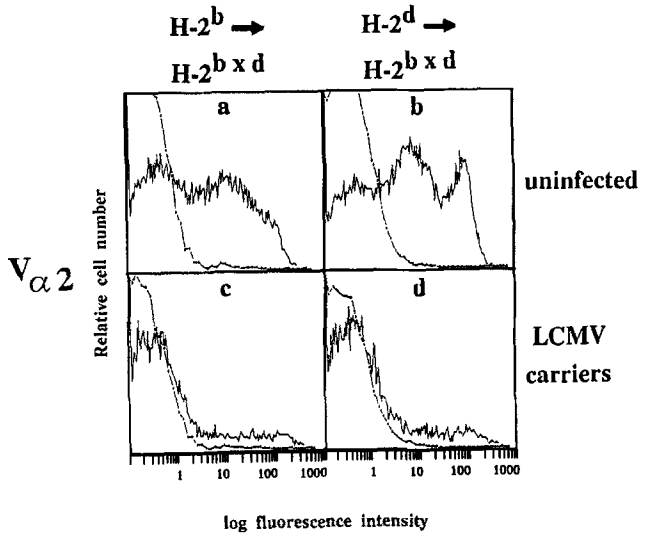

Figure 1. LCMV/H-2D^b-specific α/β TCR transgenic thymocytes developing in the absence (uninfected; *a* and *b*) or presence (LCMV carriers; *c* and *d*) of the viral antigen. Unseparated thymocytes were stained with mAb B20.1 specific for the transgenic Vα2 chain. Both H-2^b (*c*) and H-2^d (*d*) bone marrow–reconstituted (C57BL/6 × BALB/c)F$_1$ chimeras carrying the LCM-virus deleted transgenic Vα2$^+$ cells at an early stage of differentiation. Comparable histograms were obtained from stainings with KJ16 specific for the transgenic β chain (data not shown). Broken lines indicate control thymocytes stained with fluorescein-conjugated goat anti–rat antibodies alone. Comparable results were obtained from at least four individual chimeras per group. We have been unable to explain the reproducibly higher amount of TCR^{hi} cells in group *b* as compared with *a*.

bone marrow chimeras or the engraftment of thymectomized mice with parental thymus implants. However, positive selection of the transgenic α/β TCR depended on the expression of H-2D^b by the thymic epithelium, thus excluding the possibility to study negative selection in H-2D^b-negative thymi. This experimental problem could be circumvented as follows.

H-2^{bm13} mice express a mutated H-2D^b molecule with three amino acid changes at the bottom of the peptide antigen binding groove (42). The P14 transgenic α/β TCR can be positively selected in the context of H-2D^{bm13} products (P. S. Ohasi and H. P. Pircher, manuscript in preparation). However, H-2D^{bm13} molecules are unable to present the viral epitope (LCMV glycoprotein aa 32–42) to the transgenic TCR. Furthermore, transgenic H-2^{bm13} LCMV carrier mice are unable to clonally delete the transgenic TCR (Table 1) (unpublished results). Finally, H-2^{bm14} mice, another H-2D^b mutant mouse strain, neither promote positive nor negative selection of the P14 transgenic α/β TCR, and were therefore ideally used as closely related controls.

The unique property of H-2D^{bm13} molecules allowing positive but not negative selection of the transgenic TCR offered a new methodological approach to overcome the known problem of radioresistant or 2′-deoxyguanosine-resistant residual lymphohemopoietic cells "contaminating" the thymus of irradiation bone marrow chimeras or thymus-transplanted mice. To study the capacity of the thymic epithelium for clonal deletion, irradiation chimeras were generated by injection of transgenic T cell–depleted bone marrow into either H-2^b × ^{bm13} or H-2^{bm13} × ^{bm14} F$_1$ hosts. When analyzed 8–12 wk later, uninfected control chimeras revealed high levels of transgenic Vα2$^+$ and Vβ8.1$^+$ thymocytes (Table 2 and Fig. 2, *a*, *c*, and *e*). Chimeric virus carriers clonally deleted transgenic T cells resulting in low levels of both TCR$^{int/hi}$ thymocytes as well as of mature cells in the periphery (Table 2 and Fig. 2, *b* and *f*). Interestingly, in H-2^b → H-2^{bm13} × ^{bm14} virus carriers, a subpopulation of thymocytes expressing the transgenic TCR at low (TCR^{low}) density was found (Fig. 2 *d*) that was absent in H-2^b-expressing hosts (Figs. 1 *d* and 2 *f*). In H-2^b → H-2^{bm13} × ^{bm14} carriers, clonal deletion was induced by lymphohemopoietic tissue, since the restriction element H-2D^b capable of viral antigen presentation causing deletion was not expressed by host radioresistant tissue. Thus, deletion induced by lymphohemopoietic cells occurred at a later stage of T cell differentiation and was therefore distinguishable from deletion induced by host radioresistant cells. Thymocytes from the various chimeras showed similar forward light scatters (not shown), indicating that they were of similar size. This permitted direct comparison of the TCR expression levels measured.

To assess the tolerance status of transgenic T cells from

Table 1. *Characteristics of α/β TCR Transgenic (tg) Mice Used in This Study*

tg mice	H-2 compatible nontransgenic mouse strain	H-2 K	H-2 I	H-2 D	Positive selection of the tg TCR	Negative selection of the tg TCR in LCMV carriers
tg H-2^b	C57BL/6	b	b	b	+	+
tg H-2^{bm13}	B6.C-H2^{bm13}	b	b	bm13	+	–
tg H-2^{bm14}	B6.C-H2^{bm14}	b	b	bm14	–	–
tg H-2^d	BALB/c	d	d	d	–	–

In uninfected mice, the P14 tg α/β TCR is positively selected in the context of both H-2D^b and H-2D^{bm13} resulting in high TCR expression (~95% of T cells express the transgenes) and skewing to the CD8 lineage. In congenitally or neonatally infected LCMV carrier mice expressing the viral antigen in the thymus (39, 40), negative selection of the transgenic TCR is due to thymic clonal deletion occurring in association with H-2D^b but not H-2D^{bm13} or H-2D^{bm14} (38, and P. S. Ohashi and H. P. Pircher, manuscript in preparation).

Table 2. *TCR Expression and Proliferative/Cytotoxic Responses of TCR tg Lymphocytes from Parent→F_1 Chimeric Controls and Carriers*

		tg H-2^b→ H-2$^{b \times bm13}$		tg H-2^b→ H-2$^{bm13 \times bm14}$		tg H-2^{bm13}→ H-2$^{b \times bm13}$	
	Tissue	Uninfected	LCMV carrier	Uninfected	LCMV carrier	Uninfected	LCMV Carrier
Percent cells positive for:							
Vα2 ^{low}	Thymus	43.0 ± 8.1	6.0 ± 0.3	49.5 ± 2.4	33.3 ± 6.2	41.1 ± 6.9	9.4 ± 1.1
Vβ8.1^{low}	Thymus	30.1 ± 3.1	10.9 ± 3.2	47.6 ± 2.8	33.7 ± 8.9	43.3 ± 7.9	11.8 ± 2.9
Vα2$^{int/hi}$	Thymus	43.4 ± 6.9	2.7 ± 1.1	35.4 ± 5.6	3.3 ± 0.6	41.3 ± 12.3	3.2 ± 1.4
Vβ8.1$^{int/hi}$	Thymus	38.7 ± 3.9	2.1 ± 0.6	32.1 ± 1.9	2.9 ± 0.3	40.3 ± 11.2	1.9 ± 0.7
Vα2$^+$CD8$^+$	Lymph node	49.3 ± 4.3	1.2 ± 0.2	61.0 ± 3.1	1.7 ± 0.2	42.0 ± 4.9	0.8 ± 0.1
Vβ8.1$^+$CD8$^+$	Lymph node	56.2 ± 1.2	1.6 ± 1.3	58.3 ± 3.3	2.2 ± 0.4	44.6 ± 5.3	1.2 ± 2.5
Proliferative responses (cpm)							
to LCMV infected C57BL/6 stimulators	Spleen	37.0 × 10^3	0.9 × 10^3	72.7 × 10^3	0.2 × 10^3	42.8 × 10^3	1.0 × 10^3
Percent specific cytolysis of targets (E/T ratio 70:23:8:3)							
MC57G LCMV infected	Spleen	70:60:51:23	7:5:0:0	78:57:38:21	1:0:7:9	62:47:23:14	8:5:6:4
MC57G uninfected	Spleen	5:3:3:3	2:1:5:3	7:2:0:0	9:5:6:5	3:5:6:2	3:8:0:3
YAC-1	Spleen	4:3:0:0	1:0:1:3	8:5:1:3	3:2:7:7	7:1:0:0	1:1:5:1

Mean ± SEM are from FACS® analyses of unseparated cell preparations of three to five chimeras per group. Examples of FACS® profiles from one chimera per group are shown in Fig. 2. The percentages for low or int/hi positive cells, respectively, refer to the regions given in Fig. 2. Percent Vα2$^+$CD8$^+$ or percent Vβ8.1$^+$CD8$^+$ indicate the percentages of lymph node cells expressing both the transgenic TCR variable regions and CD8. Proliferative responses were measured by [^{3}H]thymidine uptake (10 h) after stimulation with LCMV-infected peritoneal macrophages (3 d). Cytotoxicity was analyzed 4 d after intravenous infection with 10^6 PFU LCMV-WE. Chimerism assessed with mAb K7-309 (K^b specific, positively staining all lymph node cells) and mAb H141-31 (D^b specific, staining H-2D^{b+} cells strongly but H-2D^{bm13+} lymphocytes only at low fluorescence intensities; reference 47) showed that ~90% of lymph node cells were of donor type.

the various chimeras, proliferation and cytotoxic T cell responses were tested (Table 2). While noncarrier chimeras responded vigorously to LCMV-infected stimulator or target cells, both LCMV-specific proliferative responses and cytotoxic T cell activities were low in the chimeric carrier mice (Table 2). Thus, all the noncarrier chimeras were T cell competent against LCMV and all the carriers were nonresponsive correlating with presence or absence of mature transgenic T cells in thymus and periphery.

Discussion

In the past, the technical difficulty to completely eliminate lymphohemopoietic cells from thymic tissue while preserving its capacity to promote T cell maturation has been a limiting factor to experimentally determine whether thymic epithelial cells can induce clonal deletion. Therefore, we studied negative selection in H-2D^b mutant mice. Since H-2$^{bm13 \times bm14}$ mice selected the transgenic TCR positively but not negatively (P. S. Ohasi, manuscript submitted for publication), it was not essential that lymphohemopoietic cells were completely eliminated by irradiation to assess the contribution of donor lymphohemopoietic cells to negative selection. In chimeric H-2^b → H-2$^{bm13 \times bm14}$ virus carriers, clonal deletion induced by lymphohemopoietic cells was at the TCR^{low} stage of thymocyte ontogeny. In contrast, only transgenic TCR-negative lymphocytes remained in virus carriers expressing H-2D^b on radiation-resistant thymic cells. Therefore, these cells probably including thymic epithelial cells are apparently capable to induce clonal deletion at an early stage of ontogeny.

An alternative explanation based on the assumption that deletion can only be induced by lymphohemopoietic cells can be postulated. The presence of TCR^{low} cells in H-2^b → H-2$^{bm13 \times bm14}$ carrier chimeras could possibly be due to "delayed" deletion caused by insufficient repopulation of the thymic microenvironment by donor cells. However, this assumption would imply that deletion in H-2^d → H-2$^{b \times d}$ or H-2^{bm13} → H-2$^{b \times bm13}$ carriers was due to low amounts of remaining lymphohemopoietic cells. Accordingly, one would also expect a complete and early deletion in H-2^b → H-2$^{bm13 \times bm14}$ carrier chimeras, where donor cells had repopulated the thymus (45). Thus, since we did not find early deletion in H-2^b → H-2$^{bm13 \times bm14}$ carriers where deletion was induced by lymphohemopoietic cells, we con-

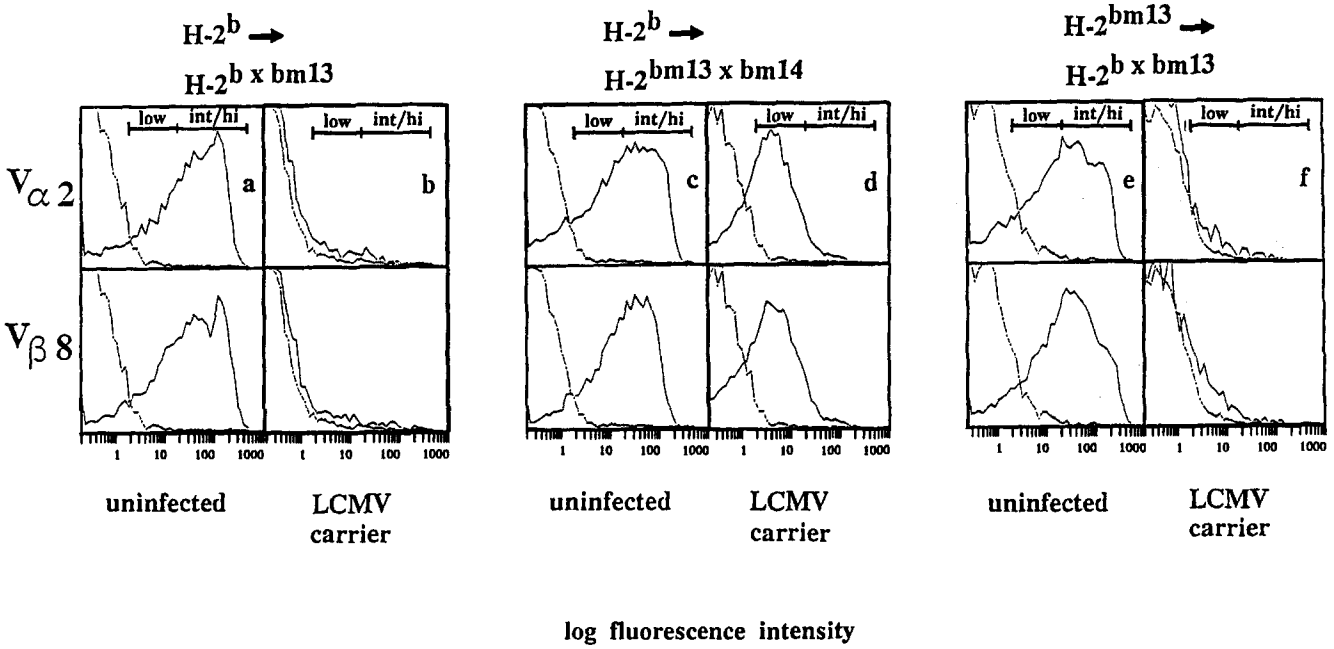

Figure 2. LCMV/H-2D^b-specific α/β TCR transgenic thymocytes developing in the absence (uninfected; *a*, *c*, and *e*) or presence (LCMV carriers; *b*, *d*, and *f*) of the viral antigen. The histograms represent examples from data given in Table 2. Unseparated thymocytes were double stained with transgenic Vα2 or Vβ8.1 chain-specific mAbs and a CD8-specific mAb. The histograms shown were software gated for the CD8$^+$ cells (including CD8$^+$ and CD4$^+$8$^+$). In chimeric LCMV carriers, expression of H-2D^b by radioresistant host tissue induced early deletion (*b* and *f*) while H-2$^{bm13 \times bm14}$ recipients deleted thymocytes at a TCR^{low} stage (*d*). The regions marked low or int/hi indicate low or intermediate/high TCR densities, respectively, and refer to the text and data given in Table 2. Additional results (not shown) were obtained from transgenic H-2^b → H-2^b or H-2^b → H-2^{bm13} chimeras, respectively, revealing comparable results to Fig. 2, *a* and *b* or *c* and *d*, respectively.

clude that the early deletion in H-2^d → H-2$^{b \times d}$ or H-2^{bm13} →H-2$^{b \times bm13}$ carriers was induced by radioresistant thymic host cells, presumably the thymic epithelium.

The results imply that the thymic epithelium may be involved in both positive and negative selection. Subpopulation of the different epithelial cells within the thymic cortex and medulla may exhibit distinct self antigens and functions (24–27, 37, 46). Alternatively, different affinity thresholds may regulate positive or negative selection (5). One may argue that our transgenic T cells were deleted early due to presumably high avidity interactions with radioresistant thymic cells. It is also possible that the observed differences in deletion may be because thymocytes first encounter epithelial cells and only later interact with lymphohemopoietic cells.

Our findings support the notion that T cells may be susceptible to clonal deletion over an extended period of thymic differentiation. Furthermore, several of the various thymic cell components may induce negative selection. Thus, the immune system may have developed a variety of ways to increase the probability of self-specific lymphocytes encountering self-antigens in order to assure efficient induction and maintenance of immunological tolerance.

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