

Tolerance of CD8⁺ T Cells Developing in Parent → F₁ Chimeras Prepared with Supralethal Irradiation: Step-Wise Induction of Tolerance in the Intrathymic and Extrathymic Environments

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Summary

Tolerance of CD8⁺ cells was examined in parent → F₁ bone marrow chimeras (BMC) prepared with supralethal irradiation; host class I expression in the chimeras was limited to non-BM-derived cells. In terms of helper-independent proliferative responses *in vitro* and induction of graft-vs.-host disease on adoptive transfer, CD8⁺ cells from long-term chimeras showed profound tolerance to host antigens irrespective of whether the cells were prepared from the thymus or from spleen or lymph nodes. By limiting dilution analysis, cytotoxic T lymphocyte (CTL) precursors specific for host antigens were rare in the extrathymic lymphoid tissues. In the thymus, by contrast, host-specific CTL precursors were only slightly less frequent than in normal parental strain mice. These host-specific CD8⁺ cells survived when BMC thymocytes were transferred intravenously to a neutral environment, *i.e.*, to donor strain mice. When transferred to further BMC hosts, however, most of the host-reactive cells disappeared. Collectively, the data suggest that tolerance of CD8⁺ cells in BMC hosts occurs in both the intrathymic and extrathymic environments. In the thymus, contact with host antigens on thymic epithelial cells deletes CD8⁺ cells controlling helper-independent proliferative responses and *in vivo* effector functions but spares typical helper-dependent CTL precursors. After export from the thymus, most of the CTL precursors are eliminated after contacting host antigens on stromal cells in the extrathymic environment.

It is generally agreed that self-tolerance induction to MHC molecules takes place largely in the thymus and reflects contact with bone marrow (BM)¹-derived cells, especially dendritic cells (1–4). Whether thymic epithelial cells (TEC) contribute to tolerance induction is controversial. As measured by skin allograft rejection, TEC seem to be capable of inducing near-complete tolerance of T cells (6–8). By contrast, TEC induce little or no tolerance of typical CD8⁺ CTL precursors. This is apparent from the finding that CTL precursors differentiating in thymus grafts treated with deoxyguanosine (which destroys BM-derived cells but spares TEC [9]) fail to show tolerance to the H-2 antigens of the graft (3, 10).

The notion that TEC play little or no role in tolerizing CTL precursors predicts that CD8⁺ cells differentiating from stem cells in parent → F₁ bone marrow chimeras

(BMC) thoroughly depleted of host BM-derived cells would not be tolerized to host class I antigens. In previous studies we investigated this possibility by preparing parent → F₁ chimeras with supralethal irradiation (1,300 cGy) and then leaving the chimeras for 6 mo before testing (11). The surprising finding was that, despite the apparent complete absence of host BM-derived cells, LN CD8⁺ cells from the chimeras showed near complete tolerance to the host in terms of short-term proliferative responses *in vitro*, even when supplemented with rIL-2; CTL responses were not examined. One explanation for this finding is that tolerance was imposed extrathymically through T cell contact with host class I antigens on stromal (non-BM-derived) cells.

To investigate this possibility we have now tested parent → F₁ BMC for intrathymic vs. extrathymic tolerance of CD8⁺ cells. The results presented here show that, in terms of short-term proliferative responses *in vitro* and induction of lethal GVHD on adoptive transfer, the tolerance of CD8⁺ cells to host antigens is marked and applies equally to thymocytes and peripheral T cells. Different results apply when tolerance is measured in terms of CTL generation. By limiting dilution analysis (LDA), CD8⁺ (CD4⁻) thymo-

¹ Abbreviations used in this paper: BM, bone marrow; BMC, bone marrow chimera; Dguo, deoxyguanosine; LDA, limiting dilution analysis; pf, precursor frequency; s/n, supernatant; TDL, thoracic duct lymph; TEC, thymic epithelial cells.

cytes from the chimeras show only minimal (20%) tolerance to the host. At the level of spleen and LN CD8⁺ cells, by contrast, tolerance by LDA is considerable (80%). These data suggest that tolerance of CD8⁺ cells in the chimeras occurs in both the intrathymic and extrathymic environments. The results of adoptive transfer experiments suggest that many of the host-reactive T cells in the thymus, of chimeras are in a tolerance-susceptible state. After export from the thymus, most of the cells die (or are functionally inactivated) when they encounter host class I antigens in the periphery. Protecting these cells from postthymic exposure to host antigens, i.e., by transferring BMC thymocytes to donor strain mice, allows the cells to survive.

Materials and Methods

Mice. C57BL/6 (B6), B6.PLThy-1⁺/Cy (B6.PL), CBA/CaJ (CBA), (B6 × CBA)F₁, B10.BR, B10.D2, (B6 × B10.D2)F₁, (B6 × B10.BR)F₁, and (B10.BR × B10.D2)F₁ mice were bred at the Research Institute of Scripps Clinic. All mice are Mls^b.

Irradiation. Mice were exposed to various doses of γ -ray irradiation from a ¹³⁷Cs source (0.8 Gy/min) delivered by a Gammacell 40 irradiator (Atomic Energy of Canada, Ottawa, Canada). To irradiate stimulator cells, spleen cells were exposed to 20 Gy of γ -ray irradiation from a ¹³⁷Cs source (4.5 Gy/min) delivered by a Gammacell 1000 irradiator (Atomic Energy of Canada).

Media. HBSS supplemented with 2.5% gamma globulin-free horse serum (Gibco Laboratories, Grand Island, NY) was used for preparation of single cell suspensions. DMEM supplemented with 10% FCS (Irving Scientific, Santa Ana, CA), 5% NCTC-109, 2 mM glutamine, 5 × 10⁻⁵ M 2-ME, and antibiotics was used for in vitro culture. In some experiments, exogenous lymphokines were added in the form of human rIL-2 or culture supernatant (s/n) of stimulated EL-4 lymphoma cells. Human rIL-2 was kindly provided by Dr. R. Maekawa (Shionogi Laboratories, Osaka, Japan). The EL-4 s/n contained 1,000 U/ml IL-2, 800 U/ml IL-4, and high amounts of IFN- γ (detected by Northern hybridization).

mAbs. We used mAbs specific for Thy-1 (T24, rat IgG) (12), Thy-1.2 (J1j, rat IgM) (13), Thy-1.1 (19E12, mouse IgG) (14), CD4 (GK1.5, rat IgG; and RL172, rat IgM) (15, 16), CD8 (YTS 169, rat IgG; and 3.168.8, rat IgM) (17, 18), heat-stable antigen (Hsd, rat IgM) (13), and I-A^b (28-16-8s, mouse IgM) (19).

Preparation of BMC. Host (B6 × CBA)F₁ mice were exposed to 1,300 cGy of irradiation and injected with 4–8 × 10⁶ anti-Thy-1 plus C-treated B6 or B6.PL BM cells (11). 2–6 d later, a cocktail of anti-CD8 (YTS 169), anti-CD4 (GK1.5), and anti-Thy-1 (T24) ascites fluid was injected intraperitoneally to eliminate radioresistant host T cells. Some BMC subsequently received a second dose of irradiation (900 cGy) and were reconstituted with T-depleted B6 BM cells, followed by further injection of anti-T cell mAb. All BMC were maintained on antibiotics added to the drinking water.

Purification of T Cell Subsets. As described elsewhere (20), CD8⁺ T cells were prepared from LN, thymus, or thoracic duct lymph (TDL) by treatment with anti-CD4 (RL172), anti-HSA, anti-I-A (28-16-8s) mAb plus C; where appropriate, cells were also treated with allele-specific anti-Thy-1 mAb (e.g., to remove host cells after transferring T cells to Thy-1-marked hosts). After mAb + C treatment, the surviving cells were usually positively panned on petri dishes coated with anti-CD8 (3.168) mAb (20). Panned cells were recovered by vigorous pipetting.

T Cell Filtration. BMC and control mice were exposed to 950

cGy of irradiation and then injected intravenously with thymocytes 4 h later. Thoracic duct cannulas were inserted 14 h after T cell injection, and TDL was collected on ice (21, 22).

Mixed Lymphocyte Reactions. Doses of 0.25–16 × 10⁴ responder cells were cultured in microtiter plates with 5 × 10⁵ 2,000-cGy-irradiated spleen cells as stimulators in a volume of 0.2 ml (20). Cultures were pulsed with 1 μ Ci [³H]TdR during the last 6 h of culture.

CTL Assays. To generate CTL in bulk culture, doses of 10⁶ purified CD8⁺ cells were cultured with 4 × 10⁶ 2,000-cGy-irradiated stimulator cells supplemented with 2% stimulated EL-4 supernatant. After 5 d, the cultures were harvested, adjusted to the required cell number, and tested for lysis of 10⁴ ⁵¹Cr-labeled target cells. To prepare Con A-stimulated target cells, B6, B10.BR, or B10.D2 spleen cells were stimulated for 64 h with 2.5 μ g/ml Con A and 10% (vol/vol) stimulated EL-4 supernatant. 0.5% triton X-100 was used for maximal release. In some experiments ⁵¹Cr-labeled 2C11 (anti-CD3) hybridoma cells (23) were used as targets. The percent specific lysis was calculated as: 100 × (experimental release – spontaneous release)/(maximal release – spontaneous release). Spontaneous release was always <20% of maximal release.

LDA (24) was performed to study CTL precursor frequency (CTL-pf). For each number of responder cells, purified CD8⁺ T cells were plated in 48 replicate cultures containing 5 × 10⁵ (B6 × CBA)F₁ or (B6 × B10.D2)F₁ stimulator cells with 2% stimulated EL-4 supernatant in U-bottomed 96-well microplates. CTL activity was tested using syngeneic and specific allogeneic target cells. The frequency was determined according to Poisson distribution.

Assay for Lethal GVHD. Host mice were exposed to 750 cGy of irradiation before intravenous transfer of donor T cells; to overcome Hh resistance in normal F₁ mice, these mice were injected with anti-NK 1.1 mAb (25) before T cell transfer. Antibiotics were given to the mice for the first 6 d. Mice were inspected daily until death or for 100 d. The day of death was defined as the day on which mice were unable to take food or water; such mice were killed to prevent suffering.

Results

Experimental Protocol. BMC were made by exposing (B6 × CBA)F₁ (H-2^b × H-2^k) mice to supralethal irradiation (1,300 cGy) and reconstituting the mice with T-depleted B6 (H-2^b, Thy-1.2) or B6.PL (H-2^b, Thy-1.1) BM cells. To remove residual radioresistant host T cells, the recipients were treated with a cocktail of anti-T cell mAbs shortly after reconstitution (see Materials and Methods). As documented elsewhere, BMC prepared under these conditions show apparent complete disappearance of host BM-derived cells within 2 mo postreconstitution (11). In cryostat sections, host class I expression in the chimeras is limited to low-level staining of vascular endothelium and moderate staining of the follicular dendritic cells of germinal centers (a population of non-BM-derived cells) (26). In the thymus there is moderate staining of cortical epithelium and strong staining of a subset of medullary epithelium (27). Unless stated otherwise, the BMC discussed below were left for at least 6 mo before testing. Some of the chimeras were conditioned with two rounds of irradiation and BM reconstitution; these double-irradiated chimeras received a total dose of 2,200 cGy.

Proliferative Responses of CD8⁺ Cells from LN. Prior

Table 1. *In Vitro* Proliferative Responses of LN CD8⁺ Cells from B6 → F₁ BMC

Exp.	Donors of CD8 ⁺ LN cells	No. of responders	Addition of lymphokines	³ H]TdR incorporation in response to stimulator cells:			
				B6	(B6 × CBA)F ₁	B10.D2	
		× 10 ⁻⁴		cpm × 10 ³			
1	Normal B6	8	rIL-2	1.9	53.4	59.7	
		4		1.1	27.1	40.4	
		2		0.3	14.1	13.8	
2	B6 → F ₁ BMC	8	rIL-2	1.6	4.0	36.2	
		Normal B6	16	-	0.8	60.1	55.1
			8		0.4	40.2	50.7
	4			0.4	11.8	29.4	
	2			0.2	3.5	7.3	
	8		EL-4 s/n	19.2	154.2	141.5	
	4			9.0	98.6	102.0	
	2			5.0	58.7	56.8	
	1			2.0	23.6	34.3	
	B6 → F ₁ BMC	16	-	1.4	2.4	51.4	
		8		0.9	1.1	33.7	
8		EL-4 s/n	17.4	40.2	108.2		
4			7.1	19.6	64.2		
2			3.5	7.9	31.0		
		1		2.1	4.8	14.9	

CD8⁺ cells were prepared by treating LN cells with anti-CD4, anti-HSA, and anti-I-A mAbs + C; stimulator cells (5 × 10⁵) were prepared from spleen and were exposed to 2,000 cGy. rIL-2 was added at 10 U/ml and EL-4 s/n at 0.5% final concentration. Cultures were harvested at 84 h, 6 h after pulsing with ³H]TdR. The data show the mean of triplicate cultures.

studies on tolerance of CD8⁺ cells in parent → F₁ BMC were limited to short-term proliferative responses of CD8⁺ cells prepared from LN. In confirmation of previous findings, LN CD8⁺ cells from long-term B6 BM → 1,300 cGy (B6

× CBA)F₁ BMC (henceforth abbreviated B6 → F₁ BMC) failed to proliferate in response to normal host-type F₁ spleen stimulator cells *in vitro*, even when supplemented with IL-2 (Table 1, Exp. 1); this contrasted with normal B6 CD8⁺

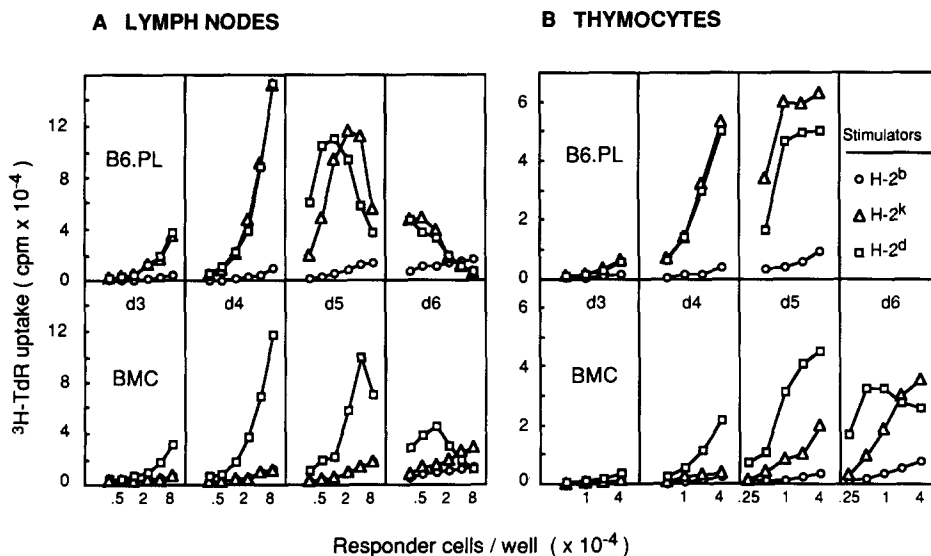


Figure 1. Proliferative responses of purified CD8⁺ cells isolated from LN (A) and thymus (B) of B6.PL → F₁ chimeras (bottom) vs. normal B6.PL mice (top). Purified CD8⁺ CD4⁻ HSA⁻ cells were prepared by a combination of mAb plus C treatment and positive panning as described in Materials and Methods. Various doses of CD8⁺ responder cells were cultured with a fixed dose of 5 × 10⁵ irradiated spleen stimulator cells. All cultures were supplemented with rIL-2 at 10 U/ml. The data show the mean of triplicate cultures pulsed with ³H]TdR 6 h before harvest.

cells, which gave strong proliferative responses even without lymphokines (Table 1, Exp. 2). Weak but significant anti-host (anti-H-2^k) responses were observed when BMC LN CD8⁺ cells were supplemented with a cocktail of lymphokines in the form of supernatant from stimulated EL-4 cells. Because of high background counts with syngeneic cells, these responses were quite low in terms of stimulation indices.

The above data refer to short-term responses measured on days 3 or 4, i.e., the time of peak responses for the control CD8⁺ cells. As shown in Fig. 1 A, similar unresponsiveness was observed when late proliferative responses were examined. In the absence of added lymphokines, BMC LN CD8⁺ cells were completely unresponsive to host antigens on days 5 and 6 of culture (data not shown). When supplemented with IL-2 (Fig. 1 A, bottom), BMC CD8⁺ cells failed to respond to host antigens on or before day 5 but gave a slight response on day 6; this response was about twofold above the background response with syngeneic stimulators.

Proliferative Responses of CD8⁺ Cells from Thymus. To study the antihost response at the level of thymocytes, BMC and control thymocytes were treated with mAb + C followed by positive panning to yield purified populations of mature CD8⁺ CD4⁻ HSA⁻ cells. Like LN CD8⁺ cells, these mature CD8⁺ BMC thymocytes gave no detectable response to host antigens in the presence of added lymphokines but responded well to third-party H-2^d antigens (data not shown). When supplemented with IL-2, mature CD8⁺

BMC thymocytes failed to respond to host antigens on day 4 (Fig. 1 B). However, low but clearly significant responses were seen on days 5 and 6. These late antihost responses were substantially higher than for BMC LN CD8⁺ cells (compare Fig. 1, A and B).

CTL Responses Generated in Bulk Cultures. Antihost CTL responses by LN and thymocytes from parent → F₁ BMC are shown in Fig. 2; using purified CD8⁺ cells as responders, CTL responses were generated in bulk culture in the presence of EL-4 supernatant and were harvested on day 5. As shown in Fig. 2 A, the CTL activity of BMC LN CD8⁺ cells was far higher against third-party target cells (B10.D2, H-2^d) than against targets expressing host antigens (B10.BR, H-2^k). CTL activity against host targets was clearly significant, however, especially at high E/T ratios, and there was no lysis of syngeneic (B6) targets. Surprisingly, in contrast to LN cells, CD8⁺ cells from BMC thymus generated strong CTL activity against host target cells. In fact, CTL activity against host targets was only slightly less than against third-party targets. In terms of "redirected" lysis, i.e., lysis of 2C11 (anti-CD3) target cells, the CTL activity of LN and thymocyte populations was quite similar (Fig. 2).

CTL Responses Assayed by LDA. LDA was used to seek quantitative information on CTLpf in thymus vs. LN. The results of LDA, using purified CD8⁺ cells as responders, are illustrated in Fig. 3 and summarized in Table 2.

In the case of LN cells, the CTLpf of normal B6 CD8⁺

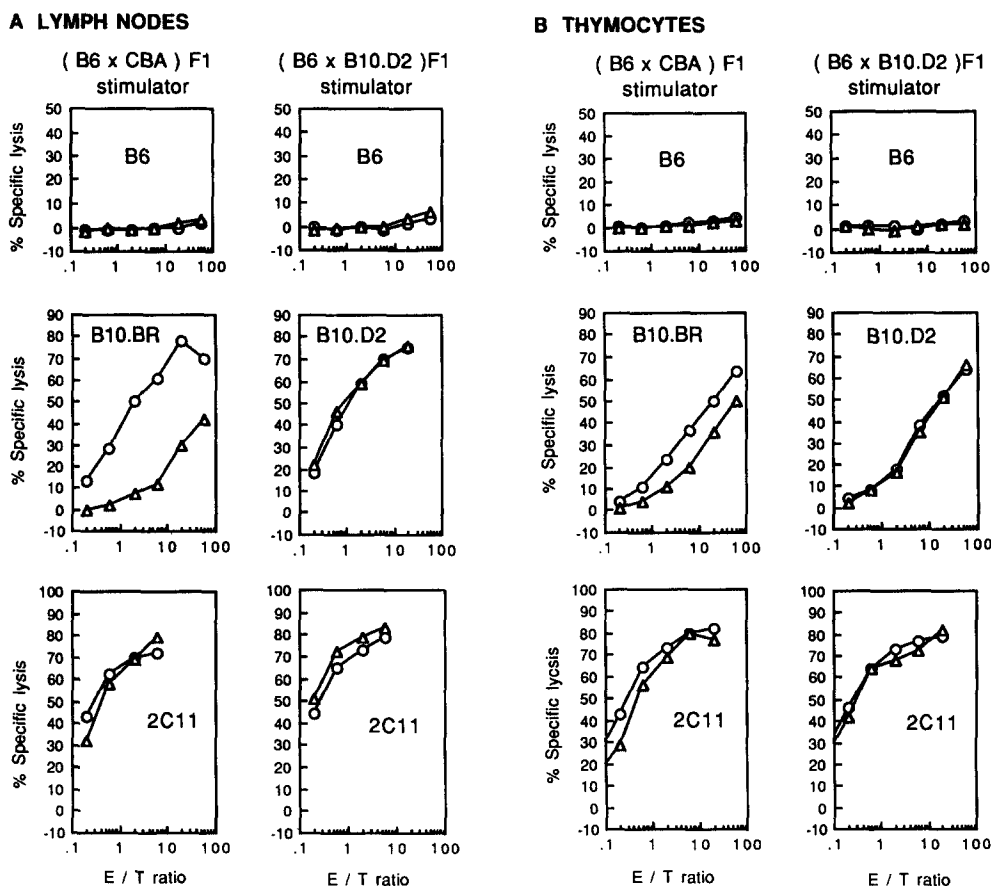


Figure 2. CTL activity of purified CD8⁺ cells prepared from LN (A) and thymus (B) of B6.PL → F₁ chimeras (Δ) vs. normal B6.PL (O) mice. As described in Materials and Methods, bulk populations of purified CD8⁺ cells were stimulated with irradiated (B6 × CBA)F₁ (H-2^k) or (B6 × B10.D2)F₁ (H-2^d) spleen stimulator cells supplemented with EL-4 supernatant as a source of lymphokines. After 5 d, CTL activity was tested on B6 (H-2^b), B10.BR (H-2^k), or B10.D2 (H-2^d) ⁵¹Cr-labeled Con A blasts as target cells; 2C11 target cells were used as a control. For each E/T ratio, the mean percent specific lysis for duplicate cultures is shown.

cells for H-2^k and H-2^d were quite similar and were generally ~1% (1/100). A typical experiment is shown in Fig. 3. For LN CD8⁺ cells from BMC, the CTLpf for host H-2^k was substantially reduced to ~0.2%, whereas the CTLpf for third-party H-2^d was unchanged or only slightly reduced. For convenience the data summarized in Table 2 are expressed in terms of the specific reduction in the CTLpf of BMC cells reactive to host H-2^k vs. third-party H-2^d: the ratio of the BMC CTLpf for H-2^k vs. H-2^d is expressed as a percentage of the control B6 CTLpf for H-2^k vs. H-2^d. For extrathymic CD8⁺ cells, studies on several different chimeras tested at ≥6 mo postreconstitution revealed a 70–80% specific reduction in host-reactive CTL precursors; this applied irrespective of whether the cells were prepared from LN, spleen, or TDL. The possibility that the tolerance of CTL precursors reflected contact with residual radioresistant BM-derived host cells was tested by comparing BMC prepared with a single dose of 1,300 cGy vs. two divided doses amounting to 2,200 cGy. Significantly, the level of tolerance in these two groups of BMC was usually indistinguishable (Fig. 3 A, Table 2).

The above data refer to extrathymic CD8⁺ cells. When mature CD8⁺ cells were prepared from BMC thymocytes, the level of specific tolerance of CTL precursors was quite limited, i.e., only 20–30% at ≥2 mo postreconstitution. This applied to BMC prepared with either one or two doses of irradiation (Fig. 3 B, Table 2). To exclude the possibility that the thymus of BMC hosts is somehow not conducive to full tolerance induction, we examined BMC reconstituted with BM cells taken from both parental strains (B6.PL + CBA/Ca BM cells). When separated with mAb + C, the H-2^b-derived mature thymocytes from these P1 + P2 → F₁ BMC showed near-complete tolerance to H-2^k (Fig. 3 C, Table 2).

The above findings indicate that, in terms of CTLpf, the tolerance of CD8⁺ cells in parent → F₁ BMC is much less prominent in the thymus than in the extrathymic environment. These data on LDA thus show a close correlation with the results of bulk culture CTL.

Stability of Tolerance of BMC CD8⁺ Cells after Adoptive Transfer to a Neutral Environment. The finding that the anti-host reactivity of BMC CD8⁺ cells is higher in the thymus

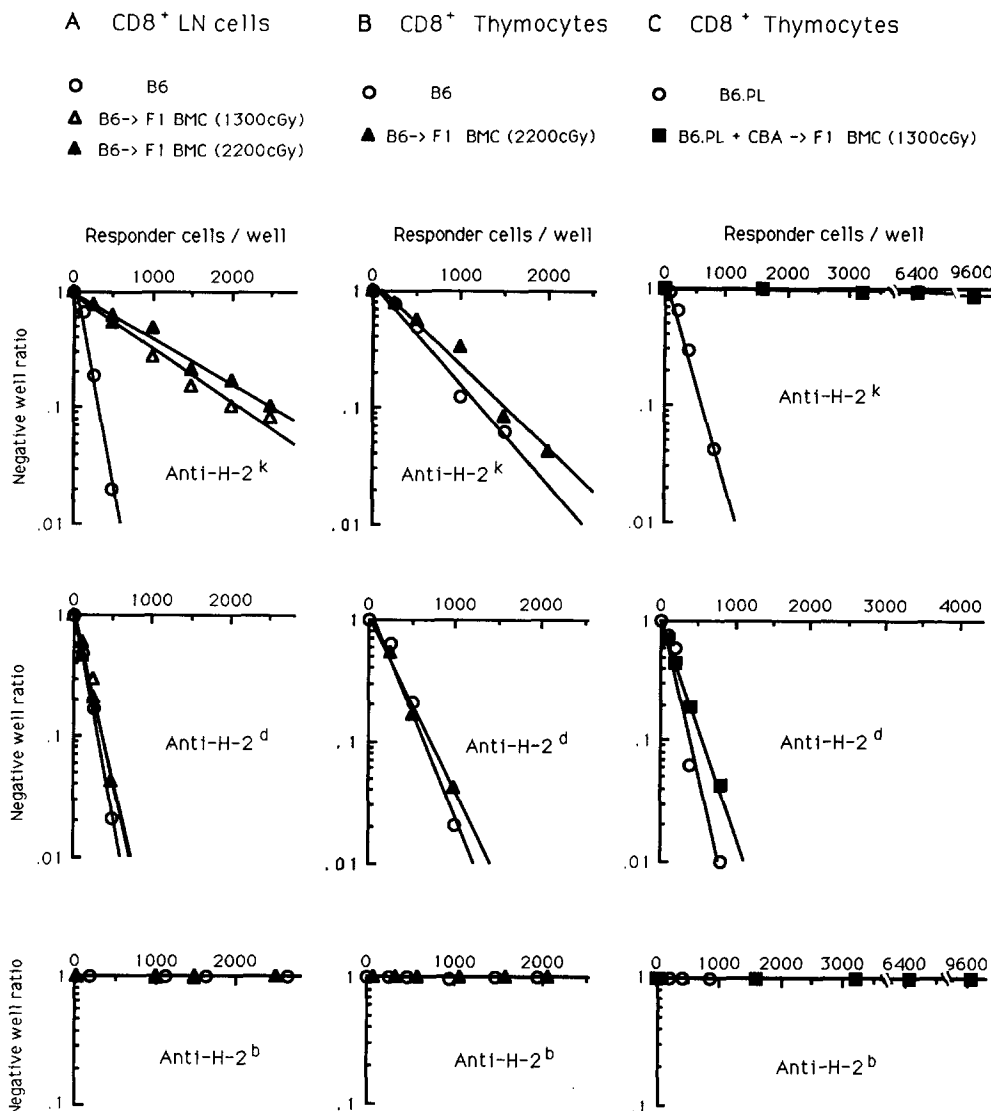


Figure 3. LDA of purified CD8⁺ cells isolated from LN (A) or thymus (B) of B6 → F₁ chimeras vs. normal B6 mice. Chimeras were prepared with either a single dose of 1,300 cGy (Δ) or two consecutive doses (4 mo apart) amounting to 2,200 cGy (▲). (C) Control chimeras were prepared by injecting F₁ mice (1,300 cGy) with a mixture of B6.PL (H-2^b) and CBA (H-2^k) BM cells (■); B6.PL-derived CD8⁺ cells from these chimeras were isolated with the aid of anti-H-2^k + anti-Thy-1.2 mAb plus C. LDA with purified CD8⁺ cells was performed as described in Materials and Methods. Precursor frequencies (×10⁻⁴) were as follows. (A) Anti-H-2^k: 63 for B6, 12 for BMC (1,300 cGy), and 10 for BMC (2,200 cGy); anti-H-2^d: 139 for B6, 105 for BMC (1,300 cGy), and 100 for BMC (2,200 cGy); (B) anti-H-2^k: 18 for B6, 14 for BMC (2,200 cGy); anti-H-2^d: 31 for B6, 32 for BMC (2,200 cGy); (C) anti-H-2^k: 33 for B6.PL, 0.23 for BMC; (C) anti-H-2^d: 51 for B6.PL, 40 for BMC. For all groups, precursor frequencies for H-2^b were all <0.20.

Table 2. Tolerance of CD8⁺ Cells from B6 → F₁ BMC: Higher Relative CTLpf for Host H-2^k in Thymus than in Peripheral Lymphoid Tissues

BMC tested	Time after BM reconstitution	Mean percent specific reduction of CTLpf for H-2 ^k (vs. H-2 ^d) in CD8 ⁺ CD4 ⁻ HSA ⁻ cells prepared from:			
		Thymus	LN	Spleen	TDL
	<i>mo</i>				
B6 → F ₁ (1,300 cGy)	≥6	33 (10)*	76 (18)	69 (3)	72 (3)
B6 → F ₁ (1,300 cGy)	1	37 (4)	90 (4)		
	2	28 (4)			
B6 → F ₁ (2,200 cGy)	≥6	17 (10)	80 (15)		
B6.PL + CBA → F ₁ (1,300 cGy)	6	99 (4)	98 (8)		

The data are summarized from 10 different experiments. In each experiment, LDA for H-2^k vs. H-2^d on cells pooled from one to four BMC was performed in parallel with LDA on cells from control B6 (or B6.PL) mice; the ratio of CTLpf for H-2^k vs. H-2^d from BMC cells was then expressed as a percentage of the ratio of CTLpf for H-2^k vs. H-2^d for the control cells. The data show the mean percentages of one to five experiments per group. With regard to third-party reactivity, the difference in CTLpf for H-2^d between BMC cells and control B6 cells was always <30%.

* No. of mice tested.

than the periphery implies that, in situ, many of the host-reactive thymocytes disappear after export to the periphery. Does this reflect exposure to host antigens in the postthymic environment? One approach to this question is to test whether the high host reactivity of BMC thymocytes is retained when the cells are transferred to a neutral environment lacking host H-2^k antigens. To examine this question, bulk populations of thymocytes taken from Thy-1-marked B6.PL → F₁ BMC vs. normal B6.PL mice were transferred intravenously to irradiated (700 cGy) B6 hosts. Donor-derived mature CD8⁺ cells (CD8⁺ Thy-1.2⁻ CD4⁻ Ia⁻ cells) pooled from two to four mice per group were recovered from LN of the host mice 9 d later. As measured by LDA, the CTLpf of these "parked" CD8⁺ cells for H-2^k and H-2^d was high for both groups of T cells. Relative to the response of the control T cells, the specific reduction in the CTLpf of the BMC CD8⁺ cells for host H-2^k (vs. H-2^d) was minimal, i.e., 28%. Parking BMC CD8⁺ thymocytes in a neutral environment thus caused little or no reduction in the reactivity of the cells for host class I antigens. In parallel experiments, the much lower host reactivity of BMC LN CD8⁺ cells remained stable when these cells were parked in B6 mice.

In other experiments, we tested whether parking BMC thymocytes in a neutral environment would allow the cells to regain antihost reactivity in terms of helper-independent proliferative responses. No such recovery was seen. Thus, when B6.PL → F₁ BMC thymocytes were parked in B6 hosts for 15 d, the proliferative response of the donor CD8⁺ cells to H-2^k (normal F₁) spleen cells in vitro was undetectable in the absence of added IL-2 (data not shown); by contrast, normal B6.PL thymocytes parked in B6 hosts produced strong helper-independent responses to H-2^k in vitro. (We use the term helper independent to refer to responses of CD8⁺ cells occurring in the absence of added lymphokines and in the ab-

sence of CD4⁺ cells. As discussed elsewhere [20], helper-independent CD8⁺ cells are presumed to produce [and utilize] their own IL-2.)

This retention of tolerance of BMC CD8⁺ cells after parking was also seen for the induction of GVHD. As shown in Fig. 4 A, normal B6.PL thymocytes caused 100% incidence of GVHD after transfer to irradiated normal F₁ hosts; this also applied to purified donor-derived CD8⁺ cells (CD8⁺ CD4⁻ Thy-1.2⁻ cells) recovered from B6 mice given bulk populations of normal B6.PL thymocytes 15 d before (Fig. 4 D). In marked contrast, even after parking in a neutral environment, B6.PL → F₁ BMC CD8⁺ thymocytes remained totally unable to cause lethal GVHD in normal F₁ hosts (Fig. 4, A and D). The same finding applied to spleen and LN CD8⁺ cells (Fig. 4, B, C, E, and F): after parking in neutral B6 hosts, BMC LN CD8⁺ cells failed to cause lethal GVHD in normal F₁ hosts (Fig. 4 E) but retained the capacity to cause GVHD in third-party class I-different (B6 × bm1)F₁ hosts (Fig. 4 F).

Collectively, the above data indicate that the level of host tolerance of BMC CD8⁺ thymocytes remains unchanged when the cells are parked in a neutral environment. The cells remain strongly tolerant in terms of helper-independent proliferative responses and GVHD induction but continue to contain a high frequency of host-specific CTL precursors. With regard to CTL precursors, the persistence of these cells after parking in a neutral environment supports the notion that the disappearance of CTL precursors after export from the thymus in situ is a reflection of direct contact with host antigens in the postthymic environment. The problem with this idea is that host class I expression in the chimeras is quite low and is restricted to non-BM-derived cells (see above). Two questions arise: Is the level of host class I expression in the chimeras sufficient to be recognized by normal parental strain

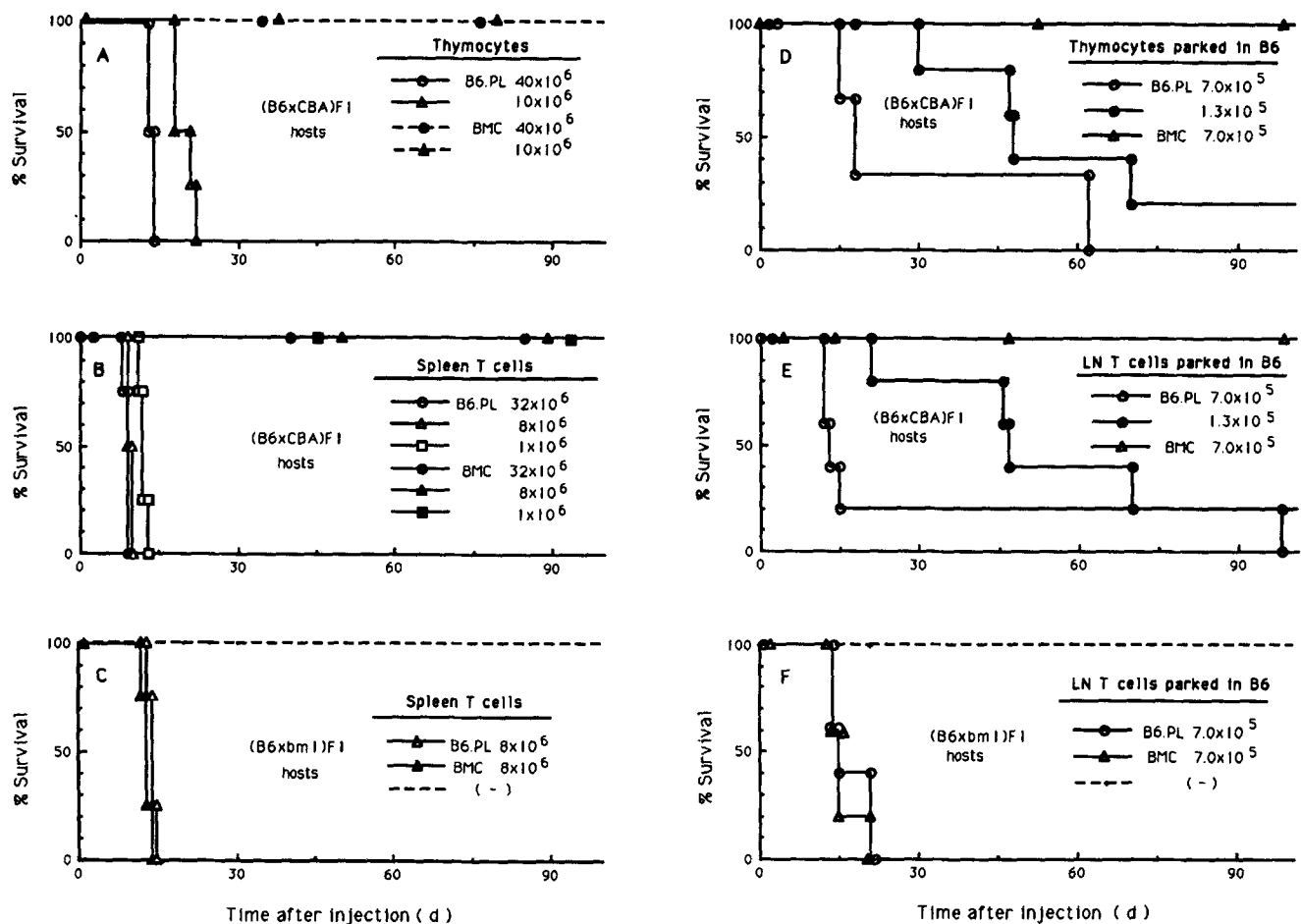


Figure 4. Capacity of $CD8^+$ cells from $B6.PL \rightarrow F_1 BMC$ vs. normal $B6.PL$ mice to cause lethal GVHD after adoptive transfer to irradiated (750 cGy) F_1 hosts. (A-C) Unseparated thymocytes or spleen T cells were transferred. (D-F) Unseparated thymocytes or LN T cells were initially transferred intravenously to Thy-1-marked B6 mice; after 15 d, purified donor (Thy-1.1)-derived $CD8^+$ cells were prepared from TDL and transferred to irradiated F_1 hosts to examine GVHD induction.

thymocytes after intravenous injection? What happens when BMC thymocytes are transferred intravenously to further BMC hosts? These questions are addressed below.

Extrathymic Expression of Host Class I Antigens in BMC Hosts Is Immunogenic for Normal Parental Strain Thymocytes. To test whether host class I expression in the chimeras is sufficient to be seen by normal T cells, the approach we used was to transfer normal B6 thymocytes to $B6 \rightarrow F_1 BMC$ hosts and then monitor the host reactivity of the donor $CD8^+$ cells recovered from TDL. As discussed elsewhere (22, 26, 28), filtering parental strain T cells from blood to lymph through normal F_1 hosts (where host antigen is presented primarily by BM-derived cells) causes selective trapping of host-reactive T cells in the lymphoid tissues. During this period of trapping (which lasts for 1-2 d) the donor cells recirculating into TDL are selectively depleted of host-reactive T cells. Subsequently, the trapped cells reenter the lymph as blast cells displaying strong antihost effector function. Does this sequence of trapping and blast cell formation apply when normal B6 thymocytes are transferred to $B6 \rightarrow F_1 BMC$?

In the experiment shown in Table 3, Exp. 1, large doses

of normal B6 thymocytes were transferred intravenously to $B6 \rightarrow F_1 BMC$ hosts, with normal F_1 mice and B6 mice as control hosts; the recipients were conditioned with irradiation (900 cGy) a few hours before T cell transfer. When B6 thymocytes were filtered from blood to lymph through B6 hosts, the few $CD8^+$ ($CD4^-$) cells recovered in TDL 1 d (18-32 h) later had a high CTLpf for both $H-2^k$ and $H-2^d$. By contrast, with filtration of B6 thymocytes through normal F_1 ($H-2^k$) hosts, the CTLpf for $H-2^k$ was specifically reduced by 99%. Significantly, this selective trapping of $H-2^k$ -reactive cells was almost as extensive when normal B6 thymocytes were filtered through $B6 \rightarrow F_1 BMC$ hosts. Here, the CTLpf for $H-2^k$ was reduced by 95%. These findings refer to donor thymocytes recovered at 1-d posttransfer. In the case of normal F_1 hosts, large numbers of B6 $CD8^+$ blasts with direct $H-2^k$ -specific CTL activity appeared in the lymph after day 3 (data not shown). These cells were also recovered in the lymph of $B6 \rightarrow F_1 BMC$ hosts, although the number of these cells was much lower. No blasts with CTL activity were recovered from B6 hosts.

The above data indicate that host class I expression in the

Table 3. LDA of CD8⁺ Thymocytes after Blood-to-Lymph Recirculation through Intermediate Hosts

Exp.	Donors of thymocytes	Irradiated recipients of thymocytes	Time of collection of TDL	CTLpf for target cells			
				H-2 ^k (B10.BR)	H-2 ^d (B10.D2)	Ratio of CTLpf for H-2 ^k vs H-2 ^d	Percent specific reduction in response to H-2 ^k
				$\times 10^{-4}$			
1	Normal B6	B6	Day 1	105	91	1.15	-
	Normal B6	Normal F ₁	Day 1	1.3	91	0.01	99
	Normal B6	B6 → F ₁ BMC	Day 1	4.5	83	0.05	95
2	Normal B6	B6.PL	Day 1	67	59	1.13	-
			Day 2	83	142	0.58	-
	B6 → F ₁ BMC	B6.PL	Day 1	8.3	37	0.23	80
			Day 2	18	71	0.26	55
	B6 → F ₁ BMC	B6.PL → F ₁ BMC	Day 1	1.5	45	0.03	97
			Day 2	2.1	63	0.03	95

Unseparated thymocytes pooled from several donors were injected intravenously (2×10^8 /mouse) into intermediate hosts (two to three mice/group) exposed to 900 cGy several hours before. The pooled lymph-borne cells collected on day 1 (16–32 h) and day 2 (40–56 h) after initial injection were treated with anti-CD4 mAb + C to prepare purified CD8⁺ cells; for Exp. 2, the cells were also treated with anti-Thy-1.1 mAb to eliminate residual host T cells. LDA on the lymph-borne cells was performed as described in Materials and Methods.

lymphoid tissues of parent → F₁ BMC is sufficient to cause extensive trapping of normal parental-strain thymocytes and to cause a small proportion of the trapped cells to undergo overt activation and differentiation.

Recognition of Host Class I Antigens by BMC Thymocytes Transferred to Secondary BMC Hosts. In view of the above findings, the question arises whether the thymocytes from B6 → F₁ BMC would be able to recognize host H-2^k antigens after intravenous transfer to further B6 → F₁ BMC. If so, would the cells be tolerated?

To examine this question, thymocytes from B6 → F₁ BMC were transferred intravenously to irradiated B6.PL hosts vs. B6.PL → F₁ BMC hosts (both Thy-1.1); as a control, normal B6 thymocytes were transferred to B6.PL hosts. The donor cells were recovered from TDL 1–2 d later and tested for H-2^k vs. H-2^d reactivity by LDA after treatment with mAb + C to prepare purified donor-derived CD8⁺ cells. The results are illustrated in Fig. 5 and summarized in Table 3, Exp. 2. With blood-to-lymph recirculation of BMC thymocytes through neutral B6.PL (H-2^b) hosts, the ratio of anti-H-2^k to anti-H-2^d CTL precursors entering TDL was unexpectedly low at day 1 posttransfer but rose to appreciable levels at day 2; at this stage there was a 55% specific reduction in H-2^k-reactive CTL precursors relative to control B6 thymocytes filtered through B6.PL hosts; this compared with the 20–30% specific reduction in H-2^k-reactive precursors found for fresh thymocytes (Table 2). These data indicate that the H-2^k (host)-reactive CTL precursors in the injected BMC populations were able to recirculate in a neutral envi-

ronment, but rather less effectively than third-party H-2^d-reactive precursors (see Discussion).

Very different results occurred when BMC thymocytes were filtered through secondary BMC hosts (Fig. 5, Table 3). In this situation the vast majority of the H-2^k-reactive thymocytes failed to recirculate. Thus, even at day 2 posttransfer, there was a 95% specific reduction in the CTLpf for H-2^k vs. H-2^d. Although LDA of the lymph-borne cells was not extended beyond day 2, the lymph was examined daily for another 4 d to determine whether the trapped H-2^k-reactive T cells differentiated into blast cells. But no blast cells were detected. This contrasted with the results of transferring control normal B6 thymocytes to BMC hosts where a proportion of the injected cells differentiated into blast cells displaying H-2^k-reactive CTL activity (see above).

The above results lead to two conclusions. First, after adoptive intravenous transfer of B6 → F₁ BMC thymocytes to secondary BMC hosts, the majority of CD8⁺ thymocytes were able to recognize host class I antigens expressed on stromal cells in the extrathymic environment. Second, such recognition caused most of these cells to disappear.

Discussion

The main aim of the experiments in this paper was to examine tolerance of CD8⁺ cells developing in parent → F₁ chimeras that were thoroughly depleted of host BM-derived cells. In this situation the donor-derived CD8⁺ cells would encounter host class I antigens in the thymus and also in the

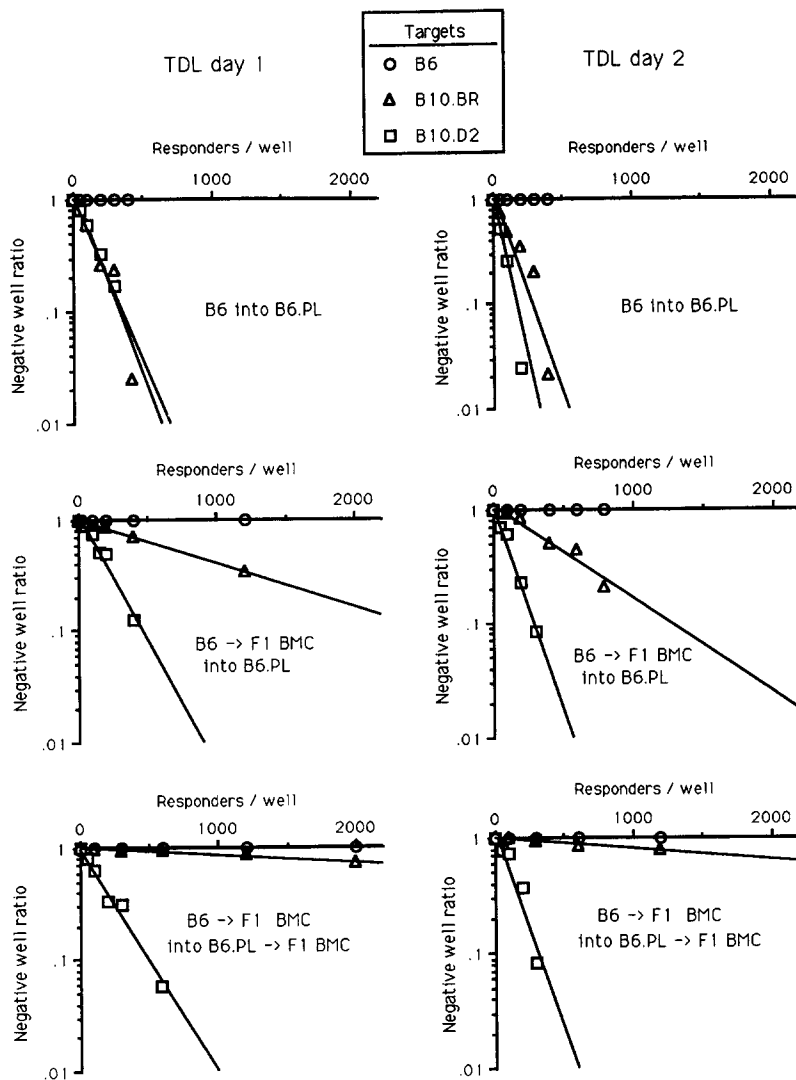


Figure 5. LDA of CD8⁺ BMC thymocytes after blood-to-lymph recirculation through intermediate irradiated hosts. Unseparated thymocytes pooled from several B6 → F₁ BMC were transferred intravenously in a dose of 10⁸ cells to B6.PL hosts vs. B6.PL → F₁ hosts exposed to 900 cGy 4 h before; as controls, thymocytes from normal B6 mice were transferred to irradiated B6.PL hosts. Thoracic duct cannulas were inserted 12 h later, and lymph was collected on ice for the following 6 d. For LDA, purified donor-derived CD8⁺ cells were prepared from lymph collected at 14–32 h (TDL day 1) and 40–60 h (TDL day 2) from two mice/group. Since the injected thymocytes consisted predominantly of immature cells, yields of mature donor-derived CD8⁺ (CD4⁻) cells in the lymph samples were very low, i.e., ~3 × 10⁵ cells/d/mouse.

post-thymic environment, but only on non-BM-derived cells. What level of tolerance would one expect to see?

With the exception of one report (29), most groups find that CD8⁺ T cells developing in BMC prepared with T-depleted marrow show full tolerance to the host in terms of CTL activity (30–32). This complete tolerance probably reflects that the chimeras were prepared with intermediate doses of irradiation (900–1,000 cGy) and were thus not totally depleted of host BM-derived cells. The chimeras used in the present study were conditioned with supralethal irradiation (1,300 cGy) and were treated with a cocktail of anti-T cell antibodies to remove residual radioresistant host T cells (a source of host class I⁺ cells). By all criteria tested, these chimeras appeared to be essentially devoid of host BM-derived cells, both in the thymus and in the extrathymic environment. Host class I expression in the thymus was restricted to cortical epithelium and a subset of medullary epithelium (27); in extrathymic sites, host class I expression was low and was limited to faint staining of blood vessel endothelial

cells and to moderate staining of the follicular dendritic cells of germinal centers (26). Despite the paucity of host class I expression, tolerance of donor-derived CD8⁺ cells in the chimeras was considerable. The most striking finding, however, was that tolerance was less marked in the thymus than in the periphery.

Tolerance in the Thymus. In keeping with the conclusion that the chimeras were devoid of host BM-derived cells, suspensions of mature CD8⁺ CD4⁻ HSA⁻ thymocytes showed only marginal (20%) tolerance to the host in terms of LDA for CTL precursors. This finding is in close agreement with the data on deoxyguanosine (Dguo)-treated thymuses (see Introduction) and thus supports the view that intrathymic contact with antigen expressed selectively on TEC causes very limited tolerance of CTL precursors (3). In other assays, however, thymocytes from the chimeras showed profound tolerance. Thus, complete unresponsiveness to host antigens was found in terms of helper-independent proliferative responses in vitro and induction of lethal GVHD on adoptive transfer;

this unresponsiveness persisted after parking the cells for 2 wk in a neutral environment. In view of this strong tolerance, how can one explain the lack of tolerance found for CTL precursors?

In considering this question, it should be emphasized that the evidence that TEC play only a minor role in tolerizing CD8⁺ cells rests heavily on studies on CTL precursors. Since generating CTL precursors for LDA in vitro usually involves adding large quantities of lymphokines, it is quite likely that most of the CTL generated in these assays are low affinity cells. Because of their low affinity, one can envisage that tolerizing (deleting) these cells in the normal thymus depends heavily on the cells encountering antigen on professional BM-derived APC, contact with TEC being insufficient to cause deletion. This would explain why the T cells differentiating in Dguo-treated thymuses (and thymuses of P → F₁ chimeras) show only a marginal reduction in typical CTL precursors: these cells avoid tolerance induction because their affinity is too low. Some CD8⁺ cells, presumably a small proportion of the cells detected in LDA, would be expected to have "above average" affinity for antigen. Our suggestion is that this minor subset of high affinity CD8⁺ cells is relatively helper independent and is largely responsible for the typical in vivo effector functions of CD8⁺ cells such as allograft rejection and induction of lethal GVHD. Because of their high affinity, we envisage that these cells are hypersusceptible to tolerance induction in the thymus and, for this reason, can be driven to undergo clonal deletion through contact with antigen expressed on TEC (see reference 33). The notion that TEC can delete a minor subset of high affinity cells would explain the paradox that T cells differentiating in thymus grafts depleted of BM-derived cells show quite strong tolerance in terms of allograft rejection (see Introduction). This model would also explain the present finding that CD8⁺ thymocytes from chimeras are strongly tolerant in terms of helper-independent proliferative responses in vitro and induction of lethal GVHD on adoptive transfer.

The above model hinges on the assumption that TEC are capable of inducing clonal deletion (if only for a small subset of T cells). Although it has been argued that the tolerogenicity of TEC is restricted to anergy induction (5, 34, 35), there is now increasing evidence that TEC are able to induce at least some level of clonal deletion (11, 36). The key question is whether such deletion is restricted to high affinity T cells. Given the difficulty of defining T cell affinity, this question is not easy to address directly. However, it is of interest that recent studies with a TCR transgenic line displaying strong helper-independent class I alloreactivity have shown that marked clonal deletion occurs when stem cells from the line differentiate in Dguo-treated thymus grafts expressing the class I antigen concerned (D. Loh, personal communication).

To recapitulate, our working model for the split tolerance found in the thymus of parent → F₁ chimeras is that T cell contact with host class I antigens expressed on TEC induces clonal deletion of a small subset of high affinity cells. Deletion of these cells causes only a minor (20%) reduction in

CTL precursors but results in marked unresponsiveness in terms of helper-independent proliferative responses in vitro and induction of lethal GVHD on adoptive transfer. The majority population of CTL precursors that evade clonal deletion by TEC are low affinity cells. These cells can respond in the presence of lymphokines in vitro but function poorly under in vivo conditions. This model presupposes that the different functional properties of helper-independent and helper-dependent CD8⁺ cells are attributable to a difference in TCR affinity. As discussed elsewhere (20), however, one cannot exclude the possibility that CD8⁺ cells fall into two functionally distinct subsets.

Tolerance in the Postthymic Environment. In marked contrast to the thymus, the level of host-reactive CTL precursors in the spleen and LN of chimeras was quite low. This is unlike the situation in Dguo thymus-grafted mice where the frequency of graft-reactive CTL precursors is as high in the extrathymic tissues as in the thymic grafts (3, 37). In considering this difference, it should be pointed out that, in Dguo thymus-grafted mice, T cell contact with allo class I antigens ceases when the cells leave the thymus grafts and enter the postthymic environment. The situation in parent → F₁ chimeras is quite different. Here, the T cells surviving tolerance induction in the thymus reencounter antigen in the periphery on host stromal (non-BM-derived) cells. Such contact presumably induces a second step of tolerance induction and causes most of the host-reactive thymic migrants to be destroyed or irreversibly inactivated.

This notion implies that the host-reactive thymocytes leaving the thymus are able to recognize the low density of host antigens expressed on non-BM-derived cells. The data on blood-to-lymph recirculation of normal and chimera thymocytes through secondary hosts are consistent with this idea. When chimera thymocytes were transferred intravenously to neutral (donor) recipients, most of the host-reactive CTL precursors were able to recirculate, albeit rather slowly (perhaps reflecting that the thymocyte suspensions were contaminated with small amounts of host antigen in the form of dissociated TEC). With transfer to secondary chimera hosts, by contrast, the striking observation was that the vast majority of the host-reactive CTL precursors failed to recirculate. This finding implies that the CTL precursors were able to recognize the low level of host antigens expressed on stromal cells and were trapped in the lymphoid tissues. Although the precise fate of the trapped cells could not be established, the finding that the lymph remained devoid of blast cells implies that most of the host-reactive cells either died or were rendered functionally anergic.

Since host class I expression in the chimeras was evident in both the intrathymic and extrathymic environments, why was tolerance induction of CTL precursors delayed until after the cells were released from the thymus? There are at least three possibilities.

(a) Host class I expression in chimeras is higher in the post-thymic environment than in the thymus. This possibility seems unlikely because the thymus of chimeras shows conspicuously high host class I expression on a subset of medullary epithe-

lial cells (27). Nevertheless, it remains possible that the accessory molecules required for tolerance induction are expressed at a lower level on thymic epithelium than on the class I⁺ stromal cells in the periphery.

(b) Host class I expression in the periphery of chimeras is intrinsically tolerogenic. Since host class I expression in chimeras is limited to non-BM-derived cells, one could argue that T cell contact with antigen on these nonprofessional APC is tolerogenic (38). In the case of mature T cells we have found no evidence for this idea. In recent studies in which virgin phenotype LN CD8⁺ cells from parental strain mice were transferred to parent → F₁ chimeras, the donor CD8⁺ cells failed to become tolerant to host class I antigens (26). Instead, a proportion of the donor cells underwent activation and differentiation into effector cells. This finding was taken to indicate that some of the host class I⁺ cells in the chimeras, e.g., follicular dendritic cells, have "semiprofessional" APC function. Despite this finding with mature T cells, it remains possible that antigen expression on non-BM-derived cells is tolerogenic for recent thymic emigrants. The main problem with this idea is that it presupposes that T cells leave the thymus in a partly immature state, for which there is no evidence.

(c) CTL precursors are partly tolerized in the thymus through contact with host antigens on TEC/CTL precursors leave the thymus in a semitolerant (anergic) state and are then fully tolerized when the cells reencounter antigen in the periphery. Since thymocytes from the chimeras were able to differentiate into effector CTL in vitro, the CTL precursors were clearly not overtly tolerized in the thymus. It has to

be remembered, however, that the CTL were generated in the presence of large quantities of lymphokines. This may have rescued the cells from tolerance induction. In vivo, by contrast, the CTL precursors would recognize antigen in the postthymic environment in the relative absence of lymphokines. This might be sufficient to induce irreversible tolerance.

Without further information, it is difficult to choose between these three possibilities. Definitive evidence on the mechanism of postthymic elimination of CD8⁺ cells in chimeras will probably have to await studies with TCR transgenic mice.

As a final point, it should be emphasized that, as in the thymus, the CD8⁺ cells in the peripheral lymphoid tissues of the chimeras were fully tolerant in terms of helper-independent proliferative responses and induction of GVHD. The notion that tolerance induction depends critically on contact with BM-derived cells would thus seem to be an oversimplification. For alloreactive T cells, our view is that intrathymic contact with antigen on BM-derived cells is only essential for tolerizing weakly reactive cells, e.g., the helper-dependent CTL generated in LDA. Contact with TEC would seem to be adequate for tolerizing CD8⁺ cells with in vivo effector functions.

In summary, the key finding in this paper is that T cell tolerance developing in parent → F₁ chimeras occurs in a step-wise fashion. Some T cells are tolerized in the thymus but others are tolerized only after export to the periphery. Whether such sequential induction of tolerance in the intrathymic and extrathymic environments is applicable to normal self-tolerance induction is still unclear.

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