STRONG T CELL TOLERANCE IN PARENT → F_1 BONE MARROW CHIMERAS PREPARED WITH SUPRALETHAL IRRADIATION

Evidence for Clonal Deletion and Anergy

By ER-KAI GAO, DAVID LO, and JONATHAN SPRENT

From the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Typical mature T cells display a subtle form of specificity for the H-2 molecules encountered in the thymus during ontogeny: extrathymic T cells respond well to self H-2 molecules complexed to exogenous antigens but generally cannot be triggered by self H-2 molecules per se. T cells with overt auto-H-2 reactivity are evident during the early stages of thymocyte differentiation, but these cells are destroyed in situ and rarely if ever reach the secondary lymphoid tissues (1). The mechanisms leading to the deletion of self H-2–reactive T cells in the thymus are poorly understood, and it is still unclear which particular cell types control this process (2). There is general agreement, however, that tolerance induction is controlled largely by bone marrow (BM)[1]-derived cells, especially APC such as macrophages and dendritic cells. Whether thymic epithelial cells contribute to tolerance induction is controversial. Most groups agree that the main function of thymic epithelium is to control positive selection of T cells, i.e., the preferential survival of T cells that display significant ("physiological") specificity for the particular H-2 molecules expressed on thymic epithelium (3–7). A key question is whether thymic epithelial cells convey only positive signals to T cells or are also capable of providing negative signals. The bulk of evidence suggests that thymic epithelial cells play only a minor role in tolerance induction (2, 8–12). In particular, studies with fetal thymus grafts depleted of APC imply that thymic epithelial cells are incapable of tolerizing $CD8^+$ CTL precursors (9–11). Nevertheless, studies with other model systems are difficult to reconcile with the view that thymic epithelium is completely nontolerogenic (13–17).

Our interest in the question of which cell types control tolerance induction stemmed from the finding that T cells generated in irradiated H-2-heterozygous ($a \times b$)F_1 mice reconstituted with a mixture of parent *a* plus parent *b* BM cells showed complete tolerance to the APC of the opposite parental strain (18, 19). If the presence

This work was supported by grants CA-38355, CA-25803, and AI-21487 from the United States Public Health Service. This is publication No. 6000-IMM from the Research Institute of Scripps Clinic.

Address correspondence to Jonathan Sprent, Department of Immunology-IMM4A, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

[1] *Abbreviations used in this paper:* BM, bone marrow; CRT, cortisone-resistant thymocytes; dguo, deoxyguanosine; GVHD, graft-vs.-host disease; LN, lymph nodes; Mig, mouse Ig; MLR, mixed lymphocyte reaction; PE, phycoerythrin.

of BM-derived cells was mandatory for tolerance induction, it was reasoned that reconstituting irradiated F_1 mice with BM cells of only one parental strain would not lead to tolerance induction. In this situation, the disappearance of host APC after irradiation would preclude tolerance induction to the host alloantigens, with the result that the donor-derived T cells differentiating in the host thymus would show strong reactivity to host-type APC in vitro. In practice, these chimeras displayed a form of split tolerance (20, 21). The donor T cells showed complete tolerance to the host in terms of CTL activity, but gave low but significant antihost responses in MLR.

Since the dose of irradiation used to prepare the chimeras used in the above studies was only 900 rad, the tolerance seen in the chimeras could have reflected T cell contact with residual host APC. Alternatively, tolerance might have been induced by non-BM-derived cells, e.g., thymic epithelial cells. To try to distinguish between these two possibilities, we have studied T cell tolerance in chimeras prepared with supralethal irradiation. Despite the apparent absence of host-type APC, the donor T cells differentiating in these chimeras show considerable (though not total) tolerance to host H-2 determinants in functional assays. The chimera T cells also display extensive clonal deletion of host-reactive $V\beta11^+$ cells. Since tolerance in the chimeras is prominent at the level of thymocytes, the data support the view that a radioresistant non-BM-derived component of the thymus, presumably thymic epithelium, can play a conspicuous role in tolerance induction.

Materials and Methods

Mice. Young (6–8 wk) (B6 × CBA/J)F_1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Young (6–8 wk) CBA/Ca, BALB/c, AKR/J, C57BL/6 (B6), B6.PL Thy-1^a, B10.BR, B6.C-H-2^{bm1} (bm1), B6.C-H-2^{bm12} (bm12), and (B6 × CBA/Ca)F_1 mice were bred at the Research Institute of Scripps Clinic.

Irradiation. Mice were exposed to various doses of irradiation from a ^{137}Cs source (85 rad/min) delivered by a Gammacell 40 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada). Cells were exposed to 1,500 rad of irradiation from a ^{137}Cs source delivered by a Gammacell 1000 irradiator (Atomic Energy of Canada Ltd.).

Media. HBSS supplemented with 2.5% gamma globulin-free horse serum (Gibco Laboratories, Grand Island, NY) was used for preparation of single cell suspensions. RPMI 1640 supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 5% NCTC 109, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics was used for culturing cells in vitro. HBSS supplemented with 1% gamma globulin-free horse serum (Gibco Laboratories) and 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) was used for immunofluorescent staining.

mAbs. The following mAbs were used: anti-Thy-1.2 (J1j, rat IgG, ascites) (22); non-allele-specific anti-Thy-1 (T24, rat IgM, ascites) (23); anti-B cell (J11d, rat IgM, culture supernatant) (22); anti-CD4 (GK1.5, rat IgG2b, ascites) (24); anti-CD8 (3.168.8, rat IgM, ascites) (25); anti-I-$A^{b(d)}$ (28-16-8S, mouse IgM, ascites) (26); anti-I-$A^{k(r,f,s)}$ (10.2.16, mouse IgG2b, ascites) (27); anti-class I K^kD^k (16-1-2N, mouse IgG2a, ascites) (28); anti-$V\beta11$ (RR3-15, rat IgG, ascites) (29); anti-$V\beta8.1$ + 8.2 (KJ16-133, rat IgG_{2a}, ascites) (30); anti-CD3 (145-2C11, hamster, ascites) (31).

Preparation of Chimeras. BM chimeras were prepared by injecting $5–10 \times 10^6$ anti-Thy-1 mAb plus C-treated (32) parental H-2-type BM cells into (B6 × CBA/J)F_1 mice subjected to an unfractionated dose of 1,300 rad irradiation 4–10 h before. Some mice received a second dose of irradiation (850–1,000 rad) plus T-depleted BM cells at 2–5 mo after initial reconstitution. In some experiments, chimeras were reconstituted with day 13 fetal liver cells; these cells were treated with a mixture of anti-Thy-1, anti-CD4, and anti-CD8 mAb + C before transfer. All chimeras were maintained on antibiotics added to the drinking water.

Purification of T Cell Subsets. Extrathymic T cells were purified from lymph nodes (LN)

using pooled cervical, axillary, inguinal, and mesenteric nodes. Cell populations containing 90–95% $CD4^+$ cells were prepared by treating LN cells with J11d plus anti-CD8 mAb + C (33); similarly, populations enriched for $CD8^+$ cells were prepared by treating LN with J11d plus anti-CD4 mAb + C. $CD8^-$ thymocytes were prepared by treating thymocytes with anti-CD8 mAb + C.

Stimulators for Mixed Lymphocyte Reactions (MLR). In most experiments, anti-Thy-1 plus C-treated spleen cells were used as stimulators (33). In some experiments, thymus suspensions were enriched for APC by separation on Percoll gradients (33, 34).

MLR. Doses of 0.5–2 × 10^5 responder cells were cultured in flat-bottomed microtiter plates with 5 × 10^5 irradiated (1,500 rad) anti-Thy-1 + C-treated spleen cells as stimulators in a volume of 200 μl, and then pulsed with 1 μCi [^{3}H]TdR 18 h before harvest (33). The data shown in the tables refer to the mean responses of triplicate cultures.

Blocking of MLR with Anti-I-A mAb. To seek information on the relative affinity of the residual host-reactive $CD4^+$ cells in parent → F_1 chimeras, doses of 2 × 10^5 $CD4^+$ cells from k → (b × k)F_1 chimeras were cultured with APC (5 × 10^5 irradiated T-depleted spleen) expressing host-type H-2^b antigens in the presence of various concentrations of anti-I-A^b mAb. In parallel, graded concentrations of anti-I-A^b mAb were added to control cultures containing H-2^b APC plus 5 × 10^4, 10^5, or 2 × 10^5 normal H-2^k $CD4^+$ cells. MLR were harvested on days 4, 5, and 6. Since the response of the chimera $CD4^+$ cells was substantially lower than the response of the control $CD4^+$ cells, the inhibitory effect of the anti-I-A^b mAb was analyzed only in cultures in which the control responses for each cell type (the responses obtained in the absence of mAb) were approximately the same in terms of change in (Δ) cpm. Thus, to compare the inhibition seen with the dose of 2 × 10^5 chimera $CD4^+$ cells, it was necessary to make a comparison with cultures containing a lower dose of the control $CD4^+$ cells, e.g., 5 × 10^4 or 1 × 10^5. The percent inhibition of MLR by the anti-I-A^b mAb was calculated according to the formula: percent inhibition = 100 × [1 − (Δ cpm with mAb/Δ cpm without mAb)]. Control cultures in which anti-I-A^b mAb was added to cultures containing H-2^k $CD4^+$ cells and H-2^s (SJL) stimulators gave no inhibition.

Assay for Lethal Graft-vs-Host Disease (GHVD). Adult mice aged 10–12 wk were exposed to heavy irradiation (1,000 rad) 4–5 h before transfer of T cells and anti-Thy-1 plus C-treated host marrow cells intravenously. Mice were inspected three times per week until death, or for 100 d. No antibiotics were given to the mice.

Skin Grafting. Ear or tail skin grafts were applied to the flank region by the method of Billingham (35).

Preparation of Cortisone-resistant Thymocytes (CRT). Mice were injected intraperitoneally with 5 mg/mouse cortisone acetate (Merck, Sharp, and Dohme, West Point, PA) 18 h before removing the thymus.

Immunofluorescent Staining and FACS Analysis. To search for host-derived lymphohematopoietic cells in parent → F_1 chimeras, lymphoid cells (0.5–1 × 10^6) from b → (b × k)F_1 chimeras were first stained with an antibody specific for host H-2 class I molecules (anti-K^kD^k) followed by FITC-labeled goat anti-mouse Ig (anti-Mig) γ chain-specific antibody (CooperBiomedical, Inc., Malvern, PA). After extensive washing, the cells were then stained with biotinylated anti-Thy-1.2 mAb or biotinylated J11d mAb followed by phycoerythrin (PE)-streptavidin (Biomeda Corp., Foster City, PA). Doses of 10^4 fresh (unfixed) cells were analyzed on a FACS IV flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

To detect T cells expressing V_β TCR molecules, lymphoid cells were stained with various anti-V_β TCR mAbs followed by FITC-labeled H and L chain-specific affinity-purified $F(ab')_2$ fragments of mouse anti-rat IgG (Pel-Freez Biologicals, Rogers, AR) or FITC-labeled anti-CD3 mAb. In most experiments, the cells were then stained with PE-labeled anti-CD4 mAb (Becton Dickinson & Co., Mountain View, CA). In some experiments, biotinylated anti-CD8 mAb followed by PE-labeled streptavidin was used for secondary staining.

Results

Unless stated otherwise, BM chimeras were prepared by exposing adult (B6 × CBA/J)F_1 (H-2^b × H-2^k) mice to an unfractionated dose of very heavy gamma irradiation, i.e., 1,300 rad, followed by reconstitution with T-depleted parental strain

(or parental H-2 type) stem cells, usually BM cells. Some chimeras received a second dose of irradiation (850–1,000 rad) given at 2–5 mo after initial reconstitution. The survival of the chimeras was quite high (≥90%), even for twice-irradiated chimeras. T cell regeneration in twice-irradiated chimeras was slow, and it was necessary to leave these chimeras for at least 2 mo in order to obtain sufficient T cells for functional assays.

To avoid confusion, it should be mentioned that many of the chimeras were prepared across a combined H-2 plus strong Mls (Mls^a) barrier, the intention being to examine tolerance to both antigens simultaneously. For simplicity, only the data on H-2 tolerance are presented in this paper.

Depletion of Host Lymphohematopoietic Cells. When parent → F_1 chimeras prepared with a single dose of 1,300 rad were tested at ≥2 mo after reconstitution, cryostat sections of the spleen, LN, gut, and skin showed a virtual absence of cells expressing a high density of host Ia molecules (not shown). Host Ia expression was clearly detectable on the cortical epithelial cells of the thymus and was conspicuous on scattered aggregates of cells in the thymic medulla. Based on several criteria, including double staining with antikeratin reagents, the medullary cells with high host Ia expression appeared to be a subset of epithelial cells. A full description of these cells will be published elsewhere.

Although previous work with BM chimeras prepared with ~1,000 rad showed that host APC disappeared rapidly after irradiation (5, 32, 36), minor survival of host APC could not be excluded. To search for functional APC in chimeras given 1,300 rad, lymphoid cells from the chimeras were tested for their capacity to stimulate MLR by normal parental strain $CD4^+$ cells. As exemplified by the experiments shown in Table I, even high doses of spleen cells or thymocytes from the chimeras failed to stimulate normal donor strain $CD4^+$ cells, but were strongly immunogenic for $CD4^+$ cells from the opposite parent.

To search for host-derived T and B cells in the chimeras, lymphoid suspensions of long-term B6 ($H\text{-}2^b$) → F_1 chimeras were stained for expression of host $H\text{-}2K/D^k$ molecules vs. Thy-1 or J11d (a B cell marker) using two-color immunofluorescence and FACS analysis. A typical experiment is shown in Fig. 1. Host-derived $H\text{-}2K/D^{k+}$ cells were very rare in the thymus (<0.5%) (not shown) but accounted for ~4% of spleen cells and ~7% of LN cells (Fig. 1, *a–c*). These cells consisted almost entirely of radioresistant T cells, since >95% of the cells were $Thy\text{-}1^+$ and $J11d^-$. These host-derived T cells disappeared when the chimeras were subjected to a second dose of irradiation (Fig. 1, *d–f*). To establish the origin of the T cells developing in these double-irradiated chimeras, the BM cells used for secondary reconstitution carried a Thy-1 marker. For example, B6 → F_1 (Thy-1.2 → Thy-1.2) chimeras were reirradiated and reconstituted with Thy-1-incompatible B6.PL $Thy\text{-}1^a$ ($H\text{-}2^b$, Thy-1.1) BM cells. These chimeras will be abbreviated "B6.PL → (B6 → F_1)." The lymphoid cells recovered from these chimeras at 3 mo after secondary reconstitution contained ≤0.2% host-derived $H\text{-}2K/D^{k+}$ cells, although significant numbers of $Thy\text{-}1.2^+$ cells were found in spleen (3%) and LN (7%) (Fig. 1, *d* and *e*). These $Thy\text{-}1.2^+$ $H\text{-}2K/D^{k-}$ cells were almost undetectable in thymus (Fig. 1 *f*) and were presumably radioresistant cells derived from the B6 BM cells used for initial reconstitution of the chimeras. The majority of the LN cells in B6.PL → (B6 → F_1) chimeras appeared to be T cells derived from the second dose of BM cells (B6.PL), since ~70% of the cells

TABLE I

Capacity of Lymphohematopoietic Cells from Parent → F_1 BM Chimeras to Stimulate Primary MLR: Failure to Detect APC of Host Origin

Exp.	Stimulators: Strain	Stimulators: Tissue source	No. of stimulators	[^{3}H]TdR incorporation with CD4$^+$ responders: B6.PL (H-2^b)	[^{3}H]TdR incorporation with CD4$^+$ responders: CBA/Ca (H-2^k)
			× 10^{-5}	*cpm × 10^3*	
1	B6.PL	Thymus	4	2.0	65.4
	CBA/Ca	Thymus	4	34.1	3.6
	(B6 × CBA/Ca)F_1	Thymus	4	58.6	49.2
	B6.PL → F_1 BMC	Thymus	4	1.9	37.0
				B6 (H-2^b)	AKR/J (H-2^k)
2	B6	Spleen	0.2	0.3	3.8
			1	0.8	24.9
			5	2.3	99.9
			10	2.3	51.6
	AKR/J	Spleen	0.2	6.0	0.4
			1	41.7	0.9
			5	138.1	2.5
			10	79.4	3.7
	AKR/J → F_1 BMC	Spleen	0.2	1.7	0.3
			1	31.2	0.5
			5	52.7	1.7
			10	109.3	2.2

The bone marrow chimeras (BMC) were tested at 2 mo after irradiation and BM reconstitution. Responder cells were CD4$^+$ cells prepared from LN (2 × 10^5/well). The stimulator cells in Exp. 1 were prepared from thymus using separation on Percoll gradients; the cells used for stimulators were taken from the ≤1.07 band of the Percoll gradients. The stimulators in Exp. 2 were anti-Thy-1 + C-treated spleen cells. All stimulators were irradiated (1,500 rad). MLR were harvested on day 4 of culture. The data show mean responses of triplicate cultures.

were J11d$^-$ Thy-1.2$^-$ and expressed either CD4 or CD8 molecules (not shown). The cells were not typed for Thy-1.1.

For functional studies with B6.PL → (B6 → F_1) chimeras, the LN T cells from these mice were treated with anti-Thy-1.2 mAb + C before use, thereby ensuring that the T cells were derived from the second dose of BM cells. Similar treatment was used to prepare T cells from reirradiated CBA/Ca → F_1 chimeras (Thy-1.2 → Thy-1.2) reconstituted with AKR/J (H-2^k, Thy-1.1) BM cells [AKR/J → (CBA/Ca → F_1) chimeras].

MLR by LN CD8$^+$ Cells. Purified LN CD8$^+$ cells from parent → F_1 chimeras invariably showed complete tolerance to host-type H-2 determinants in primary MLR ± rIL-2. The possibility that tolerance reflected contact with class I molecules on residual host T cells seems unlikely, since full tolerance was observed with twice-irradiated chimeras, i.e., mice containing no detectable host T cells. Similar tolerance was observed when long-term chimeras were given a large dose of opsonizing anti-Thy-1 mAb (37) to remove mature T cells (including host T cells) and then allowed to regenerate a new wave of CD8$^+$ cells (Table II).

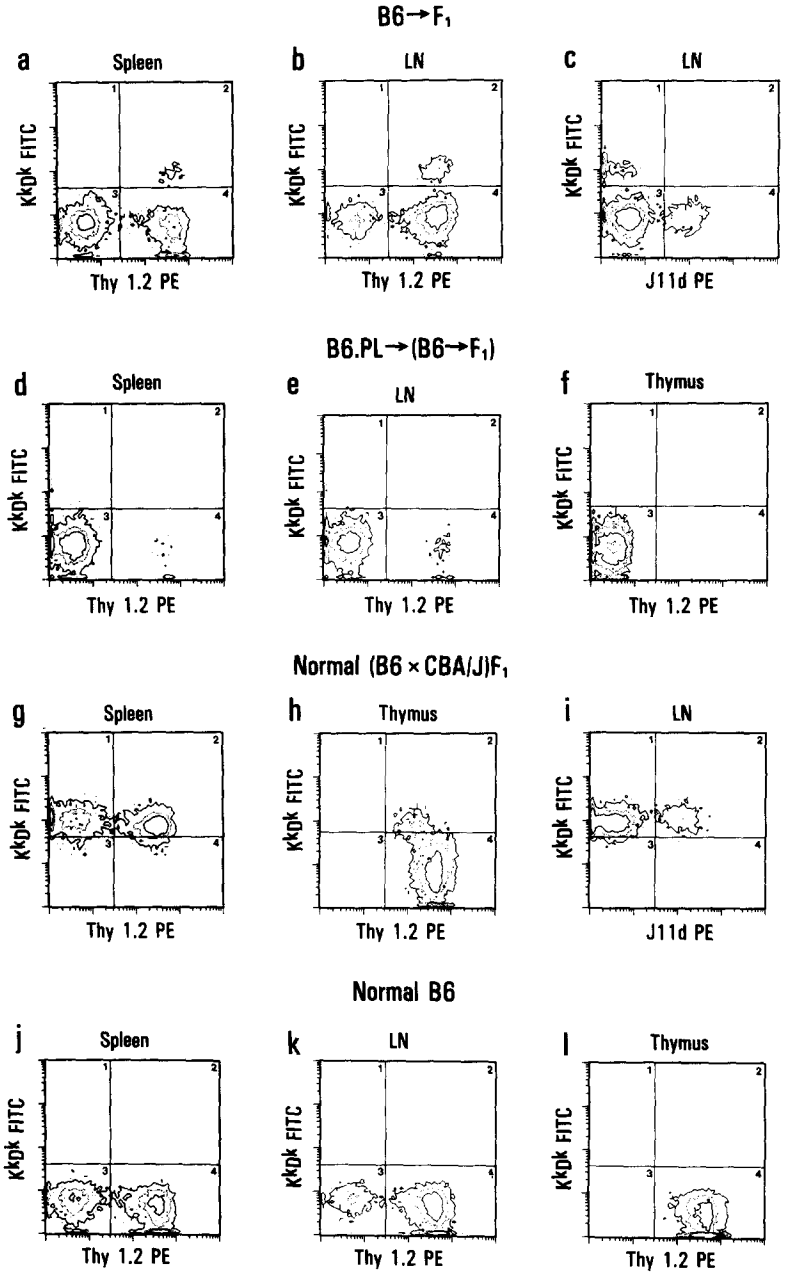

FIGURE 1. Origin of lymphoid cells in parent → F_1 chimeras. Lymphoid cells from b → (b × k)F_1 chimeras given a single dose of irradiation (*a–c*) or double irradiation (*d–f*) were stained with anti-K^kD^k (anti-host H-2 class I) mAb plus FITC anti-Mig (*y*-axis) followed by biotinylated anti-Thy-1.2 mAb or J11d mAb plus PE-streptavidin (*x*-axis) and analyzed on a FACS IV using two-channel immunofluorescence (Materials and Methods). Lymphoid cells from normal (B6 × CBA/J)F_1 mice (*g–i*) and normal B6 mice (*j–l*) were stained as controls. The B6 → F_1 chimera given a single dose of irradiation (*a–c*) was tested at 5 mo after reconstitution. It is evident that the spleen and LN of this chimera contained detectable numbers of host-derived K^kD^{k+}

TABLE II

MLR by Purified CD8⁺ Cells from B6.PL → F1 BMC: Full Tolerance to Host-type H-2 Determinants ± rIL-2

Donors of purified CD8⁺ cells	Addition of rIL-2 (3 U/ml) to MLR	[³H]TdR incorporation with spleen stimulators		
		B6.PL ($H\text{-}2^b$)	B10.BR ($H\text{-}2^k$)	bm1 ($H\text{-}2^{bm1}$)
		cpm × 10³		
B6.PL → F1 BMC	–	0.9	1.2	32.7
	+	5.5	7.9	58.6
Normal B6.PL	–	1.1	91.2	104.4
	+	8.7	164.7	152.5
Normal (B6 × CBA)F1	–	1.1	0.8	91.1
	+	10.9	10.7	149.7

BMC were depleted of mature T cells at 2 mo post-irradiation by injection of opsonizing anti-Thy-1 mAb (0.2 ml of T24 ascites fluid) (37). The mice were then left for 3 mo to generate a new wave of T cells. Purified LN CD8⁺ cells (Materials and Methods) were used as responders (2×10^5/well) in MLR using T-depleted irradiated (1,500 rad) spleen cells (5×10^5/culture) as stimulators; to inhibit the responses of any residual CD4⁺ cells in the CD8⁺ cell preparation, the cultures were supplemented with anti-CD4 mAb (0.1% of ascites fluid). rIL-2 (kindly provided by Cetus Corp., Emeryville, CA) was added to the cultures where indicated. Since CD8⁺ cells respond selectively to H-2 class I alloantigens, Ia-compatible class I (H-2K)-incompatible bm1 stimulators were used as a third-party control. MLR were measured on day 3. The data show mean responses of triplicate cultures.

MLR by LN CD4⁺ Cells. LN CD4⁺ cells from parent → F1 chimeras invariably gave low but significant MLR to APC expressing host-type H-2 determinants. However, the magnitude of this "antihost MLR" was always considerably less than the response of normal parental strain CD4⁺ cells. The features of the antihost MLR can be summarized as follows.

The magnitude of the antihost MLR by the chimera CD4⁺ cells was generally in the range of 10–50% of the response given by normal parental strain CD4⁺ cells. This was established by comparing the MLR of the chimera cells with the MLR given by graded doses of normal CD4⁺ cells or CD4⁺ cells taken from syngeneic chimeras (Table III, Exps. 1 and 3; Fig. 2). The response of the chimera CD4⁺ cells showed the typical kinetics of unprimed T cells with peak responses being observed on day 6 or later (Fig. 2 *A*). The MLR of the chimera cells to host-type H-2 determi-

cells (4% in spleen and 7% in LN). Since >95% of the K^kD^{k+} cells in the chimera were Thy-1.2⁺ and J11d⁻ (*a–c*), the cells were presumed to be radioresistant host T cells. These host cells were extremely rare in chimeras given a second dose of irradiation (*d–f*). The chimera illustrated was initially reconstituted with B6 BM, left for 2 mo, and then exposed to 850 rad followed by reconstitution with Thy-1.1⁺ B6.PL Thy-1ª BM cells; lymphoid cells were prepared at 3 mo after secondary reconstitution. It is evident that the proportion of K^kD^{k+} lymphoid cells in this twice-irradiated chimera was almost undetectable (≤0.2%) in spleen, LN, and thymus (*d–f*). However, the chimera did contain significant numbers of Thy-1.2⁺ cells (4% in spleen, 7% in LN, and 0.4% in thymus). Since these Thy-1.2⁺ cells were K^kD^{k-}, the cells were presumed to be radioresistant T cells derived from the initial inoculum of B6 (Thy-1.2) BM cells.

TABLE III

MLR by LN CD4⁺ Cells from Parent → F1 BMC: Incomplete Tolerance to Host H-2 Determinants

Exp.	Donors of purified CD4⁺ cells	No. of responders	[³H]TdR incorporation with spleen stimulators			
			AKR/J (H-2ᵏ)		B6 (H-2ᵇ)	BALB/c (H-2ᵈ)
		× 10^{-5}			cpm × 10^{-3}	
1	AKR/J → F_1 BMC	2	0.7		18.8	170.2
	AKR/J → (CBA/Ca → F_1) BMC	2	1.1		34.5	160.5
	AKR/J → AKR/J BMC	0.5	0.4		23.1	10.4
		1	0.6		94.7	42.4
		2	2.1		133.6	152.5
			CBA/Ca (H-2ᵏ)		B6 (H-2ᵇ)	BALB/c (H-2ᵈ)
2	CBA/Ca → F_1 BMC	1	2.4		12.3	106.5
	CBA/Ca → F_1 (anti-Thy-1 mAb) BMC	1	2.8		9.2	84.5
	CBA/Ca → (CBA/Ca → F_1) BMC	1	1.2		13.2	97.9
	Normal (B6 × CBA/J)F_1	1	2.1		0.9	93.3
			B10.BR (H-2ᵏ)		B6 (H-2ᵇ)	BALB/c (H-2ᵈ)
3	CBA/Ca + B6 → F_1 BMC	2	3.6		3.6	59.8
	CBA/Ca → F_1 BMC	2	1.4		18.6	109.4
	Normal CBA/Ca	0.5	0.6		4.6	6.4
		1	1.4		18.9	18.4
		2	3.6		57.2	87.3
			B10.BR (H-2ᵏ)	B6 (H-2ᵇ)	(B6 × CBA/Ca)F_1 (H-2ᵇ × H-2ᵏ)	BALB/c (H-2ᵈ)
4	CBA/J → F_1 BMC	2	3.2	35.8	11.4	109.3
	Normal CBA/J	2	6.8	94.3	60.5	105.3
5	B6 → F_1 BMC	2	8.9	2.4	15.2	89.0
	B6 → F_1 (anti-I-Aᵏ mAb) BMC	2	8.3	1.8	20.7	97.4
	Normal B6	2	39.0	7.8	69.4	70.1

The five experiments illustrated each contain data on BM chimeras prepared with a single dose of 1,300 rad; these chimeras were tested at 4 to 8 mo post-reconstitution. The twice-irradiated chimeras used in Exps. 1 and 2 received the second dose of irradiation at 3 mo after the first dose and were assayed at 3 mo after the second dose. In Exp. 1, the AKR/J → (CBA/Ca → F_1) chimeras were reconstituted with CBA/Ca BM cells after the first dose of irradiation and with AKR/J BM cells after the second dose; the CD4⁺ cells prepared from these chimeras were treated with anti-Thy-1.2 mAb + C in vitro before culture. The twice-irradiated chimeras in Exp. 2 were reconstituted with CBA/Ca BM cells after each dose of irradiation. The chimeras shown in line 2 of Exp. 2 received a single large dose of opsonizing anti-Thy-1 mAb (see Table II) at 2 mo post-irradiation and were tested 4 mo later. In Exp. 3, the chimeras reconstituted with BM cells taken from both parental strains were tested at 3 mo post-transfer, and the LN responders were treated with anti-H-2ᵇ mAb + C to obtain H-2ᵏ (CBA/Ca)-derived cells. The chimeras shown in line 2 of Exp. 5 were injected with anti-I-Aᵏ mAb (0.1 ml of 10.2.16 ascites fluid) on days 1, 4, and 7 of the first week after irradiation and tested 6 mo later. For each experiment, CD4⁺ cells were purified from individual chimeras. The data show MLR measured on day 5.

nants could be stimulated by either host-type F_1 APC or by APC of the opposite parent (Table III). With chimeras prepared with H-2ᵏ BM, responses were reproducibly higher to APC of the opposite parent than to host-type APC (e.g., Table III, Exp. 4); the reverse applied to reciprocal H-2ᵇ → F_1 chimeras (e.g., Table III,

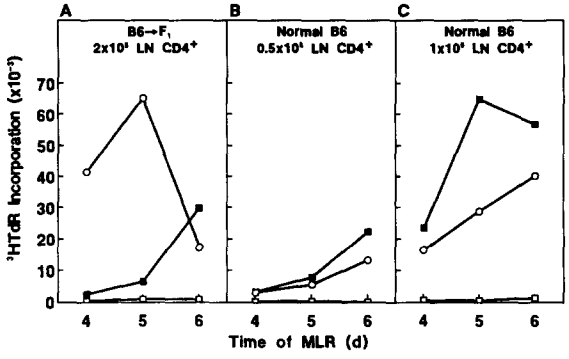

FIGURE 2. Kinetics of the antihost MLR by CD4+ cells from a B6 → F_1 chimera. LN CD4+ cells from the chimera were obtained at 4 mo after irradiation and BM reconstitution. Doses of 2 × 10^5 chimera CD4+ cells (*A*) vs. 0.5 × 10^5 or 10^5 normal B6 CD4+ cells (*B* and *C*) were cultured with 5 × 10^5 irradiated (1,500 rad) T-depleted spleen stimulator cells taken from normal B6 (□), CBA/Ca (■), or BALB/c mice (○). Cultures were harvested on days 4, 5, and 6. It can be seen that the anti-CBA/Ca response produced by the dose of 2 × 10^5 B6 → F_1 chimera CD4+ cells closely resembled the anti-CBA/Ca response given by the dose of 0.5 × 10^5 normal B6 CD4+ cells, both in terms of the magnitude of the response and the kinetics of the response.

Exp. 5). These patterns closely paralleled the responses of normal H-2^b vs. H-2^k CD4+ responders. Comparing the response to H-2-congenic B6 vs. B10.BR APC confirmed that the antihost MLR was directed to H-2 determinants rather than to non-H-2-encoded antigens (Table III). Note that none of the APC expressed strong Mls differences with respect to the responder cells.

Various manipulations designed to deplete the chimeras of any residual host-type APC had only minimal effects in increasing the magnitude of the antihost MLR. These manipulations included: (*a*) leaving the chimeras for up to 1 yr post-reconstitution and/or allowing new T cells to form in anti-Thy-1 mAb-treated chimeras; (*b*) injecting the chimeras repeatedly with anti-host Ia mAb during the first week post-reconstitution; and (*c*) subjecting the chimeras to double irradiation. None of these procedures reproducibly caused more than a minor increase in the antihost MLR (Table III). It should be noted that no antihost MLR was seen with T cells from double BM chimeras, i.e., chimeras prepared by reconstituting F_1 mice with a mixture of BM cells taken from both parental strains (Table III, Exp. 3).

To assess the possibility that the antihost MLR reflected clonal expansion of residual mature T cells in the donor marrow (which was routinely treated with a high dose of anti-Thy-1 mAb + C), we prepared chimeras with day 13 fetal liver cells. Like BM chimeras, these fetal liver chimeras showed incomplete tolerance to host H-2 determinants. In the experiment with fetal liver chimeras illustrated in Table IV, the antihost response by a dose of 2 × 10^5 chimera CD4+ cells was equivalent to the response of a two- to threefold lower dose of control CD4+ cells (CD4+ cells from syngeneic chimeras).

Inhibition of MLR by Anti-Ia mAb. To seek evidence on the relative affinity of the T cells eliciting the antihost MLR, graded doses of anti-host I-A^b mAb were added to cultures containing B6 (I-A^b I-E^-) stimulators and H-2^k-derived chimera CD4+ responders (Fig. 3). CBA/Ca → F_1 and AKR/J → F_1 chimeras were used for these studies. For controls, anti-I-A^b mAb was added to B6 stimulators cultured with normal CBA/Ca or AKR/J → AKR/J CD4+ responders. To produce responses of comparable magnitude (Δ cpm), the control CD4+ responder cells were used in two-

TABLE IV

Antihost MLR by LN CD4⁺ Cells from Twice-irradiated parent → F_1 Chimeras Prepared with Fetal Liver Cells

Donors of purified CD4⁺ cells	No. of responders	Day of assay	[³H]TdR incorporation with spleen stimulators: AKR/J (H-2ᵏ)	B6 (H-2ᵇ)	(B6 × CBA/Ca)F_1 (H-2ᵇ × H-2ᵏ)	BALB/c (H-2ᵈ)
	× 10^{-5}				cpm × 10^{3}	
AKR/J → (CBA/Ca → F_1) FLC	2	4	0.7	10.7	3.6	27.3
	2	5	2.0	23.8	11.5	78.6
AKR/J → AKR/J FLC	0.5	4	0.1	2.0	1.2	1.1
	0.5	5	0.2	11.4	3.8	3.2
	1	4	0.5	11.5	5.9	5.4
	1	5	1.6	35.4	18.9	13.9
	2	4	1.8	39.9	25.3	24.2
	2	5	4.1	80.6	65.0	64.7
Normal (B6 × CBA/J)F_1	2	4	1.4	2.0	1.7	12.1
	2	5	3.1	5.9	3.8	26.1

To prepare fetal liver chimeras (FLC), CBA/Ca → 1,300 rad (B6 × CBA/J)F_1 BM chimeras were left for a period of 5 mo, exposed to 900 rad, and reconstituted with day 13 AKR/J fetal liver (FL) cells treated with a mixture of anti-CD4, anti-CD8, and anti-Thy-1 (T24) mAb + C (Materials and Methods). CD4⁺ cells were prepared from the chimeras at 4 mo after FL reconstitution and were treated with anti-Thy-1.2 mAb + C before use. The control FLC were prepared by transferring FL cells to syngeneic AKR/J mice exposed to a single dose of 1,300 rad.

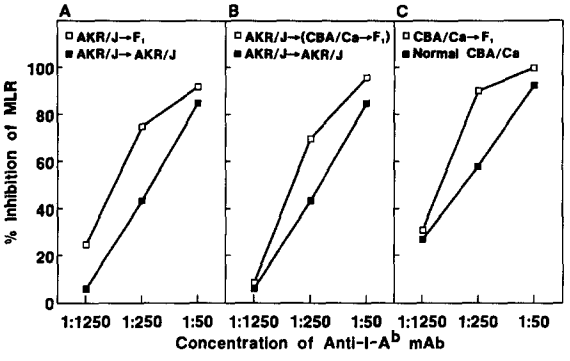

FIGURE 3. Susceptibility of the antihost MLR by chimera CD4⁺ cells to inhibition with anti-host I-A mAb. As described in Materials and Methods, doses of 2 × 10^5 chimera H-2ᵏ CD4⁺ cells were cultured with 5 × 10^5 irradiated B6 (H-2ᵇ) spleen stimulators plus graded concentrations of anti-I-Aᵇ mAb. Parallel cultures were set up with three different doses of normal B6 CD4⁺ responders, i.e., 5 × 10^4, 10^5, and 2 × 10^5. Comparison of the inhibitory effects of the anti-I-Aᵇ mAb was made only when the control response of the chimera CD4⁺ cells was comparable in magnitude (Δ cpm) with the control response given by one of the three doses of normal B6 responders. Since MLR were harvested on three different days (days 4–6), it was possible to make up to three comparisons/experiment for each chimera tested. The data shown were derived from three separate experiments conducted on a total of five chimeras (one chimera in *A*, with one comparison made on day 5 of MLR; one chimera in *B*, with two comparisons [mean shown] made on day 5 and 6 of MLR; and three chimeras in *C*, with three comparisons [mean shown] made on day 5 of MLR). In each experiment, it is evident that the antihost (anti-B6) MLR by the chimera CD4⁺ cells was more easily inhibited with anti-I-Aᵇ mAb than the anti-B6 MLR mediated by normal CBA/Ca CD4⁺ cells. The increased inhibition of the response of the chimera CD4⁺ cells was most prominent when intermediate doses of mAb were used, i.e., doses sufficient to cause ~50% inhibition of the response of the control CD4⁺ cells. It should be noted that the doses of anti-I-Aᵇ mAb used caused no inhibition of the response to SJL (H-2ˢ) APC (not shown). All of the chimeras were tested at 3–4 mo after BM reconstitution. The double-irradiated chimeras in *B* were tested at 3 mo after the second dose of irradiation.

to fourfold lower doses than the $CD4^+$ cells from the parent → F_1 chimeras (see Fig. 3 legend). The consistent finding (seen in three of three experiments) was that the anti-B6 response of the parent ($H\text{-}2^k$) → F_1 chimera $CD4^+$ cells was considerably more sensitive to inhibition with anti-$I\text{-}A^b$ mAb than the response of normal $CD4^+$ cells. The simplest explanation for this finding is that the antihost MLR by the chimera $CD4^+$ cells was mediated by low affinity cells (see Discussion).

Skin Allograft Rejection. The stimulus for allograft rejection is known to be provided by "passenger leukocytes" (APC), especially Ia^+ cells of the dendritic cell lineage (38). Since the skin of parent → F_1 chimeras was essentially devoid of cells expressing a high density of host Ia molecules (Langerhans cells), it was of interest to determine whether the chimeras could reject normal host-type skin grafts. Thus, if parent *a* → F_1 chimeras were grafted with normal parent *b* skin, would the strain *a* $CD4^+$ cells generated in the chimeras respond to the strain *b* APC of the skin grafts and lead to graft rejection? As shown in Fig. 4, experiments with both $H\text{-}2^k$ → F_1 and $H\text{-}2^b$ → F_1 chimeras showed no evidence that the chimeras could reject skin grafts of the opposite parental strain. All of the chimeras rejected control grafts expressing third-party H-2 differences or isolated H-2 class II differences (bm12) (Fig. 4). The chimeras also rejected minor H-different skin grafts, i.e., B6 → (B6 × CBA/J)F_1 chimeras rejected CBA/Ca grafts but not CBA/J grafts (tested in one experiment only; data not shown).

Induction of Lethal GVHD. The two experiments shown in Table V were designed to investigate whether $CD4^+$ cells from parent → F_1 chimeras could elicit lethal GVHD when transferred to normal host-type irradiated F_1 mice. The notable finding was that transfer of high doses, i.e., 5×10^6, of the chimera $CD4^+$ cells to host-type irradiated mice caused no mortality or signs of ill health. With transfer of normal parental strain $CD4^+$ cells, by contrast, a 10-fold lower dose of $CD4^+$ cells caused 100% mortality, and even a 50-fold lower dose of cells caused a significant incidence of GVHD, i.e., lethal GVHD in two of five mice and severe sublethal GVHD in two of the remaining three mice. The chimera $CD4^+$ cells and normal $CD4^+$ cells both caused 100% mortality when transferred to hosts expressing a third-party H-2 difference.

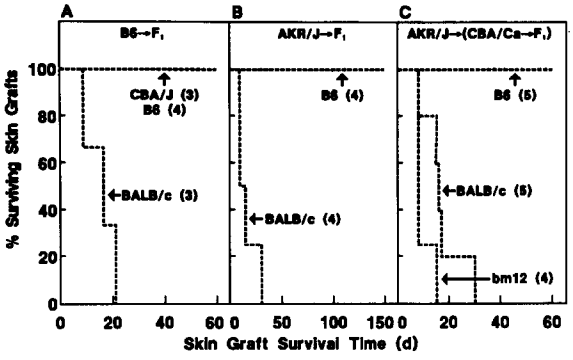

FIGURE 4. Skin graft rejection by parent → F_1 BM chimeras. Three experiments with three different batches of chimeras are shown, i.e., B6 → F_1 chimeras in *A*, AKR/J → F_1 chimeras in *B*, and AKR/J → (CBA/Ca → F_1) chimeras in *C*. For *A* and *B*, the chimeras were tested at 3 mo after irradiation and BM reconstitution; for *C*, the twice-irradiated chimeras were tested at 3 mo after the second dose of irradiation. Each chimera received two to three skin grafts taken from normal homozygous donors. The type of skin grafts applied (*arrow*) and the number of grafts examined (parenthesis) are shown in the figure. It is evident that the chimeras rapidly rejected grafts expressing third-party H-2 differences but failed to reject skin grafts of the opposite parental strain. These latter grafts remained intact until the mice were killed at 2–4 mo after grafting.

TABLE V

Lethal GVHD Mediated by CD4⁺ Cells from CBA/Ca → F1 BMC

Exp.	Donors of purified LN CD4⁺ cells transferred with host BM cells	No. of CD4⁺ cells	Recipients (1,000 rad)	H-2 stimulus	Mortality		
					Dead/ alive	Percent dead	Mean survival time
		$\times 10^{-6}$					*d*
1	CBA/Ca → F_1 BMC	5	(B6 × CBA/J)F_1	b	0/5	0	>100
		5	(CBA/Ca × BALB/c)F_1	d	5/5	100	8
	Normal CBA/Ca	0.5	(B6 × CBA/J)F_1	b	5/5	100	15
		5	(B6 × CBA/J)F_1	b	5/5	100	8
		5	(CBA/Ca × BALB/c)F_1	d	5/5	100	15
	(BM cells only)	0	(B6 × CBA/Ca)F_1	-	0/5	0	>100
		0	(CBA/Ca × BALB/c)F_1	-	0/5	0	>100
2	CBA/Ca → F_1 BMC	5	(B6 × CBA/Ca)F_1	b	0/5	0	>100
		5	(B10.D2 × B10.BR)F_1	d	5/5	100	8
	Normal CBA/Ca	0.1	(B6 × CBA/Ca)F_1	b	2/5	40	8,25, >100*
		0.5	(B6 × CBA/Ca)F_1	b	5/5	100	7
		5	(B6 × CBA/Ca)F_1	b	5/5	100	7
		5	(B10.D2 × B10.BR)F_1	d	5/5	100	19
	(BM cells only)	0	(B6 × CBA/Ca)F_1	-	0/5	0	>100
		0	(B10.D2 × B10.BR)F_1	-	0/5	0	>100

CD4⁺ cells prepared from LN of long-term (6 mo) CBA/Ca → F_1 BM chimeras were transferred intravenously into irradiated (1,000 rad 1 d before) F_1 hosts together with a dose of 5×10^6 T cell-depleted host-type BM cells.

* Two of the three surviving mice in this group developed severe GVHD before eventually recovering.

Tolerance in Thymocytes. In all of the experiments considered above, tolerance was examined at the level of extrathymic T cells, usually LN cells. To examine whether tolerance induction in the chimeras occurred intrathymically, thymocytes from the chimeras were tested for reactivity to host-type stimulators in MLR. Since MLR by unfractionated thymocytes are quite low, the thymocytes were treated with anti-CD8 mAb + C to enrich for the mature component of CD4⁺ CD8⁻ cells. Two experiments with these cells are illustrated in Table VI. The striking finding was that the CD8⁻ thymocytes from parent → F_1 chimeras responded well to third-party stimulators but showed near complete tolerance to stimulators expressing host-type H-2 determinants. This finding clearly contrasted with the significant antihost MLR mediated by CD4⁺ cells recovered from LN (Table VI, Exp. 2).

Vβ11 Expression in LN vs. Thymus. The profound host tolerance seen at the level of thymocytes raised the question whether clonal deletion of host-reactive T cells in the chimeras was greater in the thymus than in the extrathymic tissues. To examine this question, LN cells and thymocytes from parent → F_1 chimeras were tested for expression of Vβ11⁺ T cells. Vβ11⁺ T cells are selectively deleted in I-E⁺ mice (29); the deletion of these cells is near complete for CD4⁺ cells but only partial for CD8⁺ cells (39).

As shown in Table VII, Vβ11⁺ cells accounted for 5–6% of CD4⁺ cells from I-E⁻ B6 and B6.PL LN, but only 0.1% of CD4⁺ cells from I-E⁺ (B6 × CBA/J)F_1 mice

TABLE VI

MLR by CD8⁻ Thymocytes from Parent → F1 BM Chimeras: Profound Unresponsiveness to Host-type H-2 Determinants

Exp	Cell donors	Cells tested (10^5)	Day of assay	[^{3}H]TdR incorporation with spleen stimulators: CBA/Ca (H-2^k)	B6 (H-2^b)	BALB/c (H-2^d)
				cpm × 10^3		
1	CBA/Ca → F1 BMC	CD8⁻ thymus	5	0.3	0.2	18.4
	CBA/Ca → F1 (anti-Thy-1 mAb) BMC	CD8⁻ thymus	5	0.2	0.3	23.0
	CBA/Ca → (CBA/Ca → F1) BMC	CD8⁻ thymus	5	0.1	0.2	16.0
				CBA/Ca (H-2^k)	B6.PL (H-2^b)	bm12 (H-2^{bm12})
2	Normal B6.PL	CD8⁻ thymus	5	78.0	0.3	39.1
	B6.PL → (B6 → F1) BMC	CD8⁻ thymus	5	1.8	0.4	30.8
			6	3.2	0.2	62.7
		CD4⁺ LN	5	13.6	1.6	89.6
			6	63.6	1.5	10.4

In Exp. 1, line 1, the chimeras were assayed at 7 mo after reconstitution. For line 2, the chimeras were given a single large dose of anti-Thy-1 mAb (T24 ascites; 0.2 ml/mouse, i.p.) 3 mo after irradiation and BM reconstitution, and tested 4 mo after the anti-Thy-1 treatment. For line 3, CBA/Ca → F1 chimeras received a second dose of irradiation (900 rad) plus more CBA/Ca BM cells at 3 mo after initial irradiation; the chimeras were assayed at 4 mo after the second dose of irradiation. In Exp. 2, line 2, B6 → F1 chimeras received a second dose of irradiation (900 rad) plus T-depleted B6.PL BM cells at 6 mo after initial reconstitution; the chimeras were assayed at 5 mo after secondary reconstitution. For use as responder cells in MLR, thymocyte suspensions were treated with anti-CD8 mAb plus C, and LN cells were treated with anti-CD8 and Jlld plus C before culture. T-depleted spleen cells exposed to 1,500 rad were used as stimulator cells (5 × 10^5/culture).

(the hosts used for preparing the chimeras). In control experiments with double BM chimeras, i.e., CBA/Ca + B6 → F1 chimeras (Table VII, line 3), the B6-derived (H-2K^{k-}) LN CD4$^+$ cells differentiating in these chimeras showed a virtual absence of Vβ11$^+$ cells (<0.1%). By contrast, the CD4$^+$ cells differentiating in H-2^b → F1 chimeras showed incomplete deletion of Vβ11$^+$ cells. In these chimeras, Vβ11$^+$ cells accounted for 1–2% of LN CD4$^+$ cells, i.e., ~70% less than for CD4$^+$ cells from normal parental strain H-2^b mice (5–6%). The deletion of Vβ11$^+$ CD8$^+$ cells in the chimeras was variable but tended to be less extensive than for CD4$^+$ cells.

Vβ11 expression in the thymus was examined at the level of mature thymocytes, using either CRT or normal thymocytes treated with anti-CD8 mAb + C (Table VII). As in LN, mature Vβ11$^+$ CD4$^+$ cells were prominent in the thymus of I-E$^-$ H-2^b (B6.PL) mice (~4%) but were rare in I-E$^+$ (B6 × CBA/J)F1 mice (≤0.4%). Significantly, Vβ11$^+$ CD4$^+$ cells were clearly detectable in the thymus of B6.PL → F1 chimeras (1–2%), the proportion of these cells in the thymus being no lower than in LN. The marked unresponsiveness of thymocytes to host H-2 antigens in MLR thus failed to correlate with the extent of clonal deletion of Vβ11$^+$ CD4$^+$ cells.

TABLE VII

Vβ11 Expression by LN Cells and Thymocytes from Parent → F1 BM Chimeras

Cell donors	Cells tested	No. of experiments	Percent $CD4^+8^-$ cells expressing		Percent $CD4^-8^+$ cells expressing	
			Vβ11	Vβ8	Vβ11	Vβ8
B6 → F_1 BMC	Unseparated LN	2	1.6	18.4	6.2	18.4
B6.PL → (B6 → F_1) BMC	Unseparated LN	1	1.4	14.9	4.6	17.6
CBA + B6 → F_1 BMC	Unseparated LN	1	<0.1	16.9	2.8	23.9
Normal B6	Unseparated LN	3	5.7	18.0	7.5	16.4
Normal B6.PL	Unseparated LN	3	5.3	14.9	7.2	14.6
Normal (B6 × CBA/J)F_1	Unseparated LN	4	0.1	16.2	3.0	14.3
B6.PL → F_1 BMC	$Jlld^-CD8^-$ LN	3	1.2	17.9	-	-
Normal B6.PL	$Jlld^-CD8^-$ LN	3	5.3	16.7	-	-
Normal (B6 × CBA/J)F_1	$Jlld^-CD8^-$ LN	3	<0.1	14.8	-	-
B6.PL → (B6 → F_1) BMC	CRT	1	1.6	14.5	5.3	13.9
Normal B6.PL	CRT	1	3.5	17.7	6.5	11.2
Normal (B6 × CBA/J)F_1	CRT	1	<0.1	14.8	6.4	14.9
B6.PL → F_1 BMC	$CD8^-$ thymus	3	1.6	16.8	-	-
Normal B6.PL	$CD8^-$ thymus	3	4.4	16.3	-	-
Normal (B6 × CBA/J)F_1	$CD8^-$ thymus	3	0.4	18.2	-	-

Cell suspensions (pooled from one to two mice) were obtained from chimeras at 4–6 mo post-reconstitution or at 3–4 mo after the second dose of irradiation for twice-irradiated chimeras. Cell suspensions were stained with anti-Vβ mAb plus FITC-anti-Ig mAb followed by biotinylated anti-CD8 plus PE-streptavidin or PE-anti-CD4 (Materials and Methods). Stained cells were analyzed by flow cytometry. CRT were also stained with FITC-labeled anti-CD3 mAb vs. PE-anti-CD4 mAb; the data for CRT were calculated with respect to $CD3^{hi}$ cells.

Discussion

The notion that T cell tolerance to H-2 determinants reflects early T cell contact with BM-derived cells rather than thymic epithelium predicts that T cells differentiating in parent → F_1 chimeras completely depleted of host BM-derived cells would not display tolerance to host-type H-2 determinants. To test this prediction, we made concerted efforts to ensure that the chimeras examined for T cell tolerance were thoroughly depleted of host-type BM-derived cells. By all parameters tested, including staining cryostat sections with anti-Ia mAb, searching for cells able to stimulate primary MLR, and FACS analysis of lymphoid suspensions, the chimeras were essentially devoid of cells with the typical properties of APC. Radioresistant host T cells were evident in chimeras given a single dose of irradiation, but these cells disappeared after secondary irradiation. Although host-type APC were undetectable in chimeras at 2 mo after a single dose of irradiation (1,300 rad), many of the chimeras tested for tolerance were subjected to secondary irradiation and BM reconstitution.

Despite the apparent absence of host-type APC, the T cells differentiating in parent → F_1 chimeras showed profound tolerance to host-type H-2 determinants. With the exception of the antihost MLR mediated by $CD4^+$ cells, tolerance to host H-2 determinants appeared to be complete in the three other assay systems tested, i.e., MLR by $CD8^+$ cells (± IL-2), skin allograft rejection, and lethal GVHD elicited by $CD4^+$ cells. In speculating on the mechanism of tolerance induction in the chimeras, the key issue is whether tolerance was induced in the thymus or in the post-thymic environment.

For $CD8^+$ cells, tolerance could have occurred largely in the post-thymic environment, i.e., through exposure to the dense array of class I molecules expressed on host stromal (non-BM-derived) cells. Class I expression on these "nonprofessional" APC might be strongly tolerogenic, e.g., via a veto effect (40). The critical question is whether tolerance of $CD8^+$ cells in parent → F_1 chimeras is evident at the level of mature thymocytes. This question is currently under investigation.

Extrathymic tolerance of T cells in parent → F_1 chimeras is presumably less likely for $CD4^+$ cells than $CD8^+$ cells because the density of class II (Ia) expression on most non-BM-derived cells is quite low. To seek direct evidence on whether tolerance of $CD4^+$ cells can occur in the post-thymic environment, we have recently been studying tolerance in thymectomized irradiated ($a \times b$)F_1 mice given parent *a* BM cells and a parent *a* thymus graft. Provided that the thymus grafting was delayed for several months post-irradiation to allow disappearance of host APC, the strain *a* $CD4^+$ cells differentiating in the strain *a* thymus grafts showed no detectable tolerance to host strain *b* H-2 (Ia) antigens (Gao, E. K., and J. Sprent, manuscript submitted for publication). These findings make it unlikely that the tolerance of $CD4^+$ cells seen in the present study was induced extrathymically.

If the tolerance of the chimera $CD4^+$ cells occurred intrathymically, one would expect to find evidence of tolerance at the level of thymocytes. The striking finding here was that the population of mature $CD4^+$ ($CD8^-$) cells recovered from the thymus of parent → F_1 chimeras manifested almost total tolerance to host-type APC in primary MLR. Whatever the explanation for the completeness of tolerance in the thymus relative to LN (see below), these data would seem to provide firm evidence that the $CD4^+$ cells in the chimeras were tolerized within the thymus itself.

Since thymocyte suspensions from parent → F_1 chimeras were essentially devoid of typical host-type APC (Table I), we think it unlikely that the tolerance of $CD4^+$ ($CD8^-$) thymocytes to host H-2 antigens reflected intrathymic contact with residual host APC. Nevertheless, one has to consider the objection that a few host APC survived in situ but failed to enter the thymocyte suspensions used for measuring APC function in vitro. Although this remains a formal possibility, it is notable that strong intrathymic tolerance was observed in double-irradiated chimeras with a prolonged (6 mo) period between the two doses of irradiation. Since functional host APC disappeared rapidly (and apparently completely) in hosts prepared with only a single dose of irradiation, it is difficult to sustain the argument that intrathymic survival of host APC accounted for the tolerance seen in the twice-irradiated chimeras. For this reason, we think it much more likely that tolerance reflected contact with thymic epithelial cells (and/or other radioresistant non-BM-derived thymic components).

The notion that thymic epithelial cells control tolerance induction in BM chimeras clearly contrasts with the prevailing view that thymic epithelium makes little or no contribution to tolerance induction. On this point, it should be emphasized that a number of groups have claimed that thymic epithelium is at least partly tolerogenic for newly formed T cells (13–17). The main objection to these claims has been that the tolerance seen in these studies might have reflected contact with contaminating BM-derived cells. It should be stated, however, that the evidence that epithelial cells are completely nontolerogenic rests largely on studies with $CD8^+$ cells differentiating in nude mice bearing thymus grafts treated with deoxyguanosine (dguo)

(an effective method for depleting thymuses of APC). The unequivocal finding in this model is that, in the presence of exogenous lymphokines, $CD8^+$ CTL precursors differentiating in strain *a* nude mice given dguo-treated strain *b* thymus grafts show no detectable tolerance to strain *b* H-2 determinants (9, 10). We have recently confirmed this finding (Webb, S., and J. Sprent, manuscript submitted for publication). However, in marked contrast to $CD8^+$ CTL, we observed that the $CD4^+$ cells developing in nude mice bearing dguo-treated thymus grafts exhibited quite strong tolerance to graft-type APC in primary MLR. Interestingly, as in parent → F_1 chimeras (this paper), the tolerance of $CD4^+$ cells in the thymus-grafted nude mice was partial for LN $CD4^+$ cells but near complete for $CD4^+$ ($CD8^-$) thymocytes.

Collectively, the data from parent → F_1 chimeras and dguo thymus-grafted mice would seem to make a strong case that thymic epithelium is capable of inducing conspicuous tolerance at the level of $CD4^+$ cells. In speculating on the mechanism of tolerance induction by thymic epithelium, three questions arise.

Why Are Thymic Epithelial Cells Less Tolerogenic than BM-derived Cells? Although tolerance induction in parent → F_1 chimeras appeared to be complete by certain parameters, e.g., skin graft rejection and induction of lethal GVHD, $CD4^+$ LN cells from the chimeras invariably gave significant antihost responses in primary MLR. The possibility that the antihost MLR simply reflected incomplete elimination of mature T cells from the donor BM inoculum seems unlikely because the response exhibited normal kinetics and was evident with chimeras prepared with very early (day 13) fetal liver cells. Although the magnitude of the antihost MLR was variable, the responses were generally ~70% lower than the responses mediated by normal parental strain $CD4^+$ cells. In this respect, it is of interest that the $CD4^+$ cells developing in $I\text{-}E^-$ → $I\text{-}E^+$ chimeras showed ~70% reduction in $V\beta11^+$ cells. Although the correlation between the extent of $V\beta11^+$ cell deletion and the magnitude of the antihost MLR might be fortuitous, the data clearly imply that the thymic epithelial cells of the chimeras did not tolerize all host H-2-reactive $CD4^+$ cells. For full tolerance induction, contact with host H-2 determinants on APC appeared to be essential. This is evident from the finding that, in contrast to chimeras prepared with BM cells from one parental strain, T cells differentiating in double BM chimeras (*a* + *b* BM → F_1) showed complete tolerance in MLR and total elimination of $V\beta11^+$ cells (Tables III and VII).

Two models might explain why thymic epithelium is less tolerogenic than BM-derived cells. The first model rests on the assumption that H-2 tolerance is not directed to H-2 epitopes per se but to various self peptides held in the binding site of H-2 molecules. Marrack and Kappler (41) have suggested that APC and thymic epithelial cells express a different range of self peptides. Accordingly, one could argue that thymic epithelial cells are fully capable of tolerizing T cells reactive to the particular H-2/peptide complexes expressed on epithelial cells but are unable to tolerize other T cells, i.e., T cells reactive to self peptides displayed only on APC and not on thymic epithelium. In the absence of APC, these latter T cells escape tolerance induction and are allowed to exit from the thymus and reach maturity. These T cells now manifest immunity to APC and mount proliferative responses when exposed to APC in vitro. The antihost MLR mediated by LN $CD4^+$ cells from parent F_1 chimeras is thus readily explained.

The chief problem with this scenario is that it fails to explain why $CD4^+$ cells from parent → F_1 chimeras displayed complete tolerance in some assays. In light of the discrepancy between the level of tolerance seen in MLR vs. other assays, we favor the view that the cells that escape tolerance induction by thymic epithelium are simply low affinity T cells. Our suggestion here is that thymocytes with high binding affinity for H-2 epitopes (or H-2/self peptide complexes) are very sensitive to tolerance induction and can be tolerized through contact with H-2 molecules expressed either on APC or on thymic epithelium. Low affinity T cells, by contrast, are relatively resistant to tolerance induction (e.g., because of poor binding to the tolerizing cells). These T cells can only be tolerized by APC and not by thymic epithelium (perhaps because APC express a higher density of H-2 molecules and/or of certain accessory molecules). When released into the extrathymic environment of parent → F_1 chimeras, these low affinity T cells can proliferate when exposed to APC in vitro but are incapable of differentiating into effector cells, e.g., cells able to elicit GVHD. The proliferative response is thus "sterile."

Although this model accommodates the main features of the split tolerance seen in parent → F_1 chimeras, the model relies heavily on several unproven assumptions, especially the notion that high affinity T cells are required for some responses (e.g., skin graft rejection and GVHD induction), but not others (MLR). The chief problem with the model, however, is that it is basically untestable, given that there are currently no direct methods available for measuring T cell affinity. The finding that the antihost MLR by the chimera $CD4^+$ cells showed heightened susceptibility to inhibition with anti-$I\text{-}A^b$ mAb is consistent with the view that the residual host-reactive cells were of low affinity, but we do not wish to overinterpret this finding.

Why Is the Tolerance Induced by Thymic Epithelium More Marked within the Thymus than in the Extrathymic Environment? Since the deletion of $V\beta11^+$ cells in $I\text{-}E^-$ → $I\text{-}E^+$ chimeras was no more marked in thymus than LN, the profound functional tolerance observed in mature $CD4^+$ ($CD8^-$) thymocytes cannot be attributed solely to clonal deletion. The explanation we currently favor is that T cell contact with host H-2 antigens on thymic epithelial cells in the absence of host-type APC can result in two different forms of tolerance: clonal deletion and a temporary form of anergy. Since the deletion of $V\beta11^+$ cells in the chimera thymocytes was considerable (50–70%), the thymic epithelial cells of parent → F_1 chimeras presumably tolerize most host-reactive T cells by a mechanism that involves clonal deletion. We envisage that the remainder of the host-reactive T cells (perhaps low affinity cells) receive a downregulation signal from thymic epithelium. T cells affected by this signal are rendered anergic and display unresponsiveness when exposed to host APC in vitro. In the absence of host APC in the thymus, this subset of T cells is allowed to survive and exit to the periphery. Here the T cells rapidly recover from their anergic state and now manifest immunity (display an antihost MLR) when exposed to host APC.

The above scheme rests on the assumption that, when tested in MLR, the $CD4^+$ ($CD8^-$) thymocytes from the chimeras remained in a refractory (anergic) state when cultured with host-type APC. The alternative possibility is that contact with host APC in vitro caused the T cells to undergo rapid destruction. If this were the case, one could envisage that contact with thymic epithelium does not induce anergy but simply makes the T cells hypersensitive to the tolerogenic effects of APC. To assess

this second possibility, we are in the process of testing whether exposure of the chimera thymocytes to host APC in vitro results in rapid elimination of the residual $V\beta11^+$ cells.

Which Population of Thymic Epithelium Controls Tolerance Induction? Since the main function of thymic epithelium is presumably to control positive selection of T cells, the evidence that thymic epithelium also contributes to negative selection (tolerance) raises the question whether these two opposing functions operate in the same microenvironment of the thymus. Most groups have assumed that positive selection of T cells is controlled by cortical epithelial cells (3–7, 41), and direct support for this idea has come from recent studies with the ΔY line of transgenic mice (which expresses transgenic I-E molecules in cortical epithelium but not in medullary epithelium) (42). It is quite conceivable that cortical epithelial cells also induce negative selection. However, it is equally possible that the tolerogenicity of thymic epithelium is controlled by the epithelial component of the medulla (12). In this respect, it should be noted that, although some medullary epithelial cells are reported to be Ia^- (43), the medulla of long-term parent → F_1 chimeras contains dense aggregates of keratin-positive cells that coexpress a very high density of host Ia molecules (unpublished data of the authors). It is tempting to speculate that this population of medullary epithelial cells plays an important role in negative selection. The two opposing functions of thymic epithelial cells might then be strictly compartmentalized with positive selection being controlled by cortical epithelium and negative selection induced by medullary epithelium. Direct evidence on this question is needed.

Summary

T cell tolerance induction was examined in long-term H-2-heterozygous parent → F_1 chimeras prepared with supralethal irradiation (1,300 rad). Although these chimeras appeared to be devoid of host-type APC, the donor T cells developing in the chimeras showed marked tolerance to host-type H-2 determinants. Tolerance to the host appeared to be virtually complete in four assay systems: (*a*) primary mixed lymphocyte reactions (MLR) of purified lymph node (LN) $CD8^+$ cells (± IL-2); (*b*) primary MLR of $CD4^+$ ($CD8^-$) thymocytes; (*c*) skin graft rejection; and (*d*) induction of lethal graft-vs.-host disease by $CD4^+$ cells. Similar tolerance was observed in chimeras given double irradiation. The only assay in which the chimera T cells failed to show near-total tolerance to the host was the primary MLR of post-thymic $CD4^+$ cells. In this assay, LN $CD4^+$ cells regularly gave a significant antihost MLR. The magnitude of this response was two- to fourfold less than the response of normal parental strain $CD4^+$ cells and, in $I\text{-}E^- \rightarrow I\text{-}E^+$ chimeras, was paralleled by ~70% deletion of $V\beta11^+$ cells.

Since marked tolerance was evident at the level of mature thymocytes, tolerance induction in the chimeras presumably occurred in the thymus itself. The failure to detect host APC in the thymus implies that tolerance reflected contact with thymic epithelial cells (and/or other non-BM-derived cells in the thymus). To account for the residual host reactivity of LN $CD4^+$ cells and the incomplete deletion of $V\beta11^+$ cells, it is suggested that T cell contact with thymic epithelial cells induced clonal deletion of most of the host-reactive T cells but spared a proportion of these cells (possibly low affinity cells). Since these latter cells appeared to be functionally inert in the thymus (in contrast to LN), we suggest that the thymic epithelial cells

induced a temporary form of anergy in the remaining host-reactive thymocytes. This anergic state disappeared when the T cells left the thymus and reached LN.

Received for publication 7 September 1989 and in revised form 19 December 1989.

References

1. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273.
2. Sprent, J., and S. Webb. 1987. Function and specificity of T cell subsets in the mouse. *Adv. Immunol.* 41:39.
3. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 148:766.
4. Zinkernagel, R. M., G. N. Callahan, J. Klein, and G. Dennert. 1978. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature (Lond.).* 271:251.
5. Lo, D., and J. Sprent. 1986. Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature (Lond.).* 318:672.
6. Kisielow, P., H. S. Teh, H. Bluthmann, and H. von Boehmer. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature (Lond.).* 335:730.
7. Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73.
8. Jenkinson, E. J., P. Jhittay, R. Kingston, and J. J. T. Owen. 1985. Studies on the role of the thymic environment in the induction of tolerance to MHC antigens. *Transplantation (Baltimore).* 39:331.
9. von Boehmer, H., and K. Schubiger. 1984. Thymocytes appear to ignore class I major histocompatibility complex antigens expressed on thymus epithelial cells. *Eur. J. Immunol.* 14:1048.
10. von Boehmer, H., and K. Hafen. 1986. Minor but not major histocompatibility antigens of thymus epithelium tolerize precursor of cytolytic T cells. *Nature (Lond.).* 320:626.
11. Schuurman, H.-J., L. M. B. Vaessen, J. G. Vos, A. Hertogh, J. G. N. Geertzema, C. J. W. M. Brandt, and J. Rozing. 1986. Implantation of cultured thymic fragments in congenitally athymic nude rats: ignorance of thymic epithelial haplotype in generation of alloreactivity. *J. Immunol.* 137:2440.
12. Sprent, J., D. Lo, E.-K. Gao, and Y. Ron. 1988. T cell selection in the thymus. *Immunol. Rev.* 101:173.
13. Good, M. F., K. W. Pyke, and G. J. V. Nossal. 1983. Functional clonal deletion of cytotoxic T-lymphocyte precursors in chimeric thymus produced in vitro from embryonic *Analagen. Proc. Natl. Acad. Sci. USA.* 80:3045.
14. Flajnik, M. F., L. du Pasquier, and N. Cohen. 1985. Immune responses of thymus/lymphocyte embryonic chimeras: studies on tolerance and major histocompatibility complex restriction in *Xenopus. Eur. J. Immunol.* 15:540.
15. Jordan, R. K., J. H. Robinson, N. A. Hopkinson, K. C. House, and A. L. Bentley. 1985. Thymic epithelium and the induction of transplantation tolerance in nude mice. *Nature (Lond.).* 314:454.
16. Ohki, H., C. Martin, C. Corbel, M. Coley, and N. M. Le Douarin. 1987. Tolerance induced by thymic epithelial grafts in birds. *Science (Wash. DC).* 237:1032.
17. Widera, G., L. C. Burkly, C. A. Pinkert, E. C. Bottger, C. Cowing, R. Palmiter, R. L. Brinster, and R. A. Flavell. 1987. Transgenic mice selectively lacking MHC class II (I-E) antigen expression on B cells: an in vivo approach to investigate Ia gene function. *Cell.* 51:175.

18. von Boehmer, H., J. Sprent, and M. Nabholz. 1975. Tolerance to histocompatibility determinants in tetraparental bone marrow chimeras. *J. Exp. Med.* 141:322.
19. von Boehmer, H., and J. Sprent. 1976. T cell function in bone marrow chimeras: absence of host-reactive T cells and cooperation of helper T cells across allogeneic barriers. *Transplant. Rev.* 29:3.
20. Sprent, J., H. von Boehmer, and M. Nabholz. 1975. Association of immunity and tolerance to host H-2 determinants in irradiated F_1 hybrid mice reconstituted with bone marrow cells from one parental strain. *J. Exp. Med.* 142:321.
21. Iwabuchi, K., K. Ogasawara, M. Ogasawara, R. Yasumiza, M. Noguchi, L. Geng, M. Fujita, R. A. Good, and K. Onoe. 1987. A study on proliferative responses to host Ia antigens in allogeneic bone marrow chimera in mice: sequential analysis of the reactivity and characterization of the cells involved in the responses. *J. Immunol.* 138:18.
22. Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* 127:2496.
23. Fink, P. J., M. J. Bevan, and I. L. Weissman. 1984. Thymic cytotoxic lymphocytes are primed in vivo to minor histocompatibility antigens. *J. Exp. Med.* 159:436.
24. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.
25. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt antigen block T-cell-mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665.
26. Ozato, K., and D. H. Sachs. 1981. Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigen of the $H\text{-}2^b$ haplotype reveal genetic control of isotype expression. *J. Immunol.* 126:317.
27. Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigen. *Curr. Top. Microbiol. Immunol.* 81:115.
28. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
29. Bill, J., O. Kanagawa, D. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of $V_\beta 11$ bearing T cells. *J. Exp. Med.* 169:1405.
30. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.* 160:452.
31. Bluestone, J. A., D. Pardoll, S. O. Sharrow, and B. J. Fowlkes. 1987. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature (Lond.).* 362:82.
32. Ron, Y., D. Lo, and J. Sprent. 1986. T cell specificity in twice-irradiated F_1 → parent bone marrow chimeras: failure to detect a role for immigrant marrow-derived cells in imprinting intrathymic H-2 restriction. *J. Immunol.* 137:1764.
33. Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068.
34. Ratcliffe, J. J. H., and M. H. Julius. 1982. H-2-restricted T-B cell interactions involved in polyspecific B cell responses mediated by soluble antigen. *Eur. J. Immunol.* 12:634.
35. Billingham, R. E. 1961. Free skin grafting in mammals. *In* Transplantation of Tissues and Cells. R. E. Billingham and W. K. Silvers, editors. Wistar Institute Press, Philadelphia, PA. 87–106.

36. Kyewski, B. A., C. G. Fathman, and H. S. Kaplan. 1984. Intrathymic presentation of circulating non-major histocompatibility complex antigens. *Nature (Lond.).* 308:190.
37. Sprent, J., M. Schaefer, D. Lo, and R. Korngold. 1986. Properties of purified T cell subsets. II. In vivo responses to class I vs. class II H-2 differences. *J. Exp. Med.* 163:998.
38. Lafferty, K. J., S. J. Prowse, C. J. Simeonovic, and H. S. Warren. 1983. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Annu. Rev. of Immunol.* 1:143.
39. Gao, E.-K., O. Kanagawa, and J. Sprent. 1989. Capacity of unprimed $CD4^+$ and $CD8^+$ T cells expressing $V_\beta 11$ receptors to respond to I-E alloantigens in vivo. *J. Exp. Med.* 170:1947.
40. Rammensee, H.-G., P. J. Fink, and M. J. Bevan. 1984. Functional clonal deletion of class I-specific cytotoxic T lymphocytes by veto cells that express antigen. *J. Immunol.* 133:2390.
41. Marrack, P., and J. Kappler. 1987. The T cell receptor. *Science (Wash. DC).* 238:1073.
42. Berg, L. J., A. M. Pullen, B. F. de St. Groth, D. Mathis, C. Benoist, and M. M. Davis. 1989. Antigen/MHC specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell.* 58:1035.
43. Jenkinson, E. J., W. van Ewijk, and J. J. T. Owen. 1981. Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice. *J. Exp. Med.* 153:280.