SPLIT TOLERANCE INDUCED BY THE INTRATHYMIC ADOPTIVE TRANSFER OF THYMOCYTE STEM CELLS

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Thymocytes originate from multipotential hematopoietic stem cells in the bone marrow and mature in the thymus into antigen-reactive T lymphocytes which then migrate to the peripheral lymphoid organs (1, 2). In contrast to antibodies that bind native antigen, the antigen receptors expressed on T lymphocytes (TCR) recognize foreign antigen physically associated with syngeneic cell surface products of the MHC (3, 4). This genetic restriction of T cell specificity is established in the thymus, where the differentiating thymocyte population is depleted of cells bearing self-reactive TCR, while T cells which express receptors restricted in their recognition of foreign antigen in association with self-MHC class I (K, D, L) and class II (I-A, I-E) gene products are allowed to mature (5–8). The molecular and cellular events involved are presently unclear, but the most direct and tenable hypothesis suggests that thymocytes become self-tolerant and learn self MHC-restricted antigen responsiveness by means of TCR-mediated interactions with other cells that reside in the thymus (9, 10). It has been demonstrated that thymocytes can learn self-MHC class II–restricted antigen recognition on thymic stromal elements (11), presumably thymic epithelial cells. MHC class I–restricted antigen recognition by cytotoxic T lymphocytes (CTL) can be learned from radiation-resistant, long-term resident cells (12, 13), again presumably thymic epithelial cells. However, thymic epithelial cells do not appear to induce MHC class I or class II tolerance efficiently (14, 15). As a consequence, it has been speculated that bone marrow–derived cells, particularly dendritic cells and macrophages, are the tolerogens for self-MHC reactivity in the thymus (16).

Whereas peripheral murine T lymphocytes express either L3T4 (CD4) or Ly-2 (CD8), surface molecules that may have a role in antigen recognition, thymocyte stem cells are negative for both markers (17). Phenotypic analyses have shown that immature thymocytes acquire CD4 and/or CD8 as they differentiate intrathymically (18, 19). The intrathymic transfer of CD4/CD8 double-negative (DN)[1] thymocytes into lethally irradiated and syngeneic bone marrow–reconstituted adoptive hosts results in a transient wave of thymus and peripheral lymphoid organ recolonization by donor-derived cells, followed by a permanent recolonization effected by cells of host bone marrow origin (20). A consequence of this experimental protocol is that host bone marrow–derived stem cells differentiate in the presence of the intrathymi-

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[1] *Abbreviations used in this paper*: CTLp, CTL precursor; DN, CD4/CD8 double-negative thymocyte; LDA, limiting dilution analysis.

cally injected donor cells. Here we show that in radiation chimeras that were established by the intrathymic injection of semi-allogeneic DN cells, the repopulating host-derived thymocytes are tolerant of the MHC class I but not class II antigens of the allogeneic DN donor (i.e., split tolerance). These results suggest that, in addition to macrophages and dendritic cells, Thy-1$^+$ cells, i.e., T cells, can induce intrathymic tolerance.

Materials and Methods

Animals. AKR/J, BALB/cByJ, B6.PL-Thy-1a/cy (B6.PL), B6.C-H-2^{bm1} (bm1), B6.C-H-2^{bm5} (bm5), B6.C-H-2^{bm12} (bm12), C57BL/6J, (AKR/J × B6.PL)F_1, (bm5 × bm12)F_1, (bm1 × C57BL/6J)F_1, (bm12 × C57BL/6J)F_1, and (B10.BR × B10.D2)F_1 mice were bred in the Research Institute of Scripps Clinic animal facility using stock originating from The Jackson Laboratory, Bar Harbor, ME. For intrathymic injections, mice for use as donors or hosts were 4–6 wk old and sex-matched. The MHC and Thy-1 haplotypes of the mice used in these experiments are listed in Table I.

Antibodies. The rat IgM mAbs RL-172.4 (anti-CD4, reference 21) and 3.168 (anti-CD8, reference 22) were used for the complement-mediated depletion of CD4$^+$ and CD8$^+$ cells. To discriminate Thy-1 alloantigen on the FACS, 19E12 (IgG anti-Thy-1.1, reference 23) and biotinylated HO13-4 (IgM anti-Thy-1.2, reference 24) were used. The pan-Thy-1-specific mAb T24 (IgG, reference 25) was used for the enumeration of total Thy-1$^+$ cells. For detection of primary antibody staining on the FACS, an affinity-purified goat anti-mouse IgG Fc-specific fluoresceinated second reagent, which does not react with B cells or IgM, was used (Cappel Laboratories, Malvern, PA). Staining of CD4$^+$ and CD8$^+$ cells was by use of phycoerythrin-conjugated GK-1.5, (reference 26, Becton Dickinson & Co., Mountain View, CA) and fluoresceinated 3.168, respectively.

Construction of Intrathymically Established Radiation Chimeras. Four strain combinations were used in these experiments; [(AKR/J × B6.PL)F_1 → C57BL/6J], [(bm5 × bm12)F_1 → B6.PL], [(bm1 × C57BL/6)F_1 → B6.PL], and [(bm12 × C57BL/6)F_1 → B6.PL]. Thymocytes from adult donor mice were depleted of CD4$^+$ and CD8$^+$ cells by two cycles of treatment with specific antibody plus guinea pig complement. Cell yields varied in individual experiments from 0.8–1.4% of the original population. Subsequent FACS analysis for CD4 and CD8 indicated the elimination of bright-positive cells. Initial experiments were performed using total DN thymocytes as donor cells, whereas in later experiments FACS-sorted Thy-1$^+$ CD4/CD8 DN cells were transferred. For FACS sorting, DN thymocytes were stained with the T24 antibody followed by fluoresceinated anti-IgG Fc-specific second reagent. Cell sorting was performed on a Becton Dickinson & Co. FACStar using forward and side scatter gates to exclude debris and set at a rate of 2–3,000 events/second. In a typical experiment, 2–4 × 10^6 DN donor thymocytes were transferred intrathymically into adoptive host mice that had received prior irradiation (950 rad) and intravenous injection with 4–5 × 10^6 syngeneic Thy-1-depleted bone marrow cells. Controls were age- and sex-matched irradiated, bone marrow-reconstituted mice that did not receive an intrathymic injection.

Analysis of Intrathymically Established Radiation Chimeras. At the indicated times after intrathymic transfer, mice were killed and thymus, spleen, and lymph node (axial, brachial, cervical, inguinal, maxillary, and periaortic) cells were recovered. Aliquots of cells were reserved for FACS analysis before the depletion of DN donor Thy-1 alloantigen-positive cells. Complement-mediated depletion of Thy-1.1$^+$ cells was performed using the 19E12 antibody, and depletion of Thy-1.2$^+$ cells using HO13.4. The cell populations were counted before and after treatment to ascertain the extent of depletion, as were control cell populations positive for antigen expression. In every experiment, the treated cell populations were analyzed on the FACS for residual Thy-1 alloantigen-positive cells that, unless stated, were <2.0%.

Mixed Lymphocyte Culture. Bulk cultures for induction of CTL were set up with 25 × 10^6 lymph node plus spleen responders and 25 × 10^6 irradiated spleen stimulator cells (15 × 10^6 irradiated cells of each H-2 haplotype when mixed stimulators were used) in 20 ml RPMI-1640 containing 5% FCS, 50 μM 2-ME, 2 mM glutamine, 5 mM Hepes, and antibiotics

TABLE I
MHC and Thy-1 Haplotypes of Mice Used

Strain	Alleles at H-2 loci				Thy-1 allele
	K	I-A	I-E	D,L	
AKR/J	k	k	k	k	1.1
BALB/cByJ	d	d	d	d	1.2
B6.PL-Thy-la/cy	b	b	–	b	1.1
B6.C-H-2^{bm1}	bm1	b	–	b	1.2
B6.C-H-2^{bm5}	bm5	b	–	b	1.2
B6.C-H-2^{bm12}	b	bm12	–	b	1.2
(AKR/J × B6.PL)F_1	k/b	k/b	k/b	k/b	1.1
(bm1 × C57BL/6J)F_1	bm1/b	b	–	b	1.2
(bm5 × bm12)F_1	bm5/b	bm12/b	–	b	1.2
(bm12 × C57BL/6J)F_1	b	bm12/b	–	b	1.2
(B10.BR × B10.D2)F_1	k/d	k/d	k/d	k/d	1.2

in an upright culture flask. Thymocyte MLC were set up similarly, except that twice the number of responder cells were cultured and an exogenous source of IL-2 (5% supernatant of 10 ng/ml PMA-stimulated EL-4 cells) was added. Bulk MLC were harvested on day 5, counted, and assayed for cytoxicity of 3 d Con A–stimulated lymph node targets, or the tumor lines CBA.D1 (H-2^k, of CBA/J origin), EL-4 (H-2^b C57BL/6 lymphoma), and P815 (H-2^d DBA/2 mastocytoma). Cytotoxicity assays were of 4–5 h duration in 96-well round-bottomed plates using 10^4 ^{51}Cr-labeled targets/well.

Micro-MLC for cell proliferation assays were set up in 96-well flat-bottomed plates using 2–4 × 10^5 lymph node responder cells and 5 × 10^5 3,000 rad irradiated splenic stimulators per well. After 4 d culture the cells were pulsed for 8 h with 1 μCi [^{3}H]thymidine, harvested, and the cell-incorporated radioactivity was quantitated.

For limiting dilution assay (LDA) of CTL precursors, spleen plus lymph node responders were set up in 96-well round-bottomed plates at the indicated concentrations per well using 24 replicate microcultures per group. 5 × 10^5 irradiated spleen stimulator cells were added, along with 5% (final concentration) supernatant of Con A–stimulated rat spleen cell culture to which α-methyl-mannoside had been added. After 10 d of incubation, cultures were split three ways to assay cytotoxicity of 3 d Con A–stimulated lymph node blast targets as described for bulk MLC assays.

All assays (except LDA) were performed in triplicate with the standard error of the mean indicated for proliferation assays. For cytotoxicity assays the standard errors were omitted in order to maintain clarity in the figures and were always <5% of the indicated responses.

Results

Repopulation of Irradiated Adoptive Hosts by the Progeny of Semiallogeneic DN Thymocytes. These experiments were designed to study what effects, if any, the presence of MHC-allogeneic T lymphocytes might have upon the regenerating T cell repertoire. In preliminary experiments, total CD4/CD8 DN thymocytes were used as donors for intrathymic transfer. Subsequent experiments used donor DN cells that were FACS sorted on the basis of Thy-1 expression to deplete macrophages, dendritic cells, and other cell types that might complicate the interpretation of the results (see Discussion).

Intrathymic transfer of MHC heterozygous donor DN thymocytes into lethally irradiated, MHC-homozygous hosts resulted in their transient repopulation of the thymus and peripheral lymphoid organs. The progeny of DN semiallogeneic donor cells expanded to maximal numbers in adoptive host thymuses at ~10–15 d after transfer and thereafter declined in number in the thymus. DN donor cell progeny

TABLE II

Repopulation of Lethally Irradiated, Syngeneic Bone Marrow-protected C57BL/6 Mice by the Progeny of Intrathymically Injected (AKR/J × B6.PL)F_1 DN Donor Cells

	Percent Thy-1.1 alloantigen-positive cells on day post-transfer					
Organ	10	20	31	49	71	110
Thymus	67	7.0	0.9	1.0	1.6	0.0
Lymph nodes	4.2	8.1	20	11	7.0	2.9
Spleen	0	3.0	9.0	6.0	6.2	1.5

Thymus, spleen, and lymph node cells were recovered on the indicated days after transfer and stained for the Thy-1.1 alloantigen of the DN donor for analysis on the FACS. The data is a compilation of three different experiments that all used 4×10^6 total DN thymocytes as intrathymic donors. The percentages of fluorescence-positive cells are corrected for nonspecific second reagent staining, which was always <2.0%.

could be detected in the lymph nodes and spleens of adoptive hosts as early as 10 and 20 d, respectively, and persisted for ~110 d after transfer (Table II) as we have reported previously for MHC-matched transfers (20). Repopulation of adoptive hosts by the progeny of intrathymically injected FACS-sorted Thy-1$^+$ semiallogeneic DN progeny showed similar kinetics. DN thymocytes showed an accelerated repopulation in comparison to bone marrow cells used in the original studies of Goldschneider et al. (27).

CTL Tolerance Induced by Semiallogeneic DN Thymocytes. Hematopoietic cells (bone marrow and spleen) have been used extensively for inducing transplantation tolerance in neonatal and irradiated animals. We wished to study the effect on a regenerating immune system of the committed T stem cell population found in the thymus. As we have shown, DN thymocytes give a pulse of T cell production in the thymus of irradiated mice and some mature progeny populate the peripheral lymphoid organs.

DN thymocytes were prepared from adult (AKR/J × B6.PL)F_1 mice (H-2^k × H-2^b, Thy-1.1). In some experiments, the DN population was stained for the Thy-1 surface marker and FACS sorted to enrich for Thy-1$^+$ cells. Fig. 1 shows a typical FACS analysis of DN cells stained for Thy-1. Total DN cells had 82.5% cells staining brightly for Thy-1. After FACS separation, 99.6% of the cells fell within the bright range. FACS fractionation procedure enriches for cells committed to the T cell lineage and eliminates cells such as macrophages, monocytes, dendritic cells, and B cells that may contaminate the DN thymocyte population.

Total DN and FACS-sorted Thy-1$^+$ DN from the H-2 heterozygous mice were injected intrathymically into lethally irradiated, bone marrow-protected C57BL/6 hosts. 42 d after the transfer these mice and control (irradiated, bone marrow-protected) mice were killed and thymus and peripheral lymphoid cells were treated with anti-Thy-1.1 mAb plus complement to remove DN-derived T cells. Surviving cells were stimulated in MLC with irradiated (B10.BR × B10.D2)F_1 (H-2^k × H-2^d) spleen cells. Fig. 2, *C* and *F* show that control mice generate about equal levels of killing measured on H-2^k and H-2^d target cells. However, thymus and peripheral cells from the intrathymically injected mice are specifically depleted of H-2^k-specific

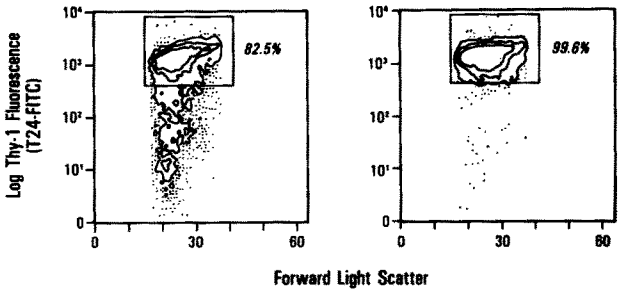

FIGURE 1. FACS sorting for Thy-1$^+$ DN thymocytes in preparation for intrathymic transfer into adoptive hosts. The left histogram shows the starting DN population stained for Thy-1 with the sorting gates indicated, and on the right is the post-sort reanalysis with the percentage of sorted Thy-1$^+$ cells.

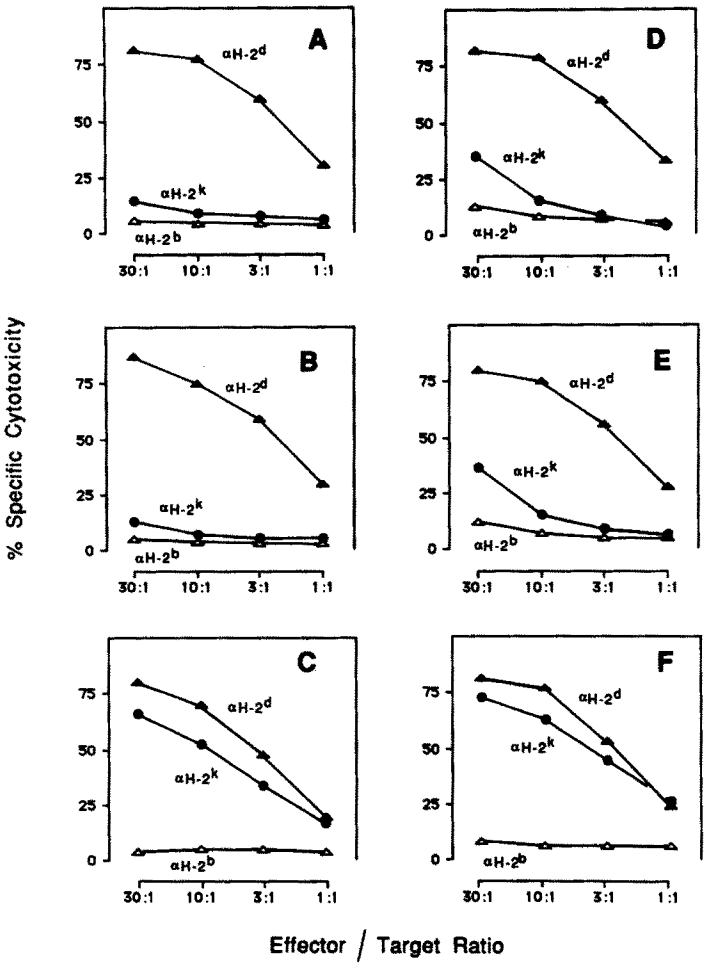

FIGURE 2. Specific CTL tolerance induced by the intrathymic injection of unfractionated or FACS-sorted Thy-1$^+$ semiallogeneic DN thymocytes. Spleen plus lymph node cells (*A-C*) or thymocytes (*D-F*) from chimeras made 42 d previously were treated with anti-Thy 1.1 antibody plus complement and the surviving cells were stimulated in MLC using irradiated (B10.BR × B10.D2)F_1 spleen cells. *A* and *D* are the responses obtained from chimeras made by the intrathymic injection of total (AKR/J × B6.PL)F_1 DN thymocytes into irradiated, bone marrow-reconstituted C57BL/6 hosts. *B* and *E* are chimeras made with the same pool of DN after FACS sorting for Thy-1$^+$ cells. *C* and *F* are control mice that did not receive DN cells intrathymically.

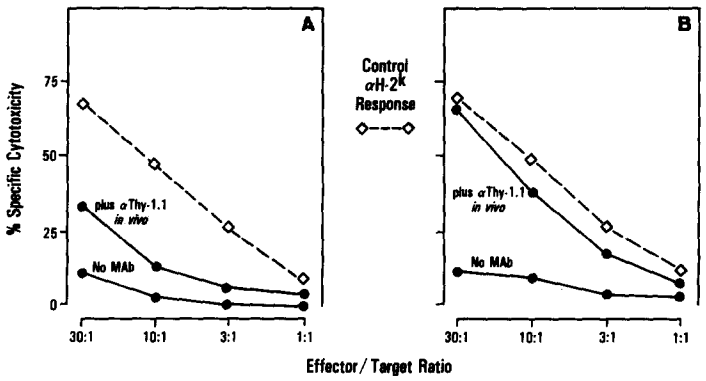

FIGURE 3. CTL tolerance in [(AKR/J × B6.PL)F_1 → C57BL/6J] intrathymic chimeras is dependent upon donor Thy-1$^+$ cells. (*A*) The lysis of H-2^k tumor targets by donor-depleted spleen plus lymph node cells cultured with (B10.BR × B10.D2)F_1 stimulators; (*B*) the lysis of H-2^k targets by donor-depleted thymocytes. Experimental animals received either no or two injections (at days 7 and 17 after transfer) of Thy-1.1-specific mAb 19E12, as indicated in the figure. Control response was that using cells recovered from irradiated, bone marrow-reconstituted animals that did not receive F_1 cells intrathymically. Animals were killed at day 50 after intrathymic transfer. At this time the animals that had received F_1 DN donor cells and 19E12 antibody had no detectable Thy-1.1$^+$ cells (donor origin) in their thymuses (0.0%), spleens (0.3% above background), or lymph nodes (0.0%). Animals that received F_1 DN donor cells but not antibody had no detectable cells of donor origin T cells in their thymuses (0.0%), 5% Thy-1.1$^+$ cells in the spleen, and 10.4% Thy-1.1$^+$ cells in the lymph nodes. The irradiated, bone marrow rescued controls showed 0.0%, 0.4%, and 0.0% Thy-1.1-specific immunofluorescence in thymus, spleen, and lymph nodes, respectively.

cytotoxic cells. This was the case with mice injected with unfractionated DN or with FACS-sorted Thy-1$^+$ DN. Tolerance to H-2^k targets in these experimental chimeras is specific since good levels of lysis of H-2^d targets were observed. These results suggested strongly that cells committed to the T cell lineage in the (H-2^k × H-2^b)F_1 DN population were capable of inducing CTL tolerance.

CTL Tolerance to Parental Allogeneic MHC Antigens Is Dependent upon the Presence of Thy-1$^+$ Cells. In a separate experiment, total DN [(AKR/J × B6.PL)F_1 → C57BL/6] intrathymic chimeras were given intraperitoneal injections of Thy-1.1-specific 19E12 antibody (0.1 ml ascites/mouse) on days 7 and 17 after intrathymic transfer. FACS analysis at day 50 after transfer showed that chimeric mice that had received antibody were completely depleted of F_1 DN donor-derived T cells, whereas chimeric mice that had not received antibody had levels of F_1 DN donor-derived cells in the peripheral lymphoid organs similar to those indicated in Table II (see Fig. 3, legend). After MLC with (B10.BR × B10.D2)F_1 (k × d) stimulator cells, T cells recovered from the spleen and lymph nodes of Thy-1.1-depleted adoptive hosts were still partially tolerant of donor H-2^k alloantigen, whereas thymocytes from these animals showed levels of anti-H-2^k cytotoxicity similar to that of control mice (Fig. 3). Thymocytes and peripheral T cells recovered from chimeras not depleted of donor cells were tolerant of H-2^k alloantigen as previously presented. All three groups, Thy-1.1-depleted intrathymic recipients, nontreated chimeras, and control animals, showed similar levels of anti-H-2^d cytotoxic activity (data not shown). This experiment confirmed that the tolerance observed in these [(AKR/J × B6.PL) → C57BL/6]

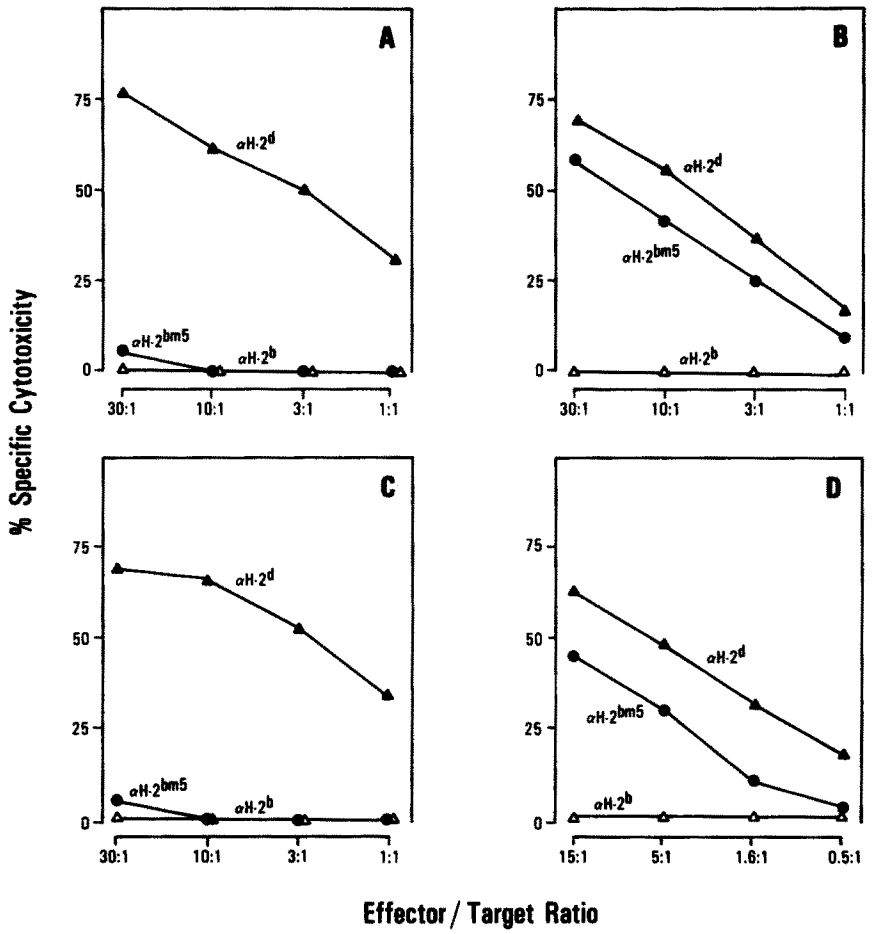

FIGURE 4. Host cells recovered from Thy-1$^+$ [(bm5 × bm12)F_1 → B6.PL] intrathymic chimeras on day 49 after transfer are nonresponsive to MHC class I (bm5) antigens of the F_1 intrathymic DN donor. (*A*) The lysis of Con A blast target cells by donor-depleted spleen plus lymph node cells initially cultured with bm5 plus BALB/c (H-2^d) stimulators; (*C*) the lysis of target cells by host-derived thymocytes cultured separately. (*B* and *D*) The lysis of targets by spleen plus lymph node cells and thymocytes, respectively, from control B6.PL mice that were irradiated and bone marrow reconstituted but not given F_1 donor cells intrathymically.

chimeras was due to the presence of Thy-1$^+$ donor cells. In addition, the data suggest that in vivo depletion of the tolerogenic stimulus by opsonizing antibody allowed the reemergence of donor alloantigen reactivity in the thymuses of treated animals.

CTL Tolerance Is Probably Due to Clonal Deletion. In further experiments to investigate the nature of the tolerance induced by the intrathymic injection of DN thymocytes we switched to a different donor/host combination. Thy-1$^+$ cells were prepared on the FACS from DN thymocytes of (bm5 × bm12)F_1 mice (Thy-1.2), and injected into the thymus lobes of lethally irradiated, bone marrow-protected B6.PL hosts (Thy-1.1). The F_1 cells differ from the host at class I, H-2K^{bm5} and at class II,

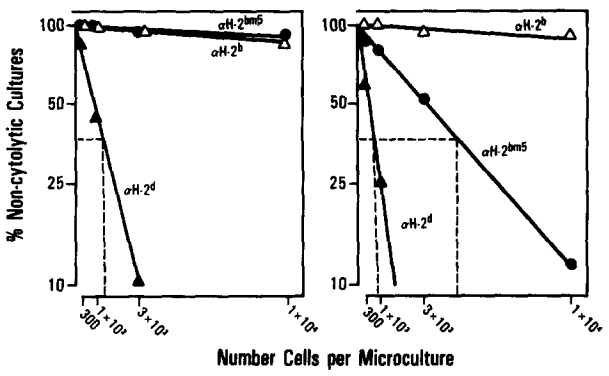

FIGURE 5. LDA of specific CTL nonresponsiveness in [(bm5 × bm12)F_1 → B6.PL] intrathymic chimeras. Host-derived (donor T cell-depleted) spleen plus lymph node cells recovered from [(bm5 × bm12)F_1 → B6.PL] chimeras are tolerant of MHC class I (bm5) antigens of the F_1 intrathymic donor (*left*). Micro-MLC stimulated with H-2^d plus bm5 spleen cells were split three ways on day 10 and assayed for cytotoxicity of Con A blasts of the indicated MHC haplotypes. Responsiveness of spleen plus lymph node cells from control B6.PL mice is shown on the right.

I-A^{bm12}. Thymus and peripheral cells were assayed for CTL responsiveness to H-$2K^{bm5}$ and to H-2^d antigens on day 49 after irradiation. Fig. 4 shows that the intrathymically injected mice respond well to H-2^d antigens but very poorly to bm5 antigens compared with control mice.

Peripheral lymphoid cells from these groups of mice were also stimulated with irradiated H-2^d and bm5 spleen cells under conditions of limiting dilution. Fig. 5 shows that control (irradiated, bone marrow-protected) mice have a frequency of ~1/1,000 cells responding to lyse H-2^d targets, and 1/5,000 responsive to bm5. The peripheral cells from intrathymically injected mice have roughly the same CTL precursor (CTLp) frequency for H-2^d antigens but have undetectable levels of CTLp for bm5 antigens. In other experiments using this combination of DN cells and host mice we have mixed chimera cells with normal B6.PL splenocyte responders and set them up in MLC against (bm5 × bm12)F_1 or bm5 plus BALB/c stimulators and observed no decrease in the anti-bm5 CTL response (data not shown). The inability of the chimeras to respond to bm5 antigens therefore appears to be the result of the depletion of antigen-reactive CTLp and not due to an ongoing suppressor mechanism.

Lack of Class II Antigen Tolerance in the Intrathymic Chimeras. The [(bm5 × bm12) → B6.PL] chimera combination allowed us to examine separately tolerance to class I, bm5 antigens and to class II, bm12 antigens. Assays for proliferation by host T cells in the peripheral lymphoid organs of the chimeras confirmed the lack of response to bm5 stimulator cells observed in the CTL assays (Table III). However, the proliferation assay indicated an ability of the cells to respond to bm12 stimulator cells to about the same level observed in control mice that had not been injected with F_1 DN cells.

Additional data for split tolerance were obtained in an experiment in which MHC class I vs. class II semiallogeneic DN cells were transferred intrathymically. Different B6.PL Thy-1.1 hosts received either (bm1 × C57BL/6)F_1 or (bm12 × C57BL/6)F_1 Thy-1.2^+ DN donor cells. Assays for proliferation at day 34 after transfer showed that lymph node cells of host origin in the [(bm1 × C57BL/6)F_1 → B6.PL] chimeras were tolerant of bm1 stimulator cells (Table IV). On the other hand, host T cells from [(bm12 × C57BL/6)F_1 → B6.PL] chimeras responded to bm12 stimulators to

TABLE III

Lack of Tolerance to Donor Allogeneic MHC Class II Antigens after the Intrathymic Transfer of FACS-sorted Thy-1$^+$ Thymocyte Stem Cells

Stimulators	[^{3}H]Thymidine incorporation (SI)* by responder cells from: (bm5 × bm12)F_1 → B6.PL	Control
	cpm ± SEM	*cpm ± SEM*
C57BL/6	2,272 ± 374 (1)	2,205 ± 512 (1)
bm5	3,029 ± 86 (1.3)	10,692 ± 1,089 (5)
bm12	14,393 ± 490 (7)	19,569 ± 3,231 (10)
(bm5 × bm12)F_1	11,932 ± 590 (6)	28,770 ± 1,155 (15)
BALB/c	52,400 ± 6,513 (25)	74,240 ± 7,091 (37)

Spleen and lymph node cells recovered from [(bm5 × bm12)F_1 → B6.PL] chimeras established 49 d previously and depleted of Thy-1.2$^+$ (intrathymic F_1 donor) cells. These data were obtained using the same experimental group of chimeric animals as that shown in Figs. 4 and 5.

* Stimulation index, calculated as the allogeneic to syngeneic proliferative response ratio.

similar levels as did control mice that had not been injected with donor F_1 DN cells. Thus, by CTL and proliferation assays, host T cells are tolerant to donor DN class I antigenic differences but are fully responsive to the class II differences in proliferation assays. This finding may reflect the fact that murine T cells do not express class II antigens, and supports our contention that donor T cells are responsible for the class I tolerance.

Discussion

The direct intrathymic adoptive transfer of MHC heterozygous thymocyte stem cells into irradiated, bone marrow-protected MHC homozygous hosts resulted in

TABLE IV

Tolerance to allogeneic MHC Class I but not Class II Antigens after the Intrathymic Transfer of FACS-sorted Thy-1$^+$ Thymocyte Stem Cells

Stimulators	[^{3}H]Thymidine incorporation (SI)* by responder cells from: (bm1 × C57BL/6)F_1 → B6.PL	(bm12 × C57BL/6)F_1 → B6.PL	Control
	cpm ± SEM	*cpm ± SEM*	*cpm ± SEM*
C57BL/6	2,981 ± 180 (1)	5,482 ± 1,059 (1)	5,499 ± 1,094 (1)
bm1	2,840 ± 366 (1)	63,241 ± 3,768 (12)	71,876 ± 1,183 (13)
bm12	25,416 ± 3,014 (9)	56,278 ± 4,514 (10)	86,106 ± 8,859 (16)
bm5	12,902 ± 941 (4)	30,078 ± 2,773 (5)	43,600 ± 5,874 (8)

Lymph node cells were recovered from [(bm1 × C57BL/6) → B6.PL] and [(bm12 × C57BL/6) → B6.PL] chimeras established 34 d previously using 2 × 10^6 Thy-1$^+$ DN donor cells. Before depletion of Thy-1.2$^+$ DN donor cells using specific antibody plus complement, 39% of [(bm1 × C57BL/6)F_1 → B6.PL] chimeric host lymph node cells were of Thy-1.2 origin. Post-depletion FACS analysis indicated no residual Thy-1.2$^+$ cells survived. For [(bm12 × C57BL/6)F_1 → B6.PL] chimeras, 21% of total lymph node cells at day 34 were of F_1 donor DN origin, and post-depletion FACS analysis indicated 2.7% Thy-1.2$^+$ cells survived (background of Thy-1.2-specific antibody staining on B6.PL control mice for this experiment was 1.1%).

* Stimulation index, calculated as the allogeneic to syngeneic response ratio.

split tolerance among host-derived T cells to donor MHC class I vs. class II alloantigen. Cytotoxicity assays showed that T cells of host origin that were recovered from [(A × B)F_1 → parent A] intrathymic chimeras and stimulated on donor plus third-party MHC stimulators were tolerant of donor MHC alloantigen. FACS sorting F_1 donor DN cells to enrich for Thy-1$^+$ cells and in vivo depletion of F_1 donor-derived cells using a donor Thy-1-specific opsonizing antibody indicated the CTL tolerance was due to the presence of the intrathymically injected thymocyte stem cells and was not due to macrophages, dendritic cells, or B cells contained in the DN population. Proliferation assays in two different donor-host combinations where MHC class I and MHC class II reactivity were dissociable showed that split tolerance had been induced. Whereas MHC class I tolerance was confirmed by the proliferation assays, reactivity by host T cells to class II donor alloantigen was always observed. The tolerance to donor class I antigens appeared to be due to clonal deletion of CTLp and not to overt mechanisms of T suppressor cell activity.

These findings suggest that murine thymocytes can deliver MHC class I tolerogenic signals. The interactions that resulted in specific tolerance to donor MHC alloantigen probably occurred as host thymocytes differentiated in the presence of the semiallogeneic donor cells. The depletion of F_1 donor-derived T cells in vivo by Thy-1.1-specific antibody resulted in the early reemergence of anti-donor reactivity among host thymocytes at a time when host peripheral T cells were still significantly tolerant of donor alloantigen.

Because tolerance breaks first in the thymus using this regimen, it is likely that tolerance is induced intrathymically in these chimeras. The peripheral tolerance observed in the Thy-1.1-depleted animals was most likely due to the delayed appearance of H-2^k-specific CTLp.

Our unpublished data indicate that tolerance in C57BL/6 animals that received unfractionated DN (AKR/J × B6.PL)F_1 intrathymic donor cells persisted for ~72–110 d. By 110 d after transfer only a few percent Thy-1.1$^+$ donor cells remained in the periphery of chimeric animals. This may reflect depletion due to normal cell turnover or elimination by reactive host T cells that differentiated in a thymic environment in which the tolerogenic stimulus was absent. We were unable to determine experimentally which of these mechanisms prevailed, but the lifetime persistence of T cells responsible for immunologic memory may favor the latter explanation.

The data presented here suggest that peripheral T cells might be important in the maintenance of tolerance. At 4–5 wk after intrathymic injection, FACS analysis indicated only 0.9–1.6% donor T cells remained in the thymuses of chimeric animals. This low level of donor-specific fluorescence might be an artifact of second reagent staining, or if real, might be due to either long-lived thymocyte stem cells (see reference 2), to the presence of differentiated T cells that stay in the thymus, or to recirculation of peripheral T cells back through the thymus. The persistence of tolerance in the thymuses of chimeric animals through day 72 after transfer suggests the result is real. We are presently investigating the mechanisms by which peripherally administered mature T cells can effect tolerance among thymocytes.

The complexity of the cellular interactions that lead to self-MHC tolerance and MHC-restricted antigen specificity has been illustrated by the recent literature. Epithelial cell grafts in the form of 2-deoxyguanosine-depleted fetal thymuses did not tolerize host animals to donor MHC antigens (14–16), whereas grafts not completely

depleted of hematopoietic cells (macrophages and dendritic cells as well as thymocytes) could tolerize (28). In similar experiments using 2-deoxyguanosine-depleted fetal thymic lobes, tolerance to donor-type minor histocompatibility antigens restricted by the host MHC class I haplotype (presumably due to antigen presentation by macrophages and dendritic cells, reference 29) has been demonstrated. Although epithelial cell interactions can influence thymocyte MHC-restricted antigen specificity (11) the literature suggests that tolerance occurs after interactions with other cell types. Surprisingly, these studies reported the long-term acceptance of 2-deoxyguanosine-depleted fetal thymus grafts by host mice in spite of demonstrable in vitro reactivity to donor allogeneic MHC antigens. Similarly, the acceptance by chick embryos of xenogeneic (quail) tissue grafts after transplantation of hematopoietic cell-free thymus grafts (30) showed that in such experimental systems even secondary grafts are accepted.

There is no evidence that T suppressor cell mechanisms are responsible for the observed tolerance to donor MHC antigens. LDA of the response to donor allogeneic MHC class I antigens showed tolerance at the CTLp level. Furthermore, mixing donor- or host-derived cells from chimeric animals with naive spleen cells did not affect the generation of allogeneic responses in MLC. The tolerance observed may be the result of interactions similar to those described as the "veto" phenomenon. Thymus, spleen or bone marrow cells from MHC class I-disparate mice can specifically tolerize adoptive hosts after intravenous transfer. The most efficient veto cells are also Thy-1$^+$, Ia$^-$ cells that apparently interact directly with and tolerize precursor lymphocytes (31, 32). It is not clear from the literature concerning "peripheral" veto cells whether committed pre-T stem cells present in the thymus, spleen, and bone marrow preparations used for in vivo injection contribute significantly to the anti-self suppressive activity observed. In addition, as mentioned previously, the effect of these protocols on intrathymic events has not been studied.

The mechanism of tolerance generation to specific antigen via T cell receptor-mediated interactions is obscure, but recent speculation suggests it might result from the lack of secondary signals normally provided by an antigen-presenting cell. Antigen-pulsed, carbodiimide-fixed, presenting cells (33) and antigen peptide-pulsed planar membranes (34) induce nonresponsiveness in immunocompetent T lymphocyte clones by the apparent failure to provide secondary "mitogenic" signals. Similar "inappropriate" T cell receptor-mediated interactions with thymic MHC molecules might contribute to the tolerization of immature thymocytes. Immature thymocytes may be inherently and uniquely sensitive to high-affinity T cell receptor-mediated interactions, leading to the deletion of self MHC-reactive cells. The intrathymic deletion in H-2^k mice of thymocytes expressing T cell receptors bearing an allotypic marker that is linked to I-E^k reactivity (10) suggests that such interactions have an important role in intrathymic T cell differentiation.

We do not conclude that MHC class I tolerance is established exclusively by interactions between thymocytes, but in this experimental system such interactions appear to be sufficient to establish tolerance. Other cells in the thymus, such as thymic dendritic cells and macrophages, most likely also play a role in self-MHC tolerance, as has previously been suspected (16, 28, 29). It might be speculated that thymic stromal elements (epithelial cells) are necessary for thymocyte selection for MHC-restricted antigen responsiveness, and that tolerance to self-MHC molecules can be

effected by various bone marrow–derived cell types. Furthermore, thymic education vs. tolerization might depend upon the thymic microenvironment in which the interactions occur, as well as the relative differentiative stage of the thymocyte. Thymocytes are, however, apparently capable of contributing to the establishment of MHC class I antigen tolerance. The experimental system described here might be useful to further elucidate these cell interactions responsible for self tolerance.

Summary

The intrathymic transfer of semiallogeneic CD4/CD8 double-negative (DN) thymocyte stem cells into irradiated host mice resulted in a transient state of chimerism in adoptive host thymus, spleen, and lymph nodes. Host-derived T cells, isolated from the thymus and periphery of the chimeric mice, were found to be specifically nonresponsive to the MHC antigens of the semiallogeneic DN donor in cytotoxicity assays. This nonresponsiveness was not permanent, but persisted as long as appreciable numbers of Thy-1 alloantigen-positive progeny of the DN donor cells could be detected in the spleen and lymph nodes of adoptive host mice. FACS sorting of DN donor cells before intrathymic transfer indicated that nonresponsiveness could be induced by Thy-1$^+$ cells and was therefore not attributable to contaminating thymic macrophages, dendritic cells, or B cells. When FACS-sorted Thy-1$^+$ (bm5 × bm12)F_1 DN cells were transferred intrathymically into C57BL/6 hosts, nonresponsiveness to DN donor MHC class I but not class II alloantigen (split tolerance) was observed. These experiments were repeated using FACS-sorted Thy-1$^+$ DN donor cells that were semiallogeneic to the irradiated adoptive host at either MHC class I or class II locus with similar results. Limiting dilution analysis showed that host-derived CTL precursors were tolerant of DN donor MHC class I alloantigen and no evidence for the involvement of suppressor T cells was found. The data indicate that murine thymocytes themselves are capable of tolerizing to MHC class I but not class II alloantigen after intrathymic transfer. The implications for intrathymic T cell differentiation and maintenance of self tolerance are discussed.

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