GENERATION OF CYTOLYTIC T LYMPHOCYTES IN THYMECTOMIZED, IRRADIATED, AND BONE MARROW-RECONSTITUTED MICE

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Products of the major histocompatibility complex (MHC)[1] play an important role in the recognition of antigen by cytolytic T lymphocytes (CTL). Viral (1) and minor histocompatibility antigens (2) or chemical modifiers (3) are recognized by CTL in association with H-2 antigens. The H-2 environment in which T cells mature, and not their genotype, determines the H-2 restriction of CTL (4, 5). This was shown by radiation bone marrow chimeras, where an A stem cell matures in an $(A \times B)F_1$ environment and, thus, recognizes antigen in association with $H\text{-}2^A$ or $H\text{-}2^B$. $(A \times B)F_1$ stem cells maturing in a parental environment (A) will only recognize antigen in association with $H\text{-}2^A$.

Initially, most data suggested (5–7) that it was the H-2 environment of the thymus that determined the specificity of the CTL repertoire. Recently, several sets of experiments suggest that the extrathymic environment can also play a role in the maturation and ultimate specificity of CTL: (*a*) nude mice that only possess a thymic rudiment (8) could generate CTL both in vitro (9–11) and in vivo (12) when interleukin 2 (IL-2) was provided; (*b*) in nude mice grafted with a thymus, the thymus determined the H-2 restriction when $(A \times B)F_1$ nude mice were grafted with a parental thymus, but in parental nude mice grafted with an allogeneic thymus (13) or an F_1 thymus (14), the parental nude environment determines the restriction; (*c*) Kruisbeek et al. (15) have shown that the extrathymic environment seems to play a role in the H-2 restriction of splenic CTL but not thymic CTL in thymus-engrafted nude mice.

There is some concern that the nude mouse is a poor model for studying extrathymic T cell differentiation because it may have other genetic defects that may influence the ultimate CTL repertoire. This concern stimulated us to develop a better defined model system for studying extrathymic T cell differentiation. If nude mice represent a model where stem cells have been able to differentiate without a thymic influence, we would expect that CTL should be generated in thymectomized, irradiated, and bone marrow-reconstituted chimeras. Previous experiments (16) have not shown the generation of CTL in such animals unless they were reconstituted with bone marrow-containing mature T cells. We hypothesized that in such animals, as in nude mice,

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[1] *Abbreviations used in this paper:* B6 Thy-1.1, B6PL Thy a/cy (Thy-1.1); B6 Thy-1.2, C57BL/6 (Thy-1.2); C, complement; CTL, cytolytic T lymphocytes; IL-2, interleukin 2; LPS, lipopolysaccharide; MHC, major histocompatibility complex; Pre-CTL, CTL precursor; TNP, trinitrophenyl.

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the addition of exogenous IL-2 might allow the generation of CTL.

In this paper, we show that the spleen cells from thymectomized, irradiated chimeras transplanted with bone marrow depleted of Thy-1^+ cells generate in vitro H-2-restricted syngeneic and allogeneic CTL when IL-2 is added to the cultures. These Thy-1^+ cells bear a more immature phenotype than the spleen cells of euthymic mice. These results suggest that Thy-1^- cells can differentiate to Thy-1^+ pre-CTL by an extrathymic differentiation pathway.

Materials and Methods

Mice. C57BL/6(B6 Thy-1.2), (B6 × DBA/2)F_1, (B6 × C3H)F_1, and BALB/c mice come from Charles River Breeding Laboratories Inc., Wilmington, MA, or from The Jackson Laboratory, Bar Harbor, ME. 6–10-wk-old animals were used in the experiments. B6 PL Thy a/cy (B6 Thy-1.1) were bred in the Redstone Building of the Sidney Farber Cancer Institute, Boston, MA.

Thymectomy. Adult thymectomy was performed on 6-wk-old animals anesthesized with ether. The thymus was removed by vacuum aspiration. Histological evaluation of thymectomized animals did not reveal any residual thymus.

Irradiation and Reconstitution. B6 Thy-1.2 mice thymectomized 1 wk before transplant or nonthymectomized B6 mice were irradiated with a Cs Source (Gamma Cell 40, Atomic Energy of Canada, Ottawa, Canada) at 940 rad and reconstituted intravenously the same day with 2 × 10^7 B6 PL Thy-1 a/Cy (Thy-1.1) bone marrow cells treated with anti-Thy-1.1 antibody and complement (C) (see below). Similarly, Thy-1.1 or Thy-1.2 mice were reconstituted with anti-Thy-1.2 antibody plus C-treated bone marrow from Thy-1.2 mice. Transplanted animals received tetracycline hydrochloride (8% solution) in their drinking water. Control animals, irradiated and nontransplanted, died in 10–13 d.

In Vitro Induction of Cytolytic T Lymphocytes. Cultures were performed in 16-mm Linbro culture plates (Flow Laboratories, Inc., Rockville, MD) in 2 ml of RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum, 2 mM L-glutamine (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 5 × 10^{-5} M 2-mercaptoethanol, penicillin (100 μg/ml), and streptomycin (100 μg/ml). 8–18 wk after transplantation, spleen cells from chimeras were used to generate primary in vitro CTL. 5 × 10^6 effector cells were incubated with 5 × 10^6 stimulator cells treated with Tris-HCl ammonium chloride and irradiated (1,200 rad). Trinitrophenyl (TNP) stimulator cells were modified with 10 mM trinitrobenzenesulfonic acid as described previously (17). Cultures were incubated for 5 d at 37°C in a 5% CO_2 incubator.

Interleukin 2 (IL-2). The term IL-2 refers to a partially purified supernatant obtained from Lewis rat spleen cells cultured for 24 h with concanavalin A (5 μg/ml). The fraction of the supernatant precipitated between 50% and 75% saturation of ammonium sulfate (18) was dialyzed against Tris-buffered saline, resuspended in RPMI 1640, and used in cultures at a final concentration of 5%.

Treatment of Cells With Monoclonal Antibody and C. 50 × 10^6 spleen cells or bone marrow cells were incubated for 30 min with 1 ml of a monoclonal anti-Thy-1.1 or anti-Thy-1.2 antibody (New England Nuclear, Boston, MA) at a dilution of 10^{-3} in L15 medium (M. A. Bioproducts) at room temperature. The cells were then pelleted, the supernatant discarded, and 1 ml of a 10^{-1} dilution of low toxic guinea pig C was added. After 40 min at 37°C, the cells were washed, and a second treatment was performed with antibody and C at the same concentration used for the first treatment but this time added together for 40 min at 37°C. The cells were then washed twice in L15. This procedure was used for the following: to eliminate Thy-1-positive cells from the bone marrow before transplanting these cells to irradiated recipients, and to deplete spleen cells before stimulation in vitro or after 5 d in culture. In the latter case, 5 × 10^6 cells were treated with 0.5 ml of serum and 0.5 ml of C.

Treatment of spleen cells with monoclonal anti-Ly-1.2 antibody (New England Nuclear) plus C was done twice using 25 × 10^6 cells and 0.5 ml of antibody at a dilution of 4 × 10^{-4}, following the same procedure used for anti-Thy-1.

Assay for Cytolytic T Cell Activity. Target cells were P815 (H-2^d), EL4(H-2^b), RDM-4(H-2^k)

tumor or lipopolysaccharide (LPS) lymphoblast cells, either unmodified or modified with 10 mM TNBS. LPS lymphoblasts were generated by culturing spleen cells with LPS (Difco Laboratories, Detroit, MI) at 10 μg/ml for 48 h. The ^{51}Cr release assay was performed as described previously (19), with some modifications. Briefly, 5×10^6 target cells were labeled with 100 μCi Na_2 $^{51}CrO_4$ (New England Nuclear) for 1 h at 37°C in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, penicillin and streptomycin, 1% nonessential amino acids, and 2 mM L-glutamine. Labeled cells were washed three times, and 10^4 cells were mixed with varying numbers of effector cells in 0.2 ml of supplemented MEM in V-bottomed plates (Linbro Chemical Co., Hamden, CT) and incubated at 37°C in 5% CO_2. 100 μl of supernatant was harvested to determine radioactivity released. The percentage of chromium released was calculated as percent specific release: ^{51}Cr released by immune cells − ^{51}Cr released by normal cells/maximum ^{51}Cr released (with 1% deoxycholate) − ^{51}Cr released by normal cells.

Results

Generation of Cytolytic Activity In Vitro in Thymectomized Chimeras. Thymectomized or nonthymectomized (control) B6 Thy-1.2 mice were lethally irradiated and transplanted with bone marrow cells from the congenic strain B6 PL Thy-1 a/Cy (Thy-1.1) depleted of Thy-1-positive cells by two treatments with anti-Thy-1.1 and C (Thy-1.1 → Thy-1.2). Similarly, lethally irradiated B6 Thy-1.1 mice were reconstituted with treated Thy-1.2 bone marrow cells (Thy-1.2 → Thy-1.1). These strain combinations were chosen to minimize possible allogeneic effects but still provide the ability to assess whether cells were from the recipient or donor. 8 wk after transplantation, the spleen cells of these chimeras were assessed for their ability to generate CTL responses in vitro. When stimulated with syngeneic B6-TNP stimulator spleen cells from normal mice, good cytolytic activity was obtained against EL4-TNP by spleen cells from control chimeras, but no cytolytic activity was generated by spleen cells from thymectomized animals (Table I). These data agree with previous reports (5); however, when 5% IL-2 was added to the cultures, thymectomized animals generated significant cytolytic activity against syngeneic TNP-modified cells. These results were obtained both for Thy-1.1 → Thy-1.2 chimeras and Thy-1.2 → Thy-1.1 chimeras. Such results have been obtained in more than 10 experiments using different batches of chimeras transplanted 8–18 wk previously. Spleen cells from thymectomized mice co-cultured with B6 syngeneic cells did not demonstrate self reactivity, i.e., the ability to lyse EL4, even in the presence of IL-2 (Table II). Similarly, when stimulated with allogeneic (B6 × DBA_2)F_1 cells, thymectomized mice generated cytolytic activity only when IL-2 was added to the culture (Table III). In all these experiments, IL-2 alone did not have any effect (Table IV). The use of (B6 × DBA/2)F_1 stimulator cells was necessary to prevent back stimulation and IL-2 production by the irradiated stimulator cells. Bone marrow cells treated with anti-Thy-1 plus C and used to reconstitute these mice did not demonstrate any response to allogeneic or TNP-modified syngeneic cells, even in the presence of IL-2 (Table V and VI), suggesting that extrathymic differentiation had occurred in these chimeras to the point where these cells now could respond to antigen plus exogenous IL-2.

Cytolytic T Lymphocytes in Chimeras Are of Donor Origin. Anti-TNP CTL generated from B6 Thy-1.1 → B6 Thy-1.2 or B6 Thy-1.2 → B6 Thy-1.1 chimeras were treated with either anti-Thy-1.1 antibody and C or anti-Thy-1.2 antibody and C. The cytolytic activity was eliminated by anti-Thy-1.1 treatment of Thy-1.1 → Thy-1.2 chimeras and by anti-Thy-1.2 treatment of Thy-1.2 → Thy-1.1 chimeras, both for

TABLE I

Generation of Anti-TNP CTL from Spleen Cells of Thymectomized Chimeras

Experiment	Responder*	Stimulator	Percent specific ^{51}Cr release of EL4-TNP‡	
			25:1§	5:1
1	Normal B6 Thy-1.1	B6-TNP	80	54
	Normal B6 Thy-1.1	B6-TNP + IL-2	63	26
	Control chimera (Thy-1.1 → Thy-1.2)‖	B6-TNP	49	16
	Control chimera (Thy-1.1 → Thy-1.2)‖	B6-TNP + IL-2	67	38
	Thymectomized chimera (Thy-1.1 → Thy-1.2)¶	B6-TNP	3	0
	Thymectomized chimera (Thy-1.1 → Thy-1.2)¶	B6-TNP + IL-2	39	16
	Thymectomized chimera (Thy-1.1 → Thy-1.2)¶	B6-TNP	0	0
	Thymectomized chimera (Thy-1.1 → Thy-1.2)¶	B6-TNP + IL-2	32	10
			50:1	10:1
2	Normal B6 Thy-1.2	B6-TNP	67	58
	Normal B6 Thy-1.2	B6-TNP + IL-2	72	60
	Control chimera (Thy-1.2 → Thy-1.1)**	B6-TNP	60	27
	Control chimera (Thy-1.2 → Thy-1.1)**	B6-TNP + IL-2	70	64
	Thymectomized chimera (Thy-1.2 → Thy-1.1)‡‡	B6-TNP	2	0
	Thymectomized chimera (Thy-1.2 → Thy-1.1)‡‡	B6-TNP + IL-2	67	47

* 5×10^6 spleen cells were cultured 5 d with 5×10^6 stimulator cells. IL-2 was used at a final concentration of 5%. IL-2 alone did not generate CTL activity.

‡ Spontaneous release was 4–19%.

§ Effector-to-target ratio.

‖ Control chimera were B6 Thy-1.2 mice irradiated and reconstituted with B6 Thy-1.1 bone marrow cells treated with anti-Thy-1.1 + C.

¶ B6 Thy-1.2 mice thymectomized, irradiated, and reconstituted with B6 Thy-1.1 bone marrow cells treated with anti-Thy-1.1 + C. Data from two mice.

** Control chimera were B6 Thy-1.1 mice irradiated and reconstituted with B6 Thy-1.2 bone marrow cells treated with anti-Thy-1.2 + C.

‡‡ B6 Thy-1.1 mice thymectomized, irradiated, and reconstituted with B6 Thy-1.2 bone marrow cells treated with anti-Thy-1.2 + C.

thymectomized animals as well as in control chimeras (Table IV). These results demonstrate that the functional cells in thymectomized animals are T cells and are of donor origin. In all experiments presented, splenic CTL were typed and found to be of donor origin.

H-2 Restriction of Anti-TNP CTL in Thymectomized Chimeras. In normal mice, anti-TNP CTL preferentially lyse autologous TNP-modified target cells but they also lyse, to a lesser degree, allogeneic TNP-coupled cells (20). It has been proposed (5) that the thymus determines the H-2 restriction of CTL, therefore, we compared the H-2 restriction of anti-TNP CTL generated from the spleens of chimeras with or without

TABLE II

Lack of CTL Reactive against Unmodified Self in Thymectomized Chimeras

Responder*	Stimulator	Target	Percent specific ^{51}Cr Release‡	
			70:1	7:1§
Normal B6 Thy-1.1	B6	EL4	20	3
Normal B6 Thy-1.1	B6 + IL-2	EL4	37	10
Normal B6 Thy-1.1	B6-TNP	EL4-TNP	78	71
Control chimera‖	B6	EL4	0	0
Control chimera‖	B6 + IL-2	EL4	7	0
Control chimera‖	B6-TNP	EL4-TNP	66	28
Thymectomized chimera‖	B6	EL4	0	0
Thymectomized chimera	B6 + IL-2	EL4	0	0
Thymectomized chimera	B6-TNP	EL4-TNP	4	0
Thymectomized chimera	B6-TNP + IL-2	EL4-TNP	61	28

* 5 × 10^6 spleen cells cultured 5 d with 5 × 10^6 stimulator cells with or without 5% IL-2.
‡ Spontaneous release for EL4 was 7% and 6–9% for EL4-TNP.
§ Effector-to-target ratio.
‖ Chimeras Thy-1.1 → Thy-1.2 (cf legend Table I).

TABLE III

Generation of Allogeneic CTL from Spleen Cells of Thymectomized Chimeras

Responder*	Stimulator	Percent specific ^{51}Cr Release of P815‡	
		20:1	4:1§
Control chimera‖	(B6 × DBA/2)F_1	67	2
Control chimera‖	(B6 × DBA/2)F_1 + IL-2	76	19
Thymectomized chimera¶	(B6 × DBA/2)F_1	0	0
Thymectomized chimera¶	(B6 × DBA/2) + IL-2	30	2
Thymectomized chimera¶	(B6 × DBA/2)F_1	4	0
Thymectomized chimera¶	(B6 × DBA/2)F_1 + IL-2	62	11
Thymectomized chimera¶	(B6 × DBA/2)F_1	13	0
Thymectomized chimera¶	(B6 × DBA/2) + IL-2	65	11

* 5 × 10^6 spleen cells were cultured for 5 d with 5 × 10^6 stimulator cells with or without 5% IL-2.
‡ Spontaneous release was from 18–21%.
§ Effector-to-target ratio.
‖ Control chimera were B6 Thy-1.2 mice irradiated and reconstituted with 2 × 10^7 Thy-1.1 bone marrow cells treated with anti-Thy-1.1 + C.
¶ B6 Thy-1.2 mice thymectomized, irradiated, and reconstituted the same as control chimera. Data from three mice.

a thymus. Table VII shows that in thymectomized animals, anti-TNP CTL lysed allogeneic TNP-modified target cells (P815-TNP) to a lesser extent than syngeneic TNP-modified targets. No difference in the H-2 restriction of anti-TNP CTL could be observed in chimeric mice with or without a thymus.

Cross-Reactivity of Alloreactive T Cells. CTL generated from normal spleen cells against allogeneic cells cross-react on TNP-modified syngeneic cells (17). Recently, it has been suggested that the subpopulation of allogeneic CTL that cross-react on

TABLE IV
Treatment of CTL with Anti-Thy-1.1 or Anti-Thy-1.2 and C

Responder*	Stimulator	Treatment‡					
		None		Anti-Thy-1.1 + C		Anti-Thy-1.2 + C	
		50:1	5:1‖	50:1	5:1	50:1	5:1
Normal B6 Thy-1.1	B6-TNP + IL-2	69	41‖	1	1	78	61
Normal B6 Thy-1.2	B6-TNP + IL-2	71	48	78	48	0	0
Control chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP	69	30	NT¶		NT	
Control chimera§ (Thy-1.1 → Thy-1.2)	IL-2	16	5	NT		NT	
Control chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	59	25	16	6	66	33
Thymectomized chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP	0	0	NT		NT	
Thymectomized chimera§ (Thy-1.1 → Thy-1.2)	IL-2	0	0	NT		NT	
Thymectomized chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	38	8	0	0	37	5
Control chimera (Thy-1.2 → Thy-1.1)§	B6-TNP + IL-2	70	59	61	44	0	2
Thymectomized chimera (Thy-1.2 → Thy-1.1)§	B6-TNP + IL-2	73	75	72	64	0	3

* 5×10^6 spleen cells were cultured for 5 d with 5×10^6 stimulator cells with or without 5% IL-2.
‡ 5×10^6 cells were treated at the end of the culture twice with anti-Thy-1 + C.
§ Chimeras were Thy-1.1 → Thy-1.2 or Thy-1.2 → Thy-1.1 (Cf Legend Table I).
‖ Specific ^{51}Cr release from EL4-TNP at the indicated effector-to-target ratios. Spontaneous release of EL4-TNP was 15%.
¶ Not tested.

TNP-modified syngeneic cells is influenced by the MHC antigens on radioresistant cells in the thymus (21). If this influence on the CTL repertoire is determined by the thymus, no cross-reactive CTL should be found in thymectomized chimeras. Table VIII shows the cross-reactivity of anti-H-2^d CTL generated by spleen cells from B6 Thy-1.1 → B6 Thy-1.2 and B6 Thy-1.2 → B6 Thy-1.1 chimeras immunized against (B6 × DBA/2)F_1 cells. The same degree of cross-reactivity by alloreactive CTL is observed on syngeneic modified EL4-TNP cells by spleen cells from thymectomized or nonthymectomized chimeras.

It has also been observed that alloreactive CTL generated from nude mice fail to demonstrate cross-reactive lysis of third-party allogeneic cells (10), again evidence suggesting that the thymus may influence the ultimate CTL repertoire. Therefore, we investigated whether thymectomized chimeras demonstrated this cross-reactivity and found that, from thymectomized or nonthymectomized chimeras, alloreactive CTL demonstrated lysis of third-party allogeneic targets (Table IX).

CTL Precursors of Thymectomized Chimeras Express the Phenotype of Immature CTL. Recent data (22) have shown that pre-CTL in normal Thy-1.1 spleens are difficult to lyse with anti-Thy-1.1 and C, and these cells have a low density of Thy-1.1 antigens on their surface, as shown by fluorescence-activated cell sorter analysis.

TABLE V

Lack of Reactivity of Bone Marrow Cells after Treatment with Anti-Thy-1.1 and C

Responder*	Stimulator	Target	Percent specific ^{51}Cr release‡	
			30:1§	3:1
Bone marrow treated with C	BALB/c	P815	63	32
Bone marrow treated with C	BALB/c + IL-2	P815	62	60
Bone marrow treated with C	B6-TNP	EL4-TNP	10	5
Bone marrow treated with C	B6-TNP + IL-2	EL4-TNP	37	10
Bone marrow-treated with Anti-Thy-1.1 + C	BALB/c	P815	0	0
Bone marrow-treated with Anti-Thy-1.1 + C	IL-2	P815	0	0
Bone marrow-treated with Anti-Thy-1.1 + C	BALB/c + IL-2	P815	0	0
Bone marrow-treated with Anti-Thy-1.1 + C	B6-TNP	EL4-TNP	1	4
Bone marrow-treated with Anti-Thy-1.1 + C	IL-2	EL4-TNP	0	2
Bone marrow-treated with Anti-Thy-1.1 + C	B6-TNP + IL-2	EL4-TNP	0	0

* Responder cells were 5 × 10^6 B6 Thy-1.1 bone marrow cells cultured 5 d with 5 × 10^6 stimulator cells. IL-2 was used at a final concentration of 5%. Before culture, responder cells were treated twice with C alone or treated twice with anti-Thy-1.1 + C.
‡ Spontaneous release was 10% for P815 and 20–23% for EL4-TNP.
§ Effector-to-target ratio.

TABLE VI

Lack of Reactivity of Bone Marrow Cells after Treatment with Anti-Thy-1.2 and C

Responder*	Stimulator	Target	Percent specific ^{51}Cr release‡	
			30:1§	3:1
Bone marrow treated with C	BALB/c	P815	62	33
Bone marrow treated with C	BALB/c + IL-2	P815	63	57
Bone marrow treated with C	B6-TNP	EL4-TNP	16	0
Bone marrow treated with C	B6-TNP + IL-2	EL4-TNP	23	7
Bone marrow treated with anti-Thy-1.2 + C	BALB/c	P815	0	0
Bone marrow treated with anti-Thy-1.2 + C	BALB/c + IL-2	P815	0	0
Bone marrow treated with anti-Thy-1.2 + C	B6-TNP	EL4-TNP	0	0
Bone marrow treated with anti-Thy-1.2 + C	B6-TNP + IL-2	EL4-TNP	0	0

* Responder cells were 5 × 10^6 B6 Thy-1.2 bone marrow cells cultured for 5 d with 5 × 10^6 stimulator cells, IL-2 was used at a final dilution of 5%. Responder cells were treated with C alone or treated twice with anti-Thy-1.2 plus C.
‡ Spontaneous release for P815 was 10% and 20–23% for EL4-TNP.
§ Effector-to-target ratio.

TABLE VII

H-2 Restriction of Anti-TNP CTL from Thymectomized Chimeras

Responder cell*	Stimulator	Target			
		EL4-TNP		P815-TNP	
Experiment 1		20:1‡	4:1	20:1	4:1
Control chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	51	26‖	27	2
Control chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	73	41	32	3
Thymectomized chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	46	21	23	1
Thymectomized chimera§ (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	51	20	46	10
Experiment 2		50:1	5:1	50:1	5:1
Normal B6 Thy-1.1	B6-TNP + IL-2	69	41	49	10
Control chimera (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	59	25	29	5
Thymectomized chimera (Thy-1.1 → Thy-1.2)	B6-TNP + IL-2	38	8	18	4
Experiment 3		10:1		10:1	
Normal B6 Thy-1.2	B6-TNP + IL-2	60		29	
Control chimera (Thy-1.2 → Thy-1.1)	B6-TNP + IL-2	63		40	
Thymectomized chimera (Thy-1.2 → Thy-1.1)	B6-TNP + IL-2	47		9	

* 5×10^6 spleen cells were cultured for 5 d with 5×10^6 stimulator cells and 5% IL-2.
‡ Effector-to-target ratio.
§ Data from two nonthymectomized and two thymectomized chimeras.
‖ Values are percent specific ^{51}Cr release.

However, once stimulated to become CTL, these cells again express high amounts of Thy-1. A correlation has been established between the sensitivity of cells to anti-Thy-1 plus C and the density of Thy-1 antigen on their cell surface: "high Thy-1" cells are easily lysed by antibody and C (i.e., require less antibody), and "low Thy-1" are more resistant to lysis (23). High Thy-1 is characteristic of T cells found in the thymus, and "low Thy-1" of T cells found in the spleen (24–26). We compared the sensitivity to anti-Thy-1 treatment of splenic pre-CTL from mice with or without a thymus to determine whether different amounts of Thy-1 could be found.

Results of Table X show a clear difference in the sensitivity of pre-CTL from thymectomized and nonthymectomized mice: two treatments with anti-Thy-1.1 antibody at a 10^{-3} dilution plus C eliminated anti-TNP CTL from the spleen cells of thymectomized animals but did not eliminate pre-CTL from normal spleen cells or from the spleen cells of nonthymectomized chimeras. Increasing the concentration of antibody in nonthymectomized animals did not result in the elimination of pre-CTL (data not shown). Thus, the splenic pre-CTL of thymectomized mice appear to have a different phenotype from that of nonthymectomized mice; that is, they have high amounts of Thy-1 antigen, whereas the spleen cells from the nonthymectomized mice appear to express low amounts of Thy-1. Fluorescence-activated cell sorter analysis

TABLE VIII

Cross-reactivity of Alloreactive CTL from Spleen Cells of Thymectomized Chimeras

Experiment	Responder*	Stimulator	Target‡ P815		Target‡ EL4-TNP	
			20:1	4:1	20:1	4:1
1	Control chimera§ (Thy-1.1 → Thy-1.2)	(B6 × DBA/2)F_1 + IL-2	76	19	36	12
	Control chimera§ (Thy-1.1 → Thy-1.2)	(B6 × DBA/2)F_1 + IL-2	72	14	32	14
	Thymectomized chimera (Thy-1.1 → Thy-1.2)§	(B6 × DBA/2)F_1 + IL-2	30	12	7	8
	Thymectomized chimera (Thy-1.1 → Thy-1.2)§	(B6 × DBA/2)F_1 + IL-2	62	11	33	16
	Thymectomized chimera (Thy-1.1 → Thy-1.2)§	(B6 × DBA/2)F_1 + IL-2	65	11	22	7
			40:1	4:1	40:1	4:1
2	Control chimera‖ (Thy-1.2 → Thy-1.1)	(B6 × DBA/2)F_1 + IL-2	70	48	27	14
	Thymectomized chimera (Thy-1.2 → Thy-1.1)‖	(B6 × DBA/2)F_1 + IL-2	88	54	35	9
	Thymectomized chimera (Thy-1.2 → 1.1)‖	(B6 × DBA/2)F_1 + IL-2	68	32	13	9

* 5×10^6 spleen cells were cultured for 5 d with 5×10^6 stimulator cells with 5% IL-2.

‡ Specific ^{51}Cr release at the indicated effector-to-target ratios. Spontaneous release for P815 was 14–27% and 11–15% for EL4-TNP.

§ Chimeras Thy-1.1 → Thy-1.2 (cf legend Table I). Data from two control and three thymectomized chimeras.

‖ Chimeras Thy-1.2 → Thy-1.1. Data from one control and two thymectomized chimeras.

demonstrated that the spleen cells of thymectomized chimeras had the same amount of Thy-1 on their surface as the thymocytes from normal Thy-1 mice (i.e., high Thy-1), whereas the spleen cells from nonthymectomized mice had the same amount of Thy-1 as spleen from normal B6 Thy-1 mice (i.e., low Thy-1) (data not shown).

It has been demonstrated (27) that early in ontogeny, allogeneic pre-CTL are Ly-1^+2^+, whereas later in development their phenotype is Ly-$1^\pm2^+$, i.e., they express low amounts of the Ly-1 antigen. Therefore, if allogeneic pre-CTL from thymectomized chimeras expressed a more immature phenotype than normal pre-CTL, one might expect this to be reflected in their Lyt phenotype. Table XI demonstrates that allogeneic pre-CTL from thymectomized chimeras were very susceptible to treatment with monoclonal anti-Ly-1.2 plus C, i.e., they express the more immature Ly-1^+2^+ phenotype, whereas those from normal spleen and control chimeras are far less susceptible, i.e., Ly-$1^\pm$. All pre-CTL were eliminated by treatment with monoclonal anti-Ly-2.2 plus C (data not shown). All TNP-specific pre-CTL were Ly-1^+2^+, as previously described (27, 28).

Discussion

An attempt has been made to develop a model system to study extrathymic T cell differentiation. Given the concerns surrounding the use of nude mice, we decided to use thymectomized, lethally irradiated, and bone marrow-reconstituted mice to

TABLE IX

Cross-reactivity of Alloreactive CTL on a Third-Party Allogeneic Target

Experiment	Responder	Stimulator	Target: RDM4 50:1	10:1	2:1	DBA/1 blasts 50:1	10:1	2:1
1	Normal B6 Thy-1.2	(B6 × C3H)F_1	40	28	6*		NT‡	
	Normal B6 Thy-1.2	(B6 × C3H)F_1 + IL-2	60	35	9	36	7	2
	Control chimera (Thy-1.2 → Thy-1.1)§	(B6 × C3H)F_1	37	18	7		NT	
	Control chimera (Thy-1.2 → Thy-1.1)§	(B6 × C3H)F_1 + IL-2	47	30	5	30	6	0
	Thymectomized chimera (Thy-1.2 → Thy-1.1)§	(B6 × C3H)F_1	11	5	2		NT	
	Thymectomized chimera (Thy-1.2 → Thy-1.1)	(B6 × C3H)F_1 + IL-2	72	68	54	43	27	12

Experiment	Responder	Stimulator	P815 50:1	5:1	RDM4 50:1	5:1
2	Normal B6 Thy-1.2	BALB/c + IL-2	60	48	41	29
	Control chimera (Thy-1.2 → Thy-1.2)‖	BALB/c + IL-2	56	25	41	22
	Thymectomized chimera (Thy-1.2 → Thy-1.2)‖	BALB/c + IL-2	59	50	66	33

* Specific ^{51}Cr release at the indicated effector-to-target ratios. Spontaneous release for RDM4 was 12% and 35% for DBA/1 LPS blasts (experiment 1), 10% for P815, and 33% for RDM4 (experiment 2).
‡ Not tested.
§ Chimera Thy-1.2 → Thy-1.1.
‖ Chimera Thy-1.2 → Thy-1.2.

address this issue. Though previous studies had proposed that spleen cells from such mice lacked functional T cells, based on the experience with nude mice, we proposed that IL-2 might provide the additional signal needed to drive pre-CTL to functional T cells. The results reported here are consistent with this proposal. We found that the spleens of these mice contain Thy-1-positive cells capable of becoming functional CTL recognizing alloantigens or TNP-modified syngeneic antigens when cultured with antigen and IL-2. By using Thy-1 congenic mice, we have been able to avoid allogeneic differences that might result in allogeneic effects but still have a marker to demonstrate the donor origin of the CTL generated.

There are several issues relating to extra-thymic differentiation that we have begun to address, namely, the stage of differentiation of these splenic pre-CTL and the CTL repertoire found in these thymectomized mice. By using Thy-1.1 donor cells, we have been able to easily use susceptibility to lysis by anti-Thy-1.1 plus C as a parameter to determine the state of maturation of the pre-CTL. It has been observed that thymocytes are high in Thy-1.1 expression but that peripheralized T cells, including pre-CTL, express little Thy-1.1 and, in fact, are difficult to lyse with anti-Thy-1.1

TABLE X

Sensitivity to Anti-Thy-1.1 and C Treatment of CTL Precursors from Thymectomized Chimeras

Experiment	Responder*	Treatment	Stimulator	Percent specific ^{51}Cr release of EL4-TNP‡		
				50:1§	10:1	2:1
1	Control chimera‖	None	B6-TNP	41	21	
	Control chimera‖	None	B6-TNP + IL-2	61	39	
	Control chimera‖	Anti-Thy-1.1 + C¶	B6-TNP + IL-2	57	35	
	Thymectomized chimera	None	B6-TNP	0	0	
	Thymectomized chimera	None	B6-TNP + IL-2	52	32	
	Thymectomized chimera	Anti-Thy-1.1 + C	B6-TNP + IL-2	13	0	
2	Control chimera	None	B6-TNP	84	79	43
	Control chimera	None	B6-TNP + IL-2	83	72	34
	Control chimera	Anti-Thy-1.1 + C	B6-TNP + IL-2	83	84	44
	Thymectomized chimera	None	B6-TNP	4	5	0
	Thymectomized chimera	None	B6-TNP + IL-2	76	61	16
	Thymectomized chimera	Anti-Thy-1.1 + C	B6-TNP + IL-2	18	10	2
	Normal B6 Thy-1.1	None	B6-TNP	86	85	60
	Normal B6 Thy-1.1	None	B6-TNP + IL-2	82	87	56
	Normal B6 Thy-1.1	Anti-Thy-1.1 + C	B6-TNP + IL-2	80	78	39

* 5×10^6 spleen cells were cultured for 5 d with 5×10^6 stimulator cells with or without 5% IL-2.

‡ Spontaneous release of EL4-TNP ranged from 7–17%.

§ Effector-to-target ratio.

‖ Chimeras Thy-1.1 → Thy-1.2 (cf legend Table I).

¶ 5×10^6 spleen cells were treated twice with anti-Thy-1.1 at a 10^{-3} dilution and complement at a dilution of 10^{-1}.

plus C. Once stimulated with antigen to become functional CTL, these cells again are high in Thy-1.1 expression and are readily susceptible to lysis by anti-Thy-1.1 plus C (22). The splenic pre-CTL from thymectomized mice were found to be high in Thy-1.1, which could place their stage of differentiation at that of thymocytes or mature T cells. Because thymocytes require exogenous IL-2 to respond to antigen (29, 30) and mature splenic T cells do not, it would appear that the splenic pre-CTL of thymectomized mice are at a stage of differentiation similar to that of thymocytes. The more immature Lyt phenotype of the allogeneic pre-CTL from these thymectomized chimeras is also consistent with this proposal. This suggests that in mice lacking a thymus, T cell differentiation occurs in the spleen and never reaches the stage of differentiation of normal splenic T cells; however, addition of exogenous IL-2 and antigen in vitro will allow them to differentiate to mature CTL. To be certain that our bone marrow inoculum did not contain a pre-CTL that required exogenous IL-2 to respond to antigen, we determined that, after our anti-Thy-1.1 plus C treatment, there were no pre-CTL capable of responding to antigen, even when IL-2 was added (Tables V and VI). This would strongly suggest that differentiation to a Thy-1$^+$ pre-CTL requiring exogenous IL-2 had occurred in these thymectomized mice.

In our initial analysis of the CTL repertoire of these mice, there are a number of similarities and several discrepancies when compared with the observations made in

TABLE XI

Sensitivity of CTL Precursors from Thymectomized Chimeras to Anti-Ly-1.2 plus C

Responder*	Stimulator	Target	Treatment: None 30:1	Treatment: None 6:1‡	Treatment: Anti-Ly-1.2 + C 30:1	Treatment: Anti-Ly-1.2 + C 6:1
Control chimera§	B6-TNP + IL-2	EL4-TNP	42	21‖	0	0
Thymectomized chimera	B6-TNP + IL-2	EL4-TNP	45	24	8	3
Normal B6 Thy-1.1	B6-TNP + IL-2	EL4-TNP	52	19	5	0
Control chimera	(B6 × DBA/2)F_1 + IL-2	P815	78	78	55	29
Thymectomized chimera	(B6 × DBA/2)F_1 + IL-2	P815	85	82	5	0
Normal B6 Thy-1.1	(B6 × DBA/2)F_1 + IL-2	P815	89	87	96	63

* Responder cells are 5×10^6 spleen cells cultured with 5×10^6 stimulator cells and 5% IL-2. Chimeras are Thy-1.1 → Thy-1.2 (cf legend Table I).

‡ Effector-target-ratio.

§ 25×10^6 spleen cells were treated twice with a monoclonal anti-Ly-1.2 antibody at a dilution of 4×10^{-4} and complement at 10^{-1}.

‖ Percent specific ^{51}Cr release. Spontaneous release was 5–7% for EL4-TNP and 13% for P815.

nude mice. (*a*) We found the CTL generated to TNP-modified syngeneic cells to be H-2 restricted. The degree of cross-reactivity to TNP-modified allogeneic cells was similar to that reported for normal B6 mice (20). There is, however, conflicting data concerning the H-2 restriction of nude mice. Gillis and Watson (31) have reported that the CTL generated in nude mice to TNP-modified syngeneic cells were unrestricted. Several groups, however, have reported H-2-restricted CTL generated in nude mice, including CTL generated to TNP-modified syngeneic cells (10, 11, 32).

(*b*) Hunig and Bevan (10) have observed that CTL generated from nude mouse spleen cells to allogeneic cells do not demonstrate cross-reactive lysis of third-party allogeneic target cells. Their data would suggest that the thymus may influence the ultimate CTL repertoire. Our thymectomized mice did demonstrate cross-reactivity and, thus, our findings are at odds with those of Hunig and Bevan. Admittedly, we examined different strain combinations, and it is possible that their strain combinations fortuitously uncovered a "hole" in the CTL repertoire that we missed. These differences, however, may reveal a difference, on a clonal level, in the T cell repertoire of nude mice compared with thymectomized mice. As we proceed with a more detailed analysis of the CTL repertoire in these mice, we might yet uncover defects or holes in their repertoire that reflect a thymic influence on the CTL repertoire.

(*c*) A third aspect of the CTL repertoire has also been studied and is also at odds with the observations of Hunig and Bevan (21). Hunig and Bevan found that when (A × B)F_1 mice were given an A thymus, CTL generated from these mice to alloantigen C would cross-react on A-TNP but not on B-TNP. They suggested that this demonstrated the influence of the thymus on the CTL repertoire, i.e., the cross-reactivity of alloreactive CTL for TNP-modified syngeneic cells, which has been observed in normal mice (17). Our thymectomized mice, however, did demonstrate this cross-reactivity. It has been argued that the reason (A × B)F_1 mice given an A thymus respond only to A + X but not B + X is due to suppressor cells that inhibit the response to B + X. Admittedly, these suppressors have not been demonstrated, but, if such cells exist, they should also suppress the clones of CTL that were cross-reactive for B-TNP, even if generated by stimulation with antigen C.

We were obviously concerned by our inability to find any holes in the T cell repertoire. Because B6 Thy-1.1 T cells express low amounts of Thy-1 antigen at certain stages of differentiation, we were concerned that some T cells recirculating to the bone marrow could escape anti-Thy-1.1 plus C treatment and account for the pre-CTL found in the spleens of the thymectomized chimeras. This, however, seems unlikely for several reasons. (*a*) The pre-CTL we detect in the spleens of thymectomized chimeras express high amounts of Thy-1.1 antigen, and, if such cells recirculated to the bone marrow, they should be eliminated easily with anti-Thy-1.1 plus C treatment. (*b*) We demonstrated that there were no detectable pre-CTL in the bone marrow inoculum after double anti-Thy-1 plus C treatment, for no cytolytic activity could be generated even when IL-2 was added with antigen (Tables V and VI). These treated bone marrow cells also failed to proliferate to the mitogen concanavalin A (data not shown). (*c*) Pre-CTL from B6 Thy-1.2 → B6 Thy-1.1 chimeras demonstrated the same requirement for IL-2, and cross-reactive CTL was found both for the B6 Thy-1.1 → B6 Thy-1.2 and B6 Thy-1.2 → B6 Thy-1.1 chimeras (Tables I, IV, VII, VIII, and IX). B6 Thy-1.2 T cells remain susceptible to treatment by anti-Thy-1.2 plus C at all stages of their differentiation: bone marrow cells, thymocytes, splenic pre-CTL, and CTL from B6 Thy-1.2 mice and B6 Thy-1.2 → B6 Thy-1.1 chimeras (control and thymectomized) were susceptible to anti-Thy-1.2 plus C treatment. Therefore, anti-Thy-1.2 plus C treatment of bone marrow should eliminate all Thy-1.2 T cells. (*d*) To be certain that we had not left a thymic remnant, we have done appropriate serial sections on thymectomized chimeras and found no thymic tissue by histological analysis. (*e*) The pre-CTL from our chimeras express the Lyt antigens, making it unlikely that the cytolytic activity we observed is the result of natural killer cell activity (Table XI).

Stutman (33) has proposed that a Thy-1-negative T cell that has undergone processing in the thymus may exist. Though such a cell has not been definitively identified, such a cell could account for the discrepancies found between nude mice and our thymectomized mice. One could speculate that the reason we cannot find holes in the repertoire is that these Thy-1-negative cells are in the bone marrow inoculum given to the thymectomized, irradiated mice, and in the recipient they differentiate to express the Thy-1 marker. Experiments using fetal liver or bone marrow from nude mice are in progress to test this hypothesis. Preliminary data with fetal liver reconstituted chimeras suggest that they give similar results (data not shown).

The model system we presented here can be used to study extrathymic differentiation but it may also provide insights into the problems one might encounter in the bone marrow transplantation of patients with thymic dysfunction. For example, older transplant patients might have involuted thymuses and their thymic function might be compromised. It is important to determine whether there will only be partial immune reconstitution of these patients, i.e., they will have holes in their repertoire. If defects in their T cell repertoire occur, especially for the recognition of viruses, these patients may be at greater risk for viral infections or malignancies. If such defects are identified, perhaps the full reconstitution of their immune system may require the addition of various interleukins together with bone marrow transplantation.

Summary

A model system has been developed to study extrathymic T cell differentiation. Mice have been thymectomized, lethally irradiated, and reconstituted with bone marrow cells depleted of Thy-1-positive cells. After 8 wk, the spleen cells of these 5athymic, bone marrow-reconstituted chimeras contain Thy-1-positive pre-cytolytic T lymphocytes (CTL) that are able to respond to antigen only when exogenous interleukin 2 is added to culture. The phenotype of these pre-CTL is similar to that of thymocytes, suggesting that they may be an immature T cell. Initial evaluation of the CTL repertoire of these athymic mice demonstrates that the CTL generated to trinitrophenyl-modified syngeneic cells are H-2 restricted and that the CTL generated to alloantigens have many of the cross-reactivities observed in normal but not in nude mice. The discrepancies observed in the CTL repertoire between these thymectomized chimeras and nude mice are discussed.

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