THYMIC CYTOTOXIC T LYMPHOCYTES ARE PRIMED IN VIVO TO MINOR HISTOCOMPATIBILITY ANTIGENS

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The thymus has been recognized as a primary site for T cell differentiation since the mid-1960s (1, 2). Despite many elegant experiments, attempts to define the relationship between organ morphology, cell kinetics, and cell function have been hindered by the complexity of thymic architecture and the dynamics of cell traffic within this organ, as well as the absence of well-defined stages in the pathway of T cell differentiation. For example, even the classical division of thymocytes into cortical and medullary cells is a controversial issue. Which compartments immunocompetent thymocytes reside in, whether a precursor-product relationship exists between cortical and medullary thymocytes, and in what way these populations contribute to the peripheral pool of functionally mature, recent thymic migrants are subjects of intense debate (reviewed in 3). In this paper, we offer evidence that questions two long-standing tenets of thymic physiology, that thymocytes are shielded from extrinsic antigen (4) and that the cell traffic within the thymus is unidirectional, moving from intrinsic stem cells or from bone marrow-derived stem cells to the thymus and to the periphery, never the reverse (5). In experiments described below, we show that thymocytes can be primed by intraperitoneal injection of cells bearing foreign minor histocompatibility (minor H) antigens, and that functional, antigen-reactive peripheral T cells can enter the thymus.

Materials and Methods

Animals. C57BL/10nSn (B10, H-2^b) and B10.D2nSn (B10.D2, H-2^d) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Lewis rats and the congenic mouse strains BALB/c (H-2^d), BALB.B (C.B, H-2^b), BALB.K (C.K, H-2^k), C57BL/Ka (Thy-1.2) [BL(1.2), H-2^b], and C57BL/Ka (Thy-1.1) [BL(1.1)] mice were obtained from our inbred colony at Stanford University. Mice of at least 6 wk of age were used in all studies.

Reagents. Rabbit anti-mouse thymocyte serum (ATS)[1] was purchased from M. A.

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[1] *Abbreviations used in this paper:* ATS, antithymocyte serum; C′, complement; Con A SN, supernatant from concanavalin A-activated rat spleen cells; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture; PHA-P, phytohemagglutinin.

Bioproducts, Walkersville, MD. Monoclonal anti-Thy-1 reagents include antibodies secreted by the rat-mouse hybridomas T24/31.7 (T24, non–allele-specific anti-Thy-1 [6]), 30-H12 (anti-Thy-1.2 [7]), and 53-2.1 (anti-Thy-1.2 [7]). T24 and 53-2.1 ascites were grown in pristane-primed, irradiated BALB/c mice and gave plateau cytotoxic titers of 3×10^5 and 3×10^4, respectively. Mouse monoclonal reagents 22.1 (anti-Thy-1.1 [8]), 13-4 (anti-Thy-1.2 [8]), 19XE5 (anti-Thy-1.1, kindly provided by R. C. Nowinski, Fred Hutchinson Cancer Research Center, Seattle, WA), C3PO (anti-Lyt-1.2 [9]), and AD4-15 (anti-Lyt-2.2 [10]) were all used in the ascites form. *Escherichia coli* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI) and the T cell mitogens concanavalin A (Con A) (Calbiochem-Behring Corp., La Jolla, CA) and phytohemagglutin (PHA-P) (Difco Laboratories) were used with mouse cells at 25, 2, and 10 μg/ml, respectively. Con A supernatant (Con A SN) was harvested from Lewis rat spleen cells cultured for 48 h with 5 μg/ml Con A. Con A SN was absorbed twice with Sephadex G25 to remove residual Con A and titered for interleukin (IL-2) activity on an IL-2-dependent T cell clone. Wherever possible, medium containing Con A SN was supplemented with 50 mM α-methyl-D-mannoside as a further precaution against residual Con A. Where indicated, hydrocortisone-21-acetate (Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally, 5 mg/mouse.

Treatment with Antibody Plus Complement. Cells at $5–20 \times 10^6$/ml were treated with antibody plus a selected rabbit complement (C′) in Hanks' balanced salt solution containing 2 mg/ml bovine serum albumin, as previously described (8). Concentrations of antibody used were in excess of those previously determined by titration to mediate plateau levels of specific lysis.

Construction of Thymus-shielded Chimeras. BL(1.2) mice were anesthetized with tribromoethanol (Aldrich Chemical Co., Milwaukee, WI), taped onto a wooden board, and their mid-sternum to head regions covered with a 2-mm lead shield. Mice were then administered 900 rad x irradiation (250 Kvp; Philips Electronic Instruments, Mahweh, NJ.), and injected intravenously 4 h later with 1.2×10^7 Thy-1 congenic BL(1.1) bone marrow cells that had been pretreated with a mixture of 22-1 and 19XE5 plus C′.

Priming for Minor H Antigens. Mice were primed 9–14 d before sacrifice by injection with 1.2×10^7 minor H different spleen cells. Spleen cells were either untreated, anti-Thy-1 plus C′ treated (intraperitoneal injection), or irradiated (intravenous injection).

Activation and Assay of Cytotoxic T Lymphocytes (CTL). Bulk mixed lymphocyte cultures consisted of 9×10^6 thymocytes or 7×10^6 spleen responder cells and 7×10^6 1,000 rad-irradiated spleen stimulator cells per well in 24-well plates (Costar, Cambridge, MA) in a total volume of 2 ml RPMI 1640 medium containing 5% fetal calf serum. Con A SN and α-methyl-D-mannoside were added where indicated. CTL activity was measured after 5–7 d by culturing serial threefold dilutions of responder cells with 10^4 ^{51}Cr-labeled target cells in 96-well, round-bottomed microtiter plates (Costar). After 4 h incubation at 37°C, the plates were centrifuged, supernatants removed, and the percent specific lysis calculated as: $100 \times$ [(cpm released by effector cells − cpm released by medium)/(cpm released by detergent − cpm released by medium)]. Effector cells to be typed for Thy-1 expression were divided into three aliquots and treated as described above with C′ alone, anti-Thy-1.1 plus C′, or anti-Thy-1.2 plus C′. After treatment and washing, surviving cells were assayed in the usual way for CTL activity. In each case, effector/target ratios were based on the number of effector cells recovered from culture on the day of assay. Target cells were 2–3 d Con A blasts prepared as previously described (11) and labeled for 1 h with sodium ^{51}Cr-chromate (New England Nuclear, Boston, MA).

For Con A induction of CTL, spleen cells and thymocytes were cultured at 3×10^6/well in 2 ml of medium containing 2 μg/ml Con A with or without Con A SN. The assay for polyclonal CTL activity was performed 3 d later using ^{51}Cr-labeled P815 with or without 10 μg/ml PHA-P as previously described (12).

Limiting dilution microcultures consisted of 24 wells each of serial threefold dilutions of thymocyte responder cells in round-bottomed microtiter plates. Each well contained 1×10^6 stimulator spleen cells in a total volume of 200 μl of RPMI 1640 with complete supplements, 20% Con A SN and 50 mM α-methyl-D-mannoside. CTL activity was

measured 7 d later in a 4-h ^{51}Cr-release assay by adding 8×10^3 target cells per well. Positive wells were those in which the amount of ^{51}Cr released exceeded by three standard deviations that released in wells with stimulators alone (reviewed in 13). Precursor frequencies were calculated both graphically and by weighted mean analysis (14).

Mitogen-induced [^{3}H]Thymidine Incorporation. Spleen cells and thymocytes were cultured at 10^5 cells per well in flat-bottomed, 96-well microtiter plates (Costar) in 200 μl medium with 25 μg/ml LPS or 2 μg/ml Con A, with or without Con A SN. As a measure of proliferation, the extent of thymidine uptake during a 6-h pulse on day 3 with [^{3}H]-thymidine (New England Nuclear) was determined using a multiple automated sample harvester (Mini-Mash; M. A. Bioproducts).

Results

Minor H-specific CTL Responses Were Generated from Thymuses and Spleens Only of Primed Mice. Minor H antigens are cell surface antigens of unknown function, an estimated 40 of which differ between the BALB and C57BL backgrounds (15). It has been known for some time that unlike the T cell responses to various haptens or to H-2 antigens, the generation of a measurable minor H-specific CTL response from spleen cells in bulk culture requires in vivo priming (16, 17). The reasons for this requirement are still somewhat controversial; suggestions include the increase in the precursor frequency of CTL or helper cells in the memory response, the generation of suppression during a primary in vitro response, or some qualitative change in the CTL precursor brought about by in vivo priming (18–20). We found that the generation of minor H-specific CTL from bulk cultures of thymocytes had a similar requirement for in vivo exposure to minor H antigens. These findings are depicted in Fig. 1, in which in vitro stimulated spleen cells and thymocytes from unprimed BL(1.2) mice (H-2^b, C57BL minors) generated active anti-H-2^k alloreactive CTL, but were unable to become activated to lyse minor H-different C.B (H-2^b, BALB minors) target cells (Fig. 1 *A* and *C*). However, good minor H-specific CTL activity was generated both by spleen cells and thymocytes from BL(1.2) mice injected 13 d previously with viable C.B spleen cells (Fig. 1 *B* and *D*).

It should be noted that the thymus-derived CTL response was quite vigorous, often more active on a per cell basis than the spleen cell response from the same animal. Both intraperitoneal and intravenous injections of whole spleen cells, anti-Thy-1–treated spleen cells, and 1,000-rad-irradiated spleen cells were able to prime thymocytes to minor H antigens. The thymocyte response to minor H antigens was generated from animals as early as 4 d and at least as late as 14 d after injection with antigen. The thymic response did not always mirror the splenic response in the extent of cross-priming (21) or the extent of T cell-mediated depletion of the direct primed response (22). Furthermore, the following results show thymus-derived CTL activity to be H-2 restricted: BL(1.2) anti-C.B CTL did not lyse BALB/c targets (which do not bear the proper H-2 antigens) nor B10 targets (which do not bear the proper minor antigens) but did lyse cells from F_1(BALB/c × B10) animals.

It is likely that very small numbers of mature T cells introduced at the time of culture can contribute overwhelmingly to responses measured from thymocyte cultures 7 d after initiation (23, 24). Realizing this, we in all cases sacrificed animals by etherization, to avoid the introduction of peripheral blood lympho-

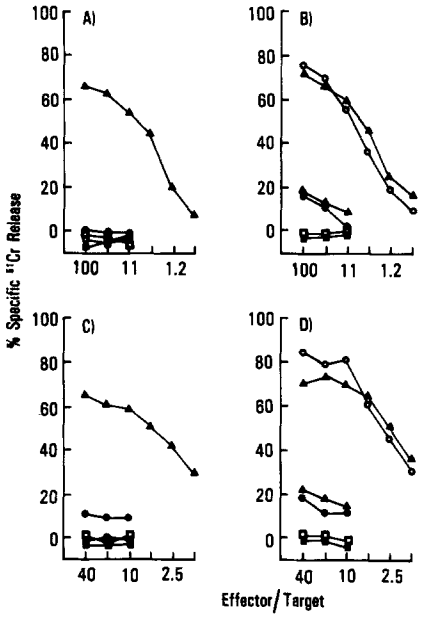

FIGURE 1. The generation of minor H-specific CTL from thymus and spleen required in vivo priming. Spleen cells and thymocytes were removed from unprimed BL(1.2) mice (*A*, *C*) or from mice injected intraperitoneally 13 d previously with 1.5×10^7 C.B spleen cells (*B*, *D*). Thymocytes (with 12% Con A SN) and spleen cells were cultured either with H-2 allogeneic C.K spleen cells (closed symbols) or minor H different C.B spleen cell stimulators (open symbols). CTL activity was measured 5 d later for spleen cell responders (*A*, *B*) and on day 7 for thymocytes (*C*, and *D*). Varying numbers of effector cells were assayed for lysis of ^{51}Cr-labeled 2-d Con A blasts of the following types: C.B (○, ●), C.K (△, ▲), and BL(1.2) (□, ■). Spontaneous release of ^{51}Cr from targets incubated in the absence of responders was 18–24%.

TABLE I

Precursor Frequencies of Thymus-derived Alloreactive and Minor H-reactive CTL

Responder Status	Reciprocal precursor frequency	
	Alloreactive	Minor H-reactive
Unprimed	32,100 ± 2,200	>400,000
Minor H Primed	27,900 ± 6,700	46,400 ± 12,600

CTL precursor frequencies were derived by weighted mean analysis of data from limiting dilution cultures of BL(1.2) thymocyte responders and stimulator spleen cells of BALB/c (alloreactive) or C.B (minor H-reactive) origin. Thymocytes were taken from unprimed mice or mice that had been primed 9–11 d previously with viable C.B spleen cells. Data represent the mean ± standard deviation from three separate experiments.

cytes, and intraperitoneally injected responder mice 1 h before sacrifice with particulate India ink, to stain parathymic lymph nodes which could then be avoided. Furthermore, limiting dilution analyses revealed that substantial numbers of precursor CTL could contribute to the minor H-specific CTL response of primed, but not unprimed, thymocytes (Table I). In fact, the precursor

frequency of minor H-specific thymic CTL from primed mice was comparable to the frequency of alloreactive CTL, although somewhat more variable. These limiting dilution data rule out the possibility of contamination of otherwise unresponsive thymocytes by small numbers of mature peripheral T cells, but do not eliminate the possibility of contamination on a larger scale. The CTL activity we measured could be due to cells from lymph nodes that do not drain the peritoneum (and therefore would not stain with India ink) or the result of the appearance in the thymus of antigen-activated peripheral T cells, a phenomenon that has recently been described for at least some proliferating T cell lines (25, 26). In the following several experiments, we attempted to test these possibilities by measuring the thymus-derived, minor H-specific CTL response from animals whose peripheral immune response has been compromised or eliminated.

Irradiation of Peripheral Cells Did Not Prevent Priming of Thymus-derived CTL. In the first set of experiments, BL(1.2) mice were x-irradiated (900–950 rad) with a lead plate shielding the upper chest and head. On the same day, mice were reconstituted with anti-Thy-1–treated bone marrow cells from Thy-1 congenic BL(1.1) mice. 2 d later, mice were injected with minor H different C.B spleen cells, from which viable T cells had been removed (to allow for unequivocal Thy-1 typing at a later stage). On this day, unreconstituted mice were found to retain 5–7% of the normal number of viable lymphoid cells in their spleens, lymph nodes, and bone marrow (data not shown). On days 11–13 after priming, spleen cells and thymocytes were typed for Thy-1 expression, and separately cultured with H-2 allogeneic (C.K) or minor H different (C.B) stimulator spleen cells. Minor H-specific effector CTL were typed for Thy-1 expression on the day of assay. Representative thymocyte data are depicted in Fig. 2. In Fig. 2*A*, the activity of minor H-specific CTL from the thymus of an unirradiated, primed BL(1.2) mouse was completely eliminated by pretreatment of effector cells with anti-Thy-1.2 plus C′, but not by pretreatment with C′ alone or with anti-Thy-1.1 plus C′. The latter treatment did, however, completely eliminate BL(1.1) effector cells (Fig. 2*B*). Such controls were included in each experiment to insure that the anti-Thy-1 pretreatments were both specific and complete. Thymocytes from thymus-shielded, irradiated chimeras generated a vigorous minor H-specific CTL response after priming with both anti-Thy-1 treated (Fig. 2*C*) and 1,300 rad-irradiated (*D*) C.B spleen cells. In three separate experiments, a total of 16 such chimeras were analyzed and all showed similar high levels of minor H-specific CTL activity. In each experiment, unprimed, thymus-shielded chimeras were included as controls and in each case generated alloreactive CTL but not minor H-specific CTL (data not shown). Thy-1 typing of the effector cells depicted in *C* and *D* reveals that all CTL activity was eliminated by pretreatment with anti-Thy-1.2 plus C′ and is therefore of host BL(1.2) origin. Furthermore, at least some of this thymic activity is resistant to in vivo exposure to cortisone, since thymocytes from animals injected with hydrocortisone-acetate 2 d before sacrifice still generated good minor H-specific CTL activity (data not shown).

The minor H-specific spleen cell responses from such chimeras were somewhat variable, lower than those found in unirradiated spleens and in the thymus, but never absent (data not shown). Evidence described below has lead us to presume that at least some of this primed splenic CTL activity is derived from thymocytes

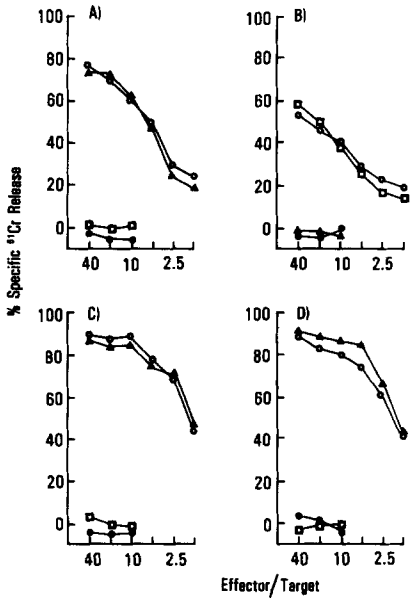

FIGURE 2. Thymic minor H-specific CTL were generated from primed, thymus-shielded chimeras. Responding cells were thymocytes from (*A*) BL(1.2), (*B*) BL(1.1), and (*C* and *D*) BL(1.2) mice that were thymus shielded, administered 900 rad, and given 1.2×10^7 anti-Thy-1-treated BL(1.1) bone marrow intravenously 14 d before sacrifice. Animals were primed to minor H antigens by intraperitoneal injection with 1.5×10^7 anti-Thy-1-treated C.B spleen cells (*A–C*) or intravenously with 1.5×10^7 1,300 rad C.B spleen cells (*D*). Animals *C* and *D* were primed 2 d after irradiation. Thymocytes were removed 12 d after priming and placed in culture for 7 d with C.B stimulators, in the presence of 12% Con A SN. CTL were treated on the day of assay with C′ alone (○), with anti-Thy-1.1 plus C′ (△), or with anti-Thy-1.2 plus C′ (□). After this treatment, CTL were assayed for lysis of 2-d Con A blasts from C.B (open symbols, 22% spontaneous release) and BL(1.2) (closed symbols, 25% spontaneous release).

that have emigrated within the period between priming and sacrifice. However, covering the upper chest and head shields not only the thymus but several neighboring lymph nodes. We attempted to minimize the contribution of any surviving peripheral T cells by intraperitoneally injecting animals 3 d before irradiation with 300 μl of commercial ATS, a treatment that depletes the periphery of T cells while leaving the thymus relatively intact (for review, see 27). Surprisingly, ATS pretreatment did not significantly decrease the magnitude of the splenic minor H-specific response at day 12 of priming, but did profoundly influence the contribution of donor bone marrow-derived cells to the thymic CTL response. In Fig. 3, both splenic (*A* and *C*) and thymic (*B* and *D*) minor H-specific responses, from ATS-pretreated, thymus-shielded, antigen-primed chimeras were typed for Thy-1 expression. Splenic T cells typed on the day of culture were all host-derived (100% susceptible to anti-Thy-1.2 plus C′) and totalled less than half the number of T cells of unirradiated control animals (data not shown). Minor H-specific effector CTL from spleens were similarly all host-derived (Fig. 3*A* and *C*). In contrast, 4–6% thymocytes typed on the day of culture from the same ATS-pretreated animals were of BL(1.1) origin; thymocytes from chimeras that did not receive ATS were 100% host-derived (data not shown). Thymic CTL showed a similar degree of chimerism (*B* and *D*). In four

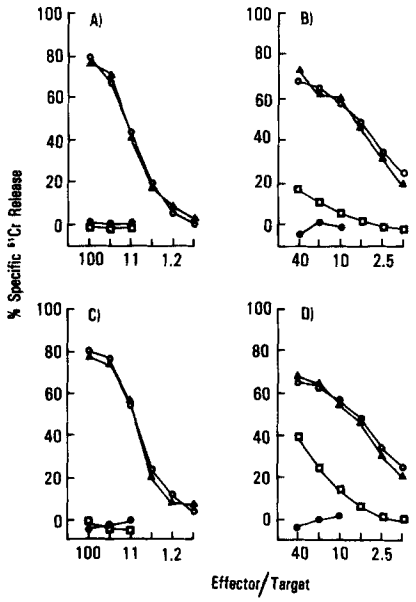

FIGURE 3. Donor bone marrow-derived CTL are generated only from the thymocytes of ATS-pretreated, thymus-shielded chimeras. Minor H-specific CTL from spleen cells (*A*, *C*) and thymocytes (*B*, *D*) from BL(1.2) animals that were injected on day −17 with 300 μl ATS, and on day −14, were thymus shielded, administered 900 rad, and given 1.2×10^7 anti-Thy-1–treated BL(1.1) bone marrow cells. Mice were given 1.2×10^7 anti-Thy-1–treated C.B spleen cells 2 d later. Spleen cells and thymocytes were removed 12 d after priming and stimulated in culture with C.B spleen cells for either 5-d (spleen cell responders) or 7-d (thymocyte responders). CTL were treated on the day of assay with C′ alone (○), anti-Thy-1.1 plus C′ (△), or anti-Thy-1.2 plus C′ (□). After treatment, responders were assayed on ^{51}Cr-labeled Con A blasts from C.B (open symbols, spontaneous release 19%) and BL(1.2) (closed symbols, spontaneous release 25–28%).

out of five ATS-pretreated animals, a small but significant CTL chimerism was detected: 1–8% of the minor H-specific CTL activity was contributed by bone marrow-derived BL(1.1) cells.

We interpret these findings in the following way: In those animals whose periphery was depleted of mature T cells by ATS and partial irradiation, the kinetics of stem cell traffic to the thymus was accelerated. This led to detectable numbers of cells within the thymus having recently arrived from the bone marrow. These recent arrivals, which had not yet left the thymus for the periphery in detectable numbers, could contribute to a primed CTL response. The fact that bone marrow-derived CTL were recovered from the thymus and not the spleen demonstrates that at least some component of the minor H-specific response in thymus-shielded chimeras was due to thymocytes. Because these irradiated and ATS-treated hosts contained significant levels of host-derived CTL, and because such cells could have been derived from either thymic or peripheral sites, we cannot estimate the extent to which host extrathymic T cells can also contribute to the thymic CTL activity.

Minor H-specific CTL Were Generated from the Thymus in the Total Absence of a Peripheral Response. To analyze further the relative contributions of peripheral

T cells and thymocytes to the "thymus-derived" CTL response, we designed a protocol for preventing peripheral activation to injected minor H antigens. Following the work of Opitz et al. (28) and J. Watson (personal communication), we subjected mice to frequent intraperitoneal injections of high-titered monoclonal anti-Thy-1 antibodies. The monoclonal reagents we used are produced by rat-mouse hybridomas T24 (an IgG2A non–allele-specific anti-Thy-1 [6]) and 53-2.1 (an IgG2A anti-Thy-1.2 [7]). As is documented in Table II, even a single injection of T24 ascites profoundly depressed the number of splenic cells expressing T cell markers and their ability to respond to T cell mitogens by proliferation or activation of polyclonal CTL. For example, 3 d after a single injection of 75 μl T24, <1% of spleen cells were susceptible to lysis by several monoclonal anti-Thy-1 antibodies plus C′ (Table II). Lyt-1- and Lyt-2-bearing cells also decreased to the expected levels (data not shown). The Con A response dropped from a stimulation index of >400 to an index of 1, with the LPS response relatively unchanged. Similarly, the Con A-induced CTL response dropped dramatically, even with the addition of Con A SN to the cultures (Table II). In contrast to the situation in the periphery, the expression in the thymus of T cell markers such as Lyt-1, Lyt-2 (data not shown), and Thy-1, and the

TABLE II

In Vivo Treatment with Monoclonal Anti-Thy-1 Antibodies Depleted Periphery but Not Thymus of Functional T Cells

Amount of antibody injected	Organ	Number of cells $\times 10^{-6}$*	Percent of untreated T cells‡	Mitogenesis§		Con A-induced CTL Percent specific lysis‖	
				Con A	LPS	−Con A SN	+Con A SN
None	Spleen	130	100	409	143	52	43
	Thymus	110	100	40	1	0	44
25 μl	Spleen	110	82	278	270	30	37
	Thymus	60	100	70	<1	1	49
75 μl	Spleen	90	0	1	90	0	14
	Thymus	90	100	54	<1	0	40
225 μl	Spleen	80	1	1	44	0	8
	Thymus	60	100	84	<1	0	42

B10 mice were injected intraperitoneally with the indicated amount of T24 ascites. Spleens and thymuses were removed 3 d later, typed with antibody plus C′ for T cell markers, cultured with the indicated mitogens and measured for proliferation on day 3, or cultured with Con A for 3 d and measured for polyclonal CTL activity in the presence of PHA. Data are from a pool of two to three mice per group.

* Total number of cells per donor organ.

‡ Percent of cells specifically lysed by three monoclonal anti-Thy-1 reagents in treated animals relative to the percent of cells lysed in uninjected control animals. In control animals, 40% of spleen cells were specifically lysed by anti-Thy-1 plus C′ (using the percent of cells lysed by anti-H-2 antibodies as 100%).

§ Figures represent stimulation indexes calculated as: (cpm incorporated with mitogen)/(cpm incorporated with medium).

‖ Percent specific lysis at an effector/target ratio of 20:1, calculated from a full titration of responders against a constant number of target cells. Cells were cultured with and without 4% Con A SN.

functional capacity of thymocytes from treated animals, remained intact. In fact, like the increase in thymic function reported after ATS treatment (29), the Con A (Table II) and PHA responses (data not shown), increased in treated animals.

This profound depletion of T cell function in the periphery was maintained for at least 10 d with several injections of T24 and 53-2.1 ascites (Table III). BALB/c mice were injected with 200 μl of T24, primed to minor H antigens the following day with irradiated or anti-Thy-1-treated B10.D2 spleen cells, and injected with T24 plus 53-2.1 the day after priming. Mice were either rested or injected every other day with anti-Thy-1 antibodies and sacrificed on day 9 or 10 after priming. Spleen cells and thymocytes were then typed with anti-H-2, anti-Thy-1, and anti-Lyt reagents, or put into culture with H-2 allogeneic or minor H different spleen cells. We attempted to avoid as much as possible the problems inherent in comparing thymic cell responses that peak at day 7 and which require the addition of factors derived from Con A SN with spleen cell responses, which peak at day 5 and do not require Con A SN. In each experiment summarized in Table III, we cultured spleen cells both with and without Con A SN and assayed both thymic and splenic CTL responses on day 6 in experiments 2 and 3.

As illustrated in Table III, the minor H CTL response required in vivo priming, even with thymocytes taken from anti-Thy-1–treated mice (experiment 3), which functionally may be more competent than cells from untreated animals (Table II and reference 29). In all 10 of the primed anti-Thy-1–treated animals tested in experiments 1 and 2 (7 of which are illustrated in Table III), spleen cells generated no minor H-specific and only low anti-H-2 CTL activity. In data not shown, typing for the expression of T cell markers identified from 0 to a maximum of 5% of T cells in these spleen cell populations, in contrast to 34–45% in the spleens of control mice typed on the same days. These data indicate that no surviving peripheral T cells were activated by the introduction of minor H antigens, even in animals that only received two anti-Thy-1 injections, the day before and the day after priming. In contrast, thymocytes in 8 of these 10 mice generated from low to moderately high levels of minor H-specific CTL activity, activity that is clearly not the result of peripheral cell influx.

We recovered from the spleens of all four of the animals analyzed in experiment 3 (three of which are shown in Table III) some memory minor H-specific CTL activity, detectable only after culture in 6% Con A SN. CTL memory cells primed in the thymus may enter the peripheral pool during the 3-d interval between the last Thy-1 injection and the day of culture, but not during the shorter intervals in experiments 1 and 2 (Table III).

Peripheral T Cells Could Contribute to the Thymic CTL Response. The previous experiments indicate that a thymic minor H response could occur in the complete absence of a peripheral response, and that at least a portion of the thymic response derived from cells that recently arrived from the bone marrow and had yet to seed the periphery. The following experiment tests whether antigen-activated peripheral cells can contribute to the thymic CTL response. BL(1.2) mice were injected with minor H different C.B spleen cells and with various numbers of nonimmune or immune lymph node cells from congenic BL(1.1) donors. Lymph node cells were used because they are relatively devoid of thymus-

TABLE III

Minor H-specific CTL Response from Thymocytes in the Absence of a Peripheral Response

Number of antibody injections	Priming protocol	Percent specific lysis*				
		Spleen − Con A SN		Spleen + Con A SN	Thymus + Con A SN	
		Allo-reactive	Minor H reactive	Minor H reactive	Allo-reactive	Minor H reactive
Experiment 1						
None	None	68	−8	−5	58	−6
None	Anti-Thy-1 spleen	76	50	62	78	74
5	Anti-Thy-1 spleen	12	−2	−3	ND	32
5	Anti-Thy-1 spleen	8	−2	−1	ND	45
5	Anti-Thy-1 spleen	26	−1	−1	ND	20
Experiment 2						
None	None	66	−1	2	78	−2
None	Anti-Thy-1 spleen	74	52	59	82	64
2	Anti-Thy-1 spleen	6	−6	−7	84	47
5	Anti-Thy-1 spleen	1	−7	−3	85	33
5	Anti-Thy-1 spleen	5	−5	−3	75	42
5	Anti-Thy-1 spleen	−1	−4	−5	73	45
Experiment 3						
5	None	ND	ND	ND	50	2
None	1,000 rad spleen	45	48	41	84	49
2	1,000 rad spleen	4	0	16	82	43
5	1,000 rad spleen	6	1	23	82	54
5	1,000 rad spleen	3	3	9	81	38

BALB/c mice were injected intraperitoneally every other day for the indicated number of times with 200 μl of T24 or 200 μl each of T24 and 53-2.1 ascites. Mice were primed 1 d after the first injection with 1.2×10^7 B10.D2 spleen cells that had been administered 1,000 rad or pretreated with anti-Thy-1 plus C′. Responder cells were cultured either with B10 or with B10.D2 stimulator cells. In experiment 1, animals were sacrificed 9 d after priming and 1 d after the last injection. Spleen cells were cultured 5 d with or without 2% Con A SN and thymocytes for 7 d with 10% Con A SN. In experiment 2, cells were removed 2 d after the last injection on day 9 of priming, and 3 d after the last injection on day 10 of priming in experiment 3. In experiments 2 and 3, spleen cells were cultured with or without 6% Con A SN, and thymocytes with 12% Con A SN, both for 6 d. Data are tabulated from individual mice.

* Percent specific lysis at an effector/target ratio of 20:1 on B10 (alloreactive CTL) or B10.D2 (minor H-specific CTL) Con A blasts. Spontaneous release in all experiments was 10–29%, with no detectable lysis of syngeneic targets in any experiment.

homing stem cells (30). Animals were sacrificed 12 d later and the relative contribution of host and donor cells to the memory CTL response determined. Representative results are summarized in Table IV. As expected, the donor contribution to the splenic minor H-specific CTL response depended both on the number of cells injected and on the immune status of the donor mouse. Of the five mice injected with unprimed lymph node cells, the donor contribution in the spleen varied from 0 to 8%, depending on the dose of injected cells. Of the 10 recipients of previously primed lymph node cells, the donor contribution in the spleen varied from 5 to 60% (Table IV and data not shown). Surprisingly, the thymus showed the same pattern of donor contribution, from 0 to 1% after

TABLE IV

Adoptively Transferred, Minor H-primed Peripheral T Cells Can Contribute to the Thymic CTL Response

BL(1.2) lymph node cells transferred	Recipient	Percent specific lysis*		Percent anti-Thy-1.1 resistant‡	
		Spleen	Thymus	Spleen	Thymus
None	BL(1.2)	53	74	100	100
None	BL(1.1)	65	75	0	0
4×10^7 unprimed	BL(1.1)	58	74	<1	<1
4×10^7 unprimed	BL(1.1)	50	73	0	<1
6×10^7 unprimed	BL(1.1)	50	52	3	1
6×10^7 unprimed	BL(1.1)	57	47	4	1
4×10^7 primed	BL(1.1)	54	71	8	5
4×10^7 primed	BL(1.1)	56	78	6	6
4×10^7 primed	BL(1.1)	58	66	5	40
4×10^7 primed	BL(1.1)	53	75	14	13
4×10^7 primed	BL(1.1)	57	72	8	7
6×10^7 primed	BL(1.1)	57	59	60	60
6×10^7 primed	BL(1.1)	48	45	50	12

BL(1.1) mice were injected intraperitoneally with 1.5×10^7 anti-Thy-1–treated C.B spleen cells and intravenously with lymph node cells from BL(1.2) donors that were either unprimed or injected 4 wk previously with C.B spleen cells. Animals were sacrificed 12 d later and responding cells cultured for 5 d (spleen cells) or 7 d (thymocytes, plus 12% Con A SN) with C.B stimulators. Responding cells were treated on the day of assay with C′ alone, anti-Thy-1.1 plus C′, or anti-Thy-1.2 plus C′, and then assayed for CTL activity on 2-d Con A blasts. Spontaneous release was 15–23%, with no detectable lysis of syngeneic target cells.

* Percent specific lysis of C.B targets at an effector/target ratio of 20:1, calculated from a full titration of responders against a constant number of target cells.

‡ A measure of the percent of the minor H-specific response mediated by donor cells, calculated from the percent of C.B-specific cytotoxicity that remained after treatment of effector cells with anti-Thy-1.1 plus C′.

the injection of unprimed cells and from 5 to 60% after the injection of previously primed lymph node cells. This relationship to the immune status of the donor animal makes it unlikely that we were detecting CTL newly differentiated within the thymus from the small number of stem cells in the injected lymph node cells. It should be stressed again that thymuses were removed in all cases in the absence of contamination by peripheral blood and after intraperitoneal injection of India ink to exclude the parathymic nodes. The CTL activity from the thymus did not always mirror the peripheral response, as seen in both the third and last recipients of primed cells listed in Table IV. These surprising results indicate that antigen-activated, mature, peripheral CTL can enter the thymus and contribute to the thymic memory CTL response (25).

Discussion

Our experiments show that the generation of measurable minor H-specific CTL responses from thymocytes require in vivo priming. We detected at least a ninefold increase in the number of thymic CTL precursors from primed relative to unprimed donors, in conditions of excess helper factors (Table I). These results strongly suggest that the CTL precursors themselves must be activated in vivo, and that the priming effect is not solely at the level of helper cells. The

linearity of our limiting dilution data indicate that the CTL precursor is the only limiting cell, and is not affected by a titratable number of suppressor cells, in contrast to the situation described for spleen cells (20).

We have presented evidence suggesting that thymocytes, including recent immigrants from the bone marrow, are primed in situ, and that mature lymph node-derived peripheral T cells activated to antigen also lodge in the thymus and contribute to the thymic CTL response. Experiments depicted in Table III indicate that a minor H-specific thymic CTL response can be generated from primed mice in the complete absence of a concomitant splenic response. The thymic CTL response is therefore not solely due to contamination by recirculating peripheral T cells. The fact that mature peripheral T cells can contribute to the thymic CTL response is illustrated in Table IV, where up to 60% of the thymic response to minor H antigens in bulk culture can be ascribed to previously primed adoptively transferred lymph node cells. These data do not allow the determination of the fraction of CTL that are donor derived. The appearance of mature T cells in the thymus has been described in the rat by Naparstek and colleagues (25, 26). In these experiments, T cell lines that proliferate in response to myelin basic protein were shown to lodge in the thymus in small numbers only after recent antigen activation in vitro (25). The fact that >99% of these injected cells end up in organs other than the thymus accords with data showing that detectable numbers of mature T cells do not specifically home to the thymus within 24 h after intravenous injection (5, 30). The significance of these experiments lies in the fact that the few cells that enter the thymus remain functional for up to 2 mo (25). It is unknown by what means and with what kinetics antigen-activated peripheral T cells enter the thymus or where they reside, facts that may in the future provide clues as to the possible intrathymic function of these persisting, mature T cells.

We know little about the properties of the CTL in the thymus that respond to minor H antigens. At least a fraction of the minor H-specific CTL response is resistant to the injection of cortisone 2 d before culture (see Results). However, this finding has little bearing on the sensitivity of responding cells at the time of antigen injection, for the necessary 10 d interval between priming and culture is more than enough time for cortisone-sensitive cells to become cortisone resistant (31). It appears that even in the presence of an excess of helper factors, cells of minority ("medullary") phenotype comprise the vast majority of thymocytes that proliferate or differentiate to CTL in the presence of Con A (23, 32) or allogeneic stimulator cells (24). It must be stressed that this minority phenotype has been defined by parameters other than intrathymic location. It is thus possible that small numbers of cells of this phenotype reside in the cortex (33), and that it is these cells which are the functionally mature thymocytes destined to seed the peripheral lymphoid organs. We have shown here that at least a part of the thymic minor H response is due to cells that have recently (within 2 d of antigen injection) arrived from the bone marrow (Fig. 3). Furthermore, we suspect that some thymic-derived, minor H-specific CTL are being exported to the periphery, as suggested by the difference in the magnitude of the splenic response in experiments 2 and 3, Table III. From all of these observations we surmise that the thymocytes which respond to minor H antigens are functionally competent

cells, at least a fraction of which turn over more rapidly than the long-lived, cortisone-resistant cell subpopulation previously described (34). Anything more than conjecture must await a means of identifying these CTL precursors independent of their response, perhaps by their in vivo association with nonlymphoid cells of the thymus (35) or by expression of a particular cell marker (36).

It must be pointed out that it is still formally possible that pre-T cells meet antigen in the periphery, either in the bone marrow or en route to the thymus. We feel that the consistently vigorous, host-derived CTL response from thymus-shielded chimeras (Fig. 2), even those pretreated with ATS (Fig. 3), favors the interpretation that resident thymocytes and not only immigrating pre-T or peripheral T cells contribute to this activity. Furthermore, priming of pre-T cells would require the expression by these cells of receptors that can bind both to the self H-2 antigens and to a diverse number of foreign minor H antigens. Given the proposed roles of the thymus in receptor selection (reviewed in 37) and the exceedingly small number of pre-T cells that can seed the thymus of an irradiated animal (38, 39; and S. Ezine, R. Rouse, and I. Weissman, manuscript submitted), we find the proposed expression by pre-T cells of receptors that bind to antigen in an H-2-restricted fashion highly unlikely. Most of the experiments interpreted as demonstrations of pre-T cell receptor expression can also be interpreted as low level expression of "environmental" antigen within the thymus (40, 41). Due to the lack of reliable anti-minor H antibodies for typing, a direct test for the presence of minor H antigens in the thymus would require a functional assay. Related experiments have shown that purified thymic dendritic cells from animals injected intravenously with either soluble protein antigens or semiallogeneic spleen cells can present antigen to T cell clones in vitro (B. A. Kyewski and H. S. Kaplan, manuscript submitted). Dendritic cells are found at the corticomedullary junction or in the medulla (42, 43) and therefore these recent findings are not contradictory to the experiments of Raviola and Karnofsky (44), demonstrating the exclusion of foreign protein antigens from the cortex but not the medulla.

If as we suspect, antigens do enter the thymus, it is unknown how and in what form this entry is allowed. We have found that priming for minor H antigens requires neither the injection of viable cells, T cells, nor cell preparations with efficient thymus-homing ability. We predict the entry into the thymus of antigen-presenting cells or their precursors in the foreign spleen cell preparation, or antigens processed by host antigen-presenting cells, perhaps in the normal course of the turnover of thymic antigen-presenting cells (35, 45). These foreign antigens must be compartmentalized and not allowed to freely interact with thymocytes in all stages of differentiation. To maintain the distinction between self and nonself, thymocytes in a tolerizable state must be secluded from this pool of foreign antigens (4, 46).

In conclusion, injected minor H antigen-bearing cells prime thymic CTL precursors specifically reactive to these antigens. Under differing experimental conditions, these primed thymic CTL were shown to be derived from three independent sources: pre-T cells, intrathymic lymphocytes, and extrathymic mature T cells. The experiments herein reported do not quantify the relative contributions of these three populations under physiologic conditions, an issue

that is not currently susceptible to experimental investigation. Nevertheless, we believe that each population experimentally revealed may contribute, perhaps in strikingly distinct ways, to the physiological response of the thymus to self and nonself antigens. Whether these responses control intrathymic maturation, extrathymic immunity, or both, awaits suitable model systems.

Summary

Potent cytotoxic T lymphocyte (CTL) activity can be derived from cultures of thymocyte responders and minor H different spleen cell stimulators. As is the case for the spleen cell response previously reported, this cytotoxic activity requires in vivo priming. We performed several experiments designed to determine whether the in vivo priming effect is due to the in situ priming of thymocyte CTL precursors, to contamination of thymus cell preparations with cells of neighboring lymph nodes, or to the appearance in the thymus of antigen-reactive peripheral T cells. We show by depletion of peripheral cells with antilymphocyte serum and part body irradiation that recent thymic immigrants derived from the bone marrow contribute to the primed thymic respnse. Thymic CTL were primed in animals in which peripheral T cell responses were completely eliminated by repeated treatment in vivo with monoclonal anti-Thy-1 reagents. Primed, antigen-activated lymph node cells were also demonstrated to contribute to the thymus-derived CTL response. Thus, the minor H-specific thymic CTL response is due both to in situ priming and the immigration of activated peripheral T cells. We discuss the possible significance for models of T cell differentiation of the presence within the thymus of antigen and antigen-reactive mature T cells.

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References

1. Miller, J. F. A. P. 1979. Experimental thymology has come of age. *Thymus.* 1:3.
2. Cantor, H., and I. Weissman. 1976. Development and function of subpopulations of thymocytes and T lymphocytes. *Prog. Allergy.* 20:1.
3. Shortman, K., and R. Scollay. 1983. Cortical and medullary thymocytes. *In* Recognition and Regulation in Cell-mediated Immunity. J. Watson and J. Marbrook, editors. Marcel Dekker, Inc., New York. In press.
4. Burnet, M. 1962. Role of the thymus and related organs in immunity. *Br. Med. J.* 2:807.
5. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. B. Biol.* 159:257.
6. Dennert, G., R. Hyman, J. Lesley, and I. S. Trowbridge. 1980. Effects of cytotoxic monoclonal antibody specific for T200 glycoprotein on functional lymphoid cell populations. *Cell. Immunol.* 53:350.
7. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
8. Marshak-Rothstein, A., P. J. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L.

Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.

9. Mark, C., F. Figueroa, Z. A. Nagy, and J. Klein. 1982. Cytotoxic monoclonal antibody specific for the Lyt-1.2 antigen. *Immunogenetics* 16:95.
10. Gottlieb, P. D., A. Marshak-Rothstein, K. Auditore-Hargreaves, D. B. Berkoben, D. August, R. Rosche, and J. Benedetto. 1980. Construction and properties of new Lyt-congeneic strains and anti-Lyt-2.2 and anti-Lyt-3.1 monoclonal antibodies. *Immunogenetics.* 10:545.
11. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 48:766.
12. Bevan, M. J., R. E. Langman, and M. Cohn. 1976. H-2 antigen-specific cytotoxic T cells induced by concanavalin A: estimation of their relative frequency. *Eur. J. Immunol.* 6:150.
13. Miller, R. G., H.-S. Teh, E. Harley, and R. A. Phillips. 1977. Quantitative studies of the activation of cytotoxic lymphocyte precursor cells. *Immunol. Rev.* 35:38.
14. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. *J. Immunol.* 126:1614.
15. Snell, G. D., J. Dausset, and S. G. Nathenson. 1976. Histocompatibility. Academic Press, Inc., New York. 401 pp.
16. Gordon, R., E. Simpson, and L. E. Samelson. 1975. In vitro cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J. Exp. Med.* 142:1108.
17. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* 142:1349.
18. Bevan, M. J. 1977. Priming for a cytotoxic response to a minor histocompatibility antigens: antigen specificity and failure to demonstrate a carrier effect. *J. Immunol.* 118:1370.
19. Pilarski, L. M. 1981. Regulation of the cytotoxic T cell response to minor histocompatibility antigens. *Transplantation (Baltimore).* 32:188.
20. Macphail, S., and O. Stutman. 1982. Suppressor T cells activated in a primary in vitro response to non-major histocompatibility alloantigens. *J. Exp. Med.* 156:1398.
21. Bevan, M. J. 1976. Minor H antigens introduced on H-2 different cells cross-react at the cytotoxic T cell level during in vivo priming. *J. Immunol.* 117:2233.
22. Fink, P. J., I. L. Weissman, and M. J. Bevan. 1983. Haplotype-specific suppression of cytotoxic T cell induction by antigen inappropriately presented on T cells. *J. Exp. Med.* 157:141.
23. Chen, W.-F., R. Scollay, and K. Shortman. 1982. The functional capacity of thymus subpopulations: limit dilution analysis of all precursors of cytotoxic lymphocytes and of all T cells capable of proliferation in subpopulations separated by the use of peanut agglutinin. *J. Immunol.* 129:18.
24. Ceredig, R., A. L. Glasebrook, and H. R. MacDonald. 1982. Phenotypic and functional properties of murine thymocytes. I. Precursors of cytolytic T lymphocytes and interleukin-2-producing cells are all contained within a subpopulation of "mature" thymocytes as analyzed by monoclonal antibodies and flow microfluorometry. *J. Exp. Med.* 155:358.
25. Naparstek, Y., J. Holoshitz, S. Eisenstein, T. Reshef, S. Rappaport, J. Chemke, A. Ben-Nun, and I. R. Cohen. 1982. Effector T lymphocyte line cells migrate to the thymus and persist there. *Nature (Lond.).* 300:262.
26. Naparstek, Y., A. Ben-Nun, J. Holoshitz, T. Reshef, A. Frenkel, M. Rosenberg, and I. R. Cohen. 1983. T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors

and accumulation in the brain and thymus of line cells. *Eur. J. Immunol.* 13:418.
27. Lance, E. M., P. B. Medawar, and R. N. Taub. 1973. Antilymphocyte serum. *Adv. Immunol.* 17:1.
28. Optiz, H. G., U. Opitz, G. Hewlett, and H. D. Schlumberger. 1982. A new model for investigations of T-cell functions in mice: differential immunosuppressive effects of two monoclonal anti-Thy-1.2 antibodies. *Immunobiology.* 160:438.
29. Cantor, H., and R. Asofsky. 1973. Paradoxical effect of anti-thymocyte serum on the thymus. *Nature (Lond.).* 243:39.
30. Lepault, F., and I. L. Weissman. 1981. An in vivo homing assay for thymus-homing bone marrow cells. *Nature (Lond.).* 293:151.
31. Weissman, I. L. 1973. Thymus cell maturation. Studies on the origin of cortisone-resistant thymic lymphocytes. *J. Exp. Med.* 137:504.
32. Herron, L. R., C. A. Abel, J. vanderWall, and P. A. Campbell. 1983. Immature thymocytes isolated using a sialic acid-specific lectin are unresponsive to concanavalin A. *Eur. J. Immunol.* 13:73.
33. Weissman, I. L., S. Baird, R. L. Gardner, V. E. Papaioannou, and W. Raschke. 1976. Normal and neoplastic maturation of T lineage lymphocytes. *Cold Spring Harbor Symp. Quant. Biol.* 41:9.
34. Elliot, E. V. 1973. A persistent lymphoid cell population in the thymus. *Nature New Biol.* 242:150.
35. Kyewski, B. A., R. V. Rouse, and H. S. Kaplan. 1982. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. *Proc. Natl. Acad. Sci. USA.* 79:5646.
36. Gallatin, W. M., I. L. Weissman, and E. C. Butcher. 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature (Lond.).* 304:30.
37. Bevan, M. J., and P. J. Fink. 1978. The influence of thymus H-2 antigens on the specificity of maturing killer and helper cells. *Immunol. Rev.* 42:3.
38. Wallis, V. J., E. Leuchars, S. Chwalinski, and A. J. S. Davies. 1975. On the sparse seeding of bone marrow and thymus in radiation chimeras. *Transplantation (Baltimore).* 19:2.
39. Micklem, H. S., C. E. Ford, E. P. Evans, and D. A. Ogden. 1975. Compartments and cell flows within the mouse hemopoietic system. I. Restricted interchange between hemopoietic sites. *Cell Tissue Kinet.* 8:219.
40. Besedovsky, H. O., A. del Rey, and E. Sorkin. 1979. Role of prethymic cells in acquisition of self-tolerance. *J. Exp. Med.* 150:1351.
41. Bradley, S. M., P. J. Morrissey, S. O. Sharrow, and A. Singer. 1982. Tolerance of thymocytes to allogeneic I region determinants encountered prethymically. *J. Exp. Med.* 155:1638.
42. Duijvstijn, A. M., and E. C. M. Hoefsmit. 1981. The ultrastructure of the rat thymus. The microenvironment of T lymphocyte maturation. *Cell Tissue Res.* 218:279.
43. Barclay, A. N., and G. Mayrhofer. 1981. Bone marrow origin of Ia-positive cells in the medulla of rat thymus. *J. Exp. Med.* 153:1666.
44. Raviola, E., and M. J. Karnovsky. 1972. Evidence for a blood thymus barrier using electron-opaque tracers. *J. Exp. Med.* 136:466.
45. Longo, D. L., and R. H. Schwartz. 1980. T-cell specificity for H-2 and Ir gene phenotype correlates with the phenotype of thymic antigen-presenting cells. *Nature (Lond.).* 287:44.
46. 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 anlagen. *Proc. Natl. Acad. Sci. USA.* 80:3045.