SEEDING OF THYMIC MICROENVIRONMENTS DEFINED BY DISTINCT THYMOCYTE–STROMAL CELL INTERACTIONS IS DEVELOPMENTALLY CONTROLLED

By BRUNO A. KYEWSKI

From the Cancer Biology Research Laboratory, Department of Radiology, Stanford University School of Medicine, Stanford, California 94305; and the Institute for Immunology and Genetics, German Cancer Research Center D-69 Heidelberg, Federal Republic of Germany

T cell differentiation passes through sequential stages in distinct organ microenvironments. Pluripotent stem cells in the bone marrow differentiate to prothymocytes that specifically home to the thymus (1, 2). Within the thymus these prothymocytes differentiate to mature T cells that unidirectionally migrate to peripheral lymphoid organs (3, 4). The intrathymic phase of T cell differentiation is of particular interest, because the generation and selection of the T cell repertoire and the differentiation of T cell subsets (helper and cytotoxic T cells) has been ascribed to this phase. Recent cloning of the genes encoding the T cell receptor (TCR)[1] polypeptides and development of TCR-specific monoclonal antibodies (mAb) allowed a more precise definition of the stage of T cell maturation at which the antigen specific receptor appears (5–7). During ontogeny, TCR gene rearrangement and TCR surface expression occurs after colonization of the thymus with prothymocytes and proceeds in organ cultures without recruitment of exogeneous prothymocytes (8, 9). Furthermore, a single thymic precursor cell can give rise to the major T cell subsets within thymic organ cultures in vitro (10, 11), implying that the thymic microenvironment itself is sufficient for inducing and maintaining these molecular and cellular differentiation events.

Despite the relatively simple architecture of the thymus we still lack a precise structure-function definition of the thymic microenvironment (12). Thus, the postulate for strict intrathymic selection mainly rests on indirect evidence. Studies on cell population dynamics indicate an imbalance between thymocyte production and cell exit (13), and functional data in chimeras demonstrate a strong bias of T cell specificity towards the MHC phenotype expressed by thymic epithelial cells (14, 15).

It is presumed that selection of T cell specificity occurs via TCR-directed cell–cell recognition. At least two types of thymic stromal cells have been invoked in

[1] *Abbreviations used in this paper:* AMC, acetone, methanol, chloroform; BM, bone marrow; CD, cell division; DC, dendritic cells; DN, double negative thymocytes; LN, lymph node; Mϕ, macrophages; PNA, peanut agglutinin; SF, seeding frequency; TCR, T cell receptor; TNC, thymic nurse cells; T-ROS, thymocyte rosettes.

T cell selection: resident thymic epithelial cells and bone marrow–derived dendritic cells (16), both of which constitutively express class I and II MHC antigens in situ. More recent data suggest that epithelial cells mediate selection for self-MHC restriction whereas medullary dendritic cells are involved in T cell tolerization (17–19). While most of these conclusions have been drawn from experimental models that either involve bone marrow radiation chimeras or thymus grafts, relatively little is known about the actual in situ interactions of T cells with these stromal cells during normal differentiation.

Recently, distinct cell–cell interaction complexes between stromal cells and T cells have been isolated and characterized. These complexes comprise associations of thymocytes with I-A$^-$ macrophages (macrophage rosettes; Mϕ-ROS), I-A$^+$ dendritic cells (dendritic cell rosettes; DC-ROS), and I-A$^+$ epithelial cells (thymic nurse cells; TNC) (20–22). Evidence has been obtained that they represent the correlate in vitro of corresponding interactions in vivo. The different location within the thymus and the different phenotypic and functional properties of the stromal cells involved in these cell–cell interactions suggest that each plays a defined role in T cell differentiation (23, 24). To substantiate the identity of these lymphostromal cell complexes as sites of T cell differentiation and selection it is important to know (a) at which stage of T cell development each recognition step occurs, and (b) whether the T cell–stromal cell interactions in situ are random or selective. The isolation of thymocytes defined by recognition of a distinct stromal cell in vivo makes these questions amenable to experimental analysis. In this study I used congenic Thy-1.1/1.2 bone marrow chimeras and followed the seeding of sites of thymocyte–stromal cell interactions by donor cells. To minimize experimental artifacts a model of nonradiation chimeras was developed in which substantial T cell chimerism was achieved by multiple intraveneous injections of donor-type bone marrow cells into normal newborn mice without prior ablation of host bone marrow or thymus. Using both nonradiation and low-dose radiation chimeras, a temporal hierarchy of the seeding of distinct thymic microenvironments during intrathymic T cell development was observed.

Materials and Methods

Animals. C57BL/Ka (Thy-1.2) and C57BL/Ka (Thy-1.1) mice bred at the animal facilities of the Department of Radiology, Stanford University were used throughout these studies. Radiation chimeras were constructed as described (21). Non-radiation chimeras were newborn Thy-1.2 mice injected at day 1 and 2 and if possible at day 3 into the preocular vein, and at days 4 and 5 intraperitoneally with 2.0×10^7 Thy-1.1 bone marrow cells/day. Cells were filtered through 20-μm pore size nylon gauze and injected in a volume of 30 μl with 30-gauge needles. Only animals that received at least two intravenous injections were used; 50–80% of the mice survived this treatment. The time points in Fig. 1 e are derived from four independently injected litters.

Isolation of Lymphostromal Cell Complexes. The isolation of T-ROS and TNC was performed as described previously (23). Pooled TNC fractions were passed through a 45-μm pore size gauze to exclude larger complexes. Highly enriched TNCs were fixed with 1% paraformaldehyde and treated with 0.1% Triton X-100 to permeabilize the epithelial membranes and allow access of the anti-Thy-1 antibodies to intra-TNC thymocytes. Labelled TNC were flattened between a slide and a cover slip to facilitate analysis of intra-TNC T cells within the focal plane. T-ROS were mounted in modified Cunningham chambers and analyzed by changing the focal plane.

Isolation of Lyt-2/L3T4⁻ Thymocytes. Unselected thymocytes of several thymuses were pooled per time point. 5 × 10⁸ cells were first incubated with saturating concentrations of anti-Lyt-2 mAb (clone 3.16 IgM, kindly provided by Dr. Fitch) for 15 min at 24°C and then 10 ml of rabbit complement (Cedarlane Laboratories, Hornby, Canada; final dilution 1:20) was added. Cells were incubated for 30 min at 37°C in the presence of 15% FCS and 25 µg/ml DNase I (Sigma Chemical Co., St. Louis, MO) then washed twice, and the same steps were repeated with mAb anti-L3T4 (clone 172.4 IgM, kindly provided by Dr. R. H. MacDonald, Ludwig Institute, Lausanne, Switzerland). Viable cells were then purified over a one-step gradient of Lymphoprep (Nyegaard, Oslo, Norway). Cell recovery ranged between 0.5 and 3%.

Isolation of PNA^{low} Thymocytes. Thymocytes were incubated for 15 min at 25°C with 0.5 mg/ml peanut agglutinin (PNA) (Sigma Chemical Co.) at 2.50 × 10⁸ cells/ml, adjusted to 5% FCS, and layered on a discontinuous gradient of 100, 50, 25, and 10% FCS/RPMI. Cell aggregates were allowed to settle for 60–90 min at 25°C. Cells in the top layer of 5% FCS (5–11% of cell input) were collected and defined as PNA^{low} thymocytes (6).

Reagents. Double staining was performed with mAb anti-Thy-1.1–biotin (clone 19E 12.1), anti-Thy-1.2–FITC (clone 30 H 12), anti-I-A^b–FITC (clone Pb 107), and avidin–TRITC (tetramethylrhodamine isothiocyanate) (Vector Laboratories, Burlingame, CA) as described (21).

Evaluation of Kinetics. For each time point, 7–10 mice of radiation chimeras and 3–4 of nonradiation chimeras were pooled. Unselected thymocytes, double-negative (DN), and PNA^{low} thymocytes and TNC were double-labeled for Thy-1.1 and Thy-1.2. T-ROS were double labeled for Thy-1.1 and I-A^b, and I-A⁻ stromal cells within ROS were equated with Mφ, and I-A⁺ stromal cells with DC (24). 100– >1,000 complexes of each type were counted according to the frequency of donor cells. The absolute counts of Thy-1.1 cells ranged from 2–3 at the earliest to >700 at the latest time points. In all experiments the calculations of the frequencies are based on the assumption of 10 T cells per Mφ-ROS or mixed T-ROS, 5 T cells per DC-ROS, and 20 T cells per TNC. These numbers represent in each case an average derived from numerous experiments. The regression curves were plotted according to best fit analysis using a computer graphics program.

Calculation of Seeding Frequencies (SF). The calculations of the seeding frequencies (Table III) are based on the following considerations. Given the two populations of Thy-1.1 and Thy-1.2 T cells seeding the thymus in nonradiation chimeras, the probability of a lymphostromal cell complex being formed only by Thy-1.2 cells is determined by (a) the overall frequency of Thy-1.2 thymocytes, and (b) the number of T cells per complex. This relationship is described in the formula: (frequency of free Thy-1.2 cells)^{(T cells/complex)} = expected frequency of complexes of Thy-1.2 only.

Given an average number of 20 thymocytes per TNC and 15 thymocytes per T-ROS, one can calculate the expected frequencies of those TNC and T-ROS that exclude Thy-1.1 cells according to this formula. This calculation is valid under the assumptions that, in situ, (a) Thy-1.1 and Thy-1.2 cells are equally likely to interact with a given stromal cell, that (b) both populations are randomly distributed, and (c) that all T cells of a complex associate independently. In the case of TNC the expected frequencies deviated from those determined experimentally. To reconcile this observation we modified one of the assumptions. Because there is no evidence that Thy-1 dictates the specificity of these interactions (23), and because we found Thy-1 cells to be randomly distributed within the thymus of nonradiation chimeras in tissue sections, we modified the third assumption. The number of T cells associating independently with a given stromal cell in situ is regarded as a variable in the above equation and termed seeding frequency (SF). Accordingly, the SF in Table III are based on the equation: (frequency of free Thy-1.2 thymocytes)^{sf} = experimental frequency of complexes of Thy-1.2 only. The number of cell divisions (CD) necessary to generate a lymphostromal complex is, accordingly: sf × 2^{cd} = number of T cells per complex.

Immunohistology. Single thymic lobes were washed in PBS, immersed for one min in 2–3 ml of freon that was precooled in an excess volume of liquid nitrogen, and then immediately transferred to 50 ml of a mixture of acetone/methanol/chloroform (AMC)

(1:2:1, vol/vol/vol) that was precooled in a slurry of ethanol and dry ice. The tissue was stored in this mixture for 4–21 d at −70°C. For further processing of the tissue, the AMC mixture was allowed to warm up to 4°C and was then replaced by ice-cold chloroform. After two further overnight changes of the cold chloroform the tissue was embedded in paraffin and tissue sections were performed according to standard procedures. 6-μm sections were stained by sequential incubation with (a) 1% BSA in PBS, (b) the rat mAb anti-Thy-1.2 (clone 30H12) or the mouse mAb anti-Thy-1.1 (clone 19 E 12.1), (c) rabbit anti–mouse IgG–biotin or horse anti–rat IgG–biotin, and (d) Vectastain avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride as a substrate (Sigma Chemical Co.). Sections were counterstained with hematoxylin.

Results

Correlation of Thymic Repopulation In Situ with Isolated Microenvironments in Low-dose Radiation Chimeras. C57BL/Ka (Thy-1.2) mice were sublethally irradiated (400 rad) and reconstituted with congenic C57BL/Ka (Thy-1.1) bone marrow cells. After initial abortive regeneration of the thymus by radioresistant host cells, donor-type cells completely replace the host cells between days 8 and 22 after reconstitution (25). This replacement of Thy-1.2 thymocytes by Thy-1.1 thymocytes was monitored in the following thymocyte subsets; Lyt-2$^-$, L3T4$^-$ (double negative, DN) thymocytes; Mϕ-, DC-, TNC-associated, PNA^{low}, and unselected thymocytes. Starting with day 6 after irradiation, T cell subsets and corresponding thymic sections were analyzed at daily or 2-d intervals. As shown in Fig. 1, *a–d*, donor type cells were found to seed the DN thymocyte subset first; after a delay of ~2 d Mϕ-associated thymocytes were repopulated, and after another delay of ~2 d TNC- and DC-associated thymocytes were replaced. Donor cells could first be detected within T-ROS at day 6, but not before day 8 within TNC (Fig. 1*d*). The PNA^{low} thymocyte subset representing mature medullary-type thymocytes was the last compartment to be seeded, again ~2 d after DC-associated T cells (Fig. 1*c*). Within the resolution limits of this method no significant difference in replacement kinetics between TNC- and DC-associated T cell subsets was discernible in two independent experimental groups. Unselected thymocytes representing 90–95% of all thymic T cells showed similar repopulation kinetics as TNC- and DC-associated thymocytes. The interval of 6 d spanning the seeding of the DN precursor cells and PNA^{low} thymocytes probably represents a minimal estimate of the overall time required for intrathymic T cell maturation. Given the slope of these kinetics, a time difference of 2 d corresponds to the relative enrichment of donor cells by a factor of about five at a given time point. The regression curves of all compartments essentially run parallel, indicating a similar rate of relative replacement.

In parallel tissue sections of the same experimental group as shown in Fig. 1*b*, donor cells first became apparent at days 8–9 as rare individual cells appearing in the inner- and mid-cortex. These cells were often found in association with capillaries. Donor cells closely apposed in doublets and clusters (a cluster is defined here as three or more donor cells) were found in random sections at days 10–12 in the mid-cortex. By day 13, donor cells had seeded the whole cortex, with prominent cluster formation in the supcapsular region. Thy-1.1$^+$ cells were not found in the medulla up to day 14. By day 15 individually scattered Thy-1.1 cells and by days 16–17 donor T cell clusters appeared in the medulla.
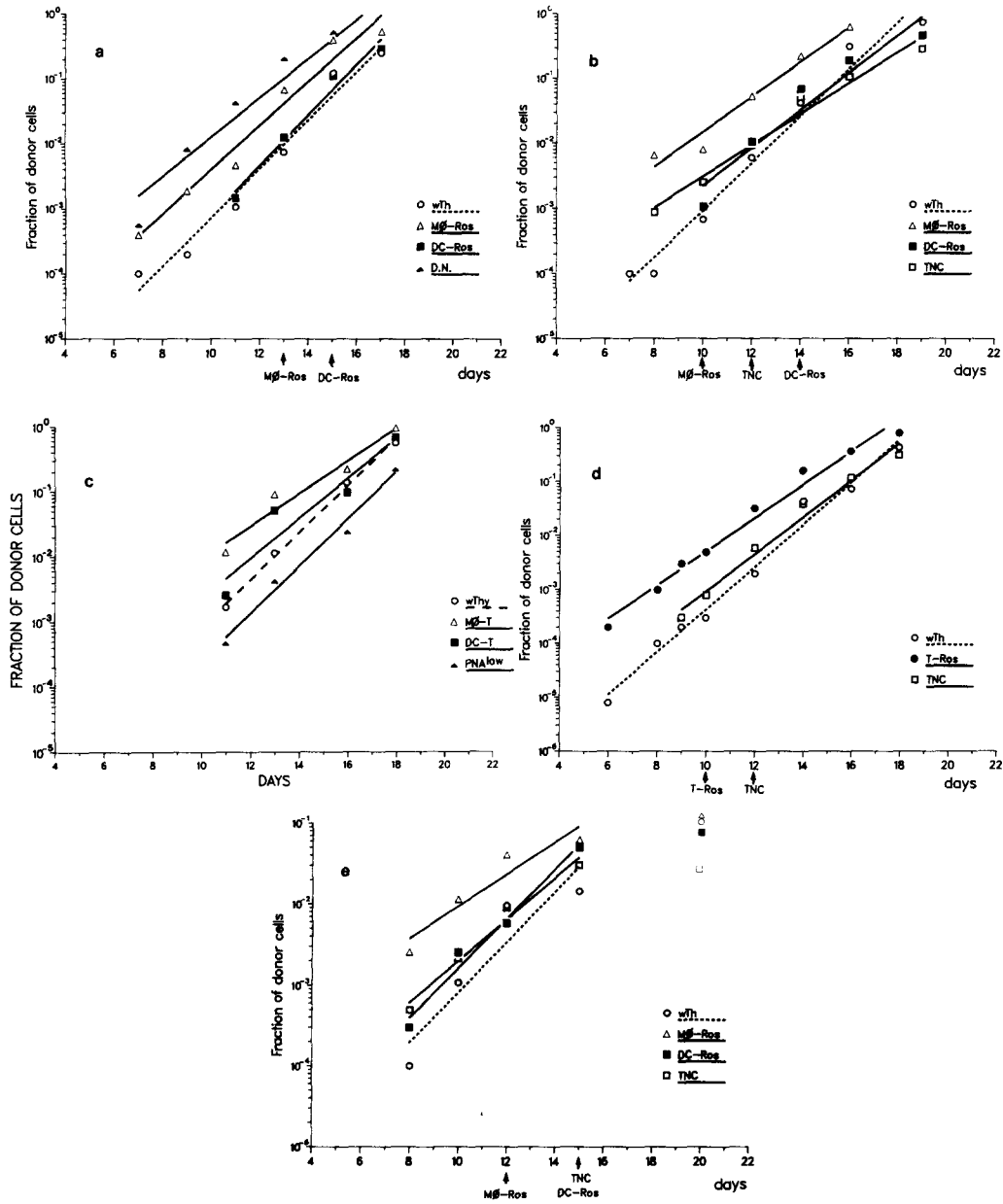
FIGURE 1.   Seeding kinetics of distinct thymic microenvironments. (a–d) Sublethally irradiated Thy-1.2 mice were injected with congenic Thy-1.1 BM cells. Four independent groups of chimeras are shown. (e) Newborn Thy-1.2 mice were injected five times with Thy-1.1 congenic BM cells. For details see Materials and Methods. The ratio of Thy-1.1 to total number of Thy-1$^+$ thymocytes in the indicated thymocyte subset is plotted against the time after reconstitution. The day of first appearance of donor cell clusters (defined as at least three donor T cells per complex) within each type of lymphostromal cell complex is indicated by arrows. The time scale refers to days after the first injection. Note the different logarithmic scales. wTh, whole thymocytes.

Thymic repopulation after radiation proceeded asynchronously with different areas of the same thymic lobe showing varying degrees of repopulation. Interestingly, scattered donor cells were detected first at the medullary side of the corticomedullary junction where the adjacent cortical region was most progressed in its repopulation (not shown).

At the level of individual lymphostromal cell complexes, single donor cells first associated with Mϕ at days 6–8 (Fig. 1 d). By days 10–11 donor cells were found in doublets and clusters within Mϕ-ROS, concomitant with the appearance of the first donor cell doublets and clusters in sections of the cortex. Likewise, the seeding of TNC occurred via individual cells (first on days 8–9) and clusters (first on day 12), the appearance of which, however, was delayed by 2 d as compared to the seeding of Mϕ. Appearance of donor cell clusters within TNC coincided with clustered distribution of donor cells in the outer cortex and subcapsular zone. Small donor-type T cells were found to interact with DC as early as days 10–12, and donor cell clusters within DC-ROS appeared by day 15 concomitantly with donor cell clusters in situ in the medulla (Fig. 2).

All three types of interactions between donor T cells and stromal cells thus seem to develop along a common scheme, namely from associations of individual small donor T cells via T cell blasts and clusters with an individual lymphostromal cell complex. Donor cells segregated into individual lymphostromal cell interaction sites rather than distributed randomly among stromal cells of a given type during repopulation (see time of appearance of donor cell clusters in Fig. 1). As a result of this nonrandom distribution, individual Mϕ-ROS, when compared to the cortex during the early seeding phase, and individual DC-ROS, when compared to the medulla during the later phase could be 50–100-fold enriched for donor T cells.

Intrathymic Traffic and Cell–Cell Interactions in Nonradiation Chimeras. To assess the physiologic validity of the aforementioned results, we aimed at studying the same events within an unperturbed thymic microenvironment. This consideration is particularly relevant when studying T cell–stromal cell interactions, because radiation may affect thymocytes and stromal cells differently. Parabiosis, part-body irradiation with thymus shielding or direct intrathymic injection were used as means to introduce donor cells without direct depletion of the thymus (26). To circumvent various artifacts associated with these experimental approaches we modified a protocol described previously (27). Newborn Thy-1.2 mice were injected several times at daily intervals with congenic Thy-1.1 bone marrow cells without previous ablation of their host bone marrow. The extent of their T cell chimerism in the thymus and mesenteric lymph nodes was assessed at different times thereafter. As shown in Fig. 3, five injections of 2×10^7 bone marrow cells led to sustained T cell chimerism after 3 wk with up to 15% donor-type T cells. The extent of chimerism depended on (a) the dose and source of donor cells injected, (b) the age of the host, and (c) the route of injection. A delay of the last three injections by 2 d resulted in a reduction of chimerism by 6%. Injections of twice the amount of bone marrow cells into adult mice led to chimerism of only 1–2% (Table I). One, three, and five injections of donor cells results in maximal chimerism of 1–3, 13, and 15%, respectively. Seeding of the cortex and significant T cell chimerism was only achieved by injection of bone
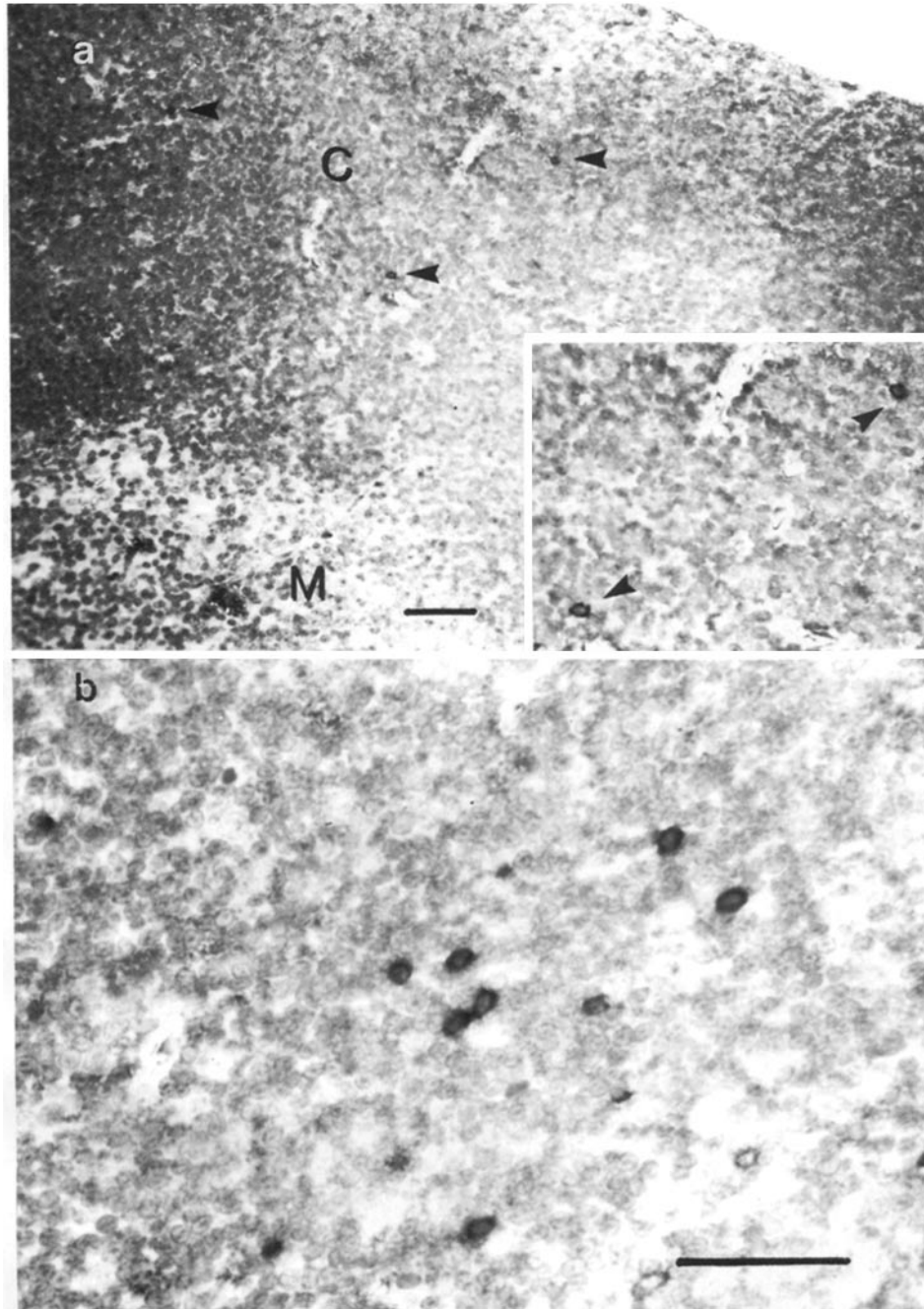
FIGURE 2. Thymic seeding in situ in nonradiation chimeras. Same experimental group as Fig. 1 e. (a) Day 9 after first donor cell injection, scattered individual donor cells in mid-cortex (arrows). Insert at higher magnification. (b) Day 11, donor cell doublet in mid-cortex. (c) Day 19, donor cell doublet in medulla (arrow), note frequency of donor cells in adjacent cortex. All sections were stained with anti-Thy-1.1 mAb (Bar = 30 μm).
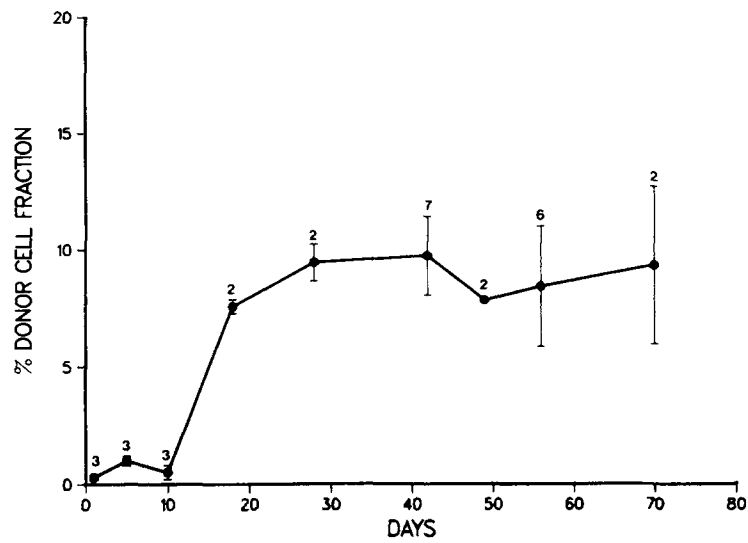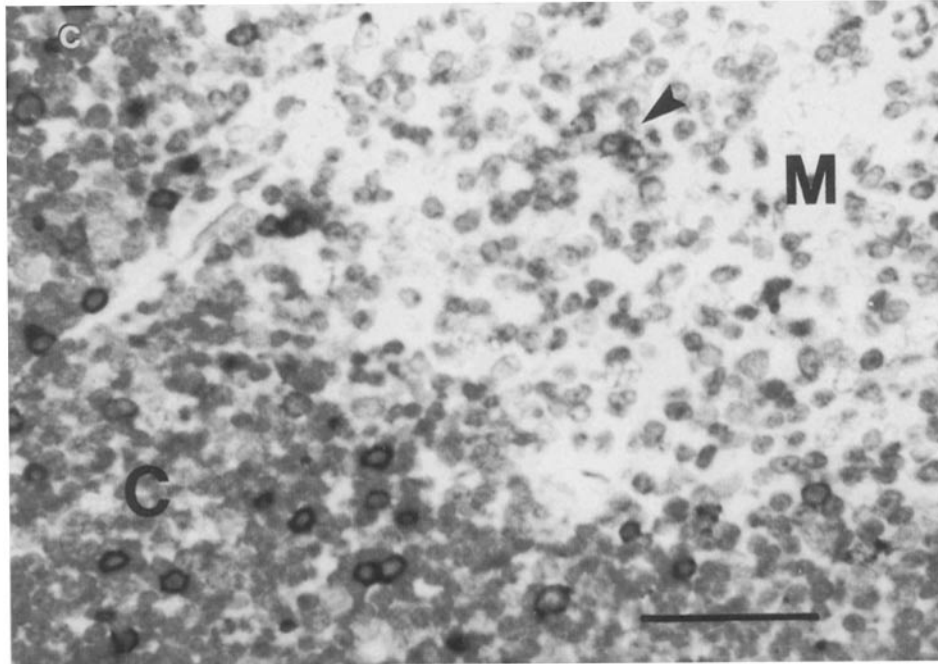
FIGURE 3. Establishment of chimerism in nonradiation model. Chimerism (given in percent of donor cells among unseparated thymocytes) develops between days 8 and 20, and remains stable thereafter. The number of animals analyzed per time point are indicated, 500 thymocytes per sample were counted.

TABLE I

Nonradiation T Cell Chimerism Is Dependent on Host Age

Host age when injected*	Time of analysis (after injection)	n	Thy-1.1+ cells‡	
			Thymocytes	Mesenteric LN
	wk		*%*	
1, 2, 3, 4, 5	6	7	10.0 ± 1.8	11.4 ± 3.3
1, 2, 5, 6, 7	8	8	4.4 ± 1.6	2.7 ± 1.2
28–35 (adult)	1	2	2.0	17.5
	2	2	1.6	<0.1
	4	2	1.5	<0.1

* Injections at days 1 and 2 were intravenous, and at days 3–5 or 5–7, intraperitoneal. Neonatal mice were injected with 2 × 10⁷ Thy-1.1 BM cells/d. Adult mice were injected intravenously with 4 × 10⁷ Thy-1.1 BM cells/d at 5 d.
‡ ≥400 Thy-1+ cells were counted.

TABLE II

Nonradiation T Cell Chimerism Is Dependent on Dose and Source of Donor Cells

Number of injections*	Source of donor cells‡	Time of analysis (after injection)	n	Thy-1.1+ cells§	
				Thymocytes	Mesenteric LN
		wk			
1	BM	6	8	3.0 ± 1.6	ND
3	BM	6	3	11 ± 2.4	6.33 ± 2.0
5	BM	6	7	10 ± 1.8	11.4 ± 3.3
5	Spleen/LN	8	2	<0.1	3.5
					2.8

* The first two injections were intravenous; the last three injections were intravenous or intraperitoneal.
‡ BM cells were untreated, splenocytes and lymph node (LN) cells were mixed in a ratio of 78/22.
§ ≥400 Thy-1+ cells were counted.

marrow cells but not by the same number of mixed spleen and lymph node cells, indicating that hematopoietic stem cells rather than postthymic T cells induce and maintain this chimerism (Table II). Development of donor cell chimerism was similar in both lobes of the thymus, indicating a significant nonclonogenic rate of seeding (data not shown). Donor cells were apparent within the thymus first at day 8, and reached a steady state of chimerism 3 wk after the first injection. The degree of chimerism was found to be reproducible and stable up to 70 d (latest point tested, Fig. 3).

To compare the pattern of donor cell seeding in situ and of isolated thymocyte subsets in this model in more detail we chose the time interval of days 8–20 after the first injection. For analysis of each time point, three or four thymuses were pooled. Donor cells first replaced the Mφ-associated host thymocytes. Concomitant replacement of intra-TNC, DC-associated and unselected thymocytes followed with a delay of ~2 d (Fig. 1e). The interval of ~2 wk between the first appearance of individual donor cells and the maximal chimerism was similar to the radiation model. Again, all four seeding kinetics ran parallel, albeit with

slopes less steep than in the postirradiation thymus (compare Fig. 1, a–d with e, note different scales on the abscissae).

The in situ appearance of donor cells in undepleted thymuses closely followed that found in sublethally irradiated thymuses. Individual donor cells appeared in the mid-cortex by day 9 (Fig. 2a), clusters in the mid-cortex were identified by day 11 (Fig. 2b) concomitant with the cluster formation of donor cells within isolated Mφ-ROS (see Fig. 1e). Up to day 16, donor cells were confined to the cortex. By day 19 donor cells were detectable in random sections in the medulla (Fig. 2c). At this time ~5% of T cells within isolated DC-ROS were already of donor type. By day 28, Thy-1.1 cells were scattered randomly throughout the cortex and the medulla, with a tendency to form clusters of three to five cells within the plane of the section. A similar distribution was found at day 70 after injection (data not shown).

Similarly to the apparent segregation of donor cells into isolated individual lymphostromal cell complexes during the course of repopulation after irradiation, during steady-state chimerism in nonirradiated thymuses, Thy-1.1$^+$ cells were found to segregate into Mφ/DC-ROS and TNC, with a tendency to form contiguous clusters (Fig. 4). We previously reported that the thymocytes making up individual lymphostromal cell complexes may be the progeny of oliogoclonal proliferation of a few cells seeding an individual stromal cell. Operationally, a seeding frequency was defined and calculated from the relative distribution of two distinguishable T cell populations within individual stromal cell complexes and among nonassociated thymocytes (23). We repeated these calculations for the nonradiation model and derived seeding frequencies of 4–5 T cells per TNC and ~15 T cells per ROS (Table III; for details of calculations see Materials and Methods), in contrast to 2 T cells per TNC and 4 T cells per ROS in the radiation model (23). The seeding frequencies of ROS are probably overestimated due to secondary crosscontamination of Thy-1.2 only ROS with Thy-1.1 cells during in vitro handling.

The seeding frequencies derived from this less artificial model were thus higher than those previously reported for a more complex radiation model. These results would support the notion that thymocytes seeding a nondepleted thymus undergo a lower extent of clonal proliferation in situ than those seeding a depleted thymus.

Discussion

In this study the in situ pattern of intrathymic colonization and traffic of thymocytes was correlated with the seeding of distinct sites of direct T cell–stromal cell interactions in two experimental models. C57BL/Ka Thy-1.1 bone marrow cells were injected either into sublethally irradiated adult or into untreated newborn Thy-1.2 congenic mice. In both models a similar sequence of events was observed. The interpretation of our results is based on the assumption that the isolated lymphostromal cell complexes (Mφ-, DC-ROS, and TNC) represent the correlate in vitro of specific cell–cell interactions in vivo. Evidence in favor of this supposition has been presented previously (21–23) and, in addition, is given by the results reported here. Thymocytes associated with each type of stromal cell display characteristic and reproducible repopulation kinetics.
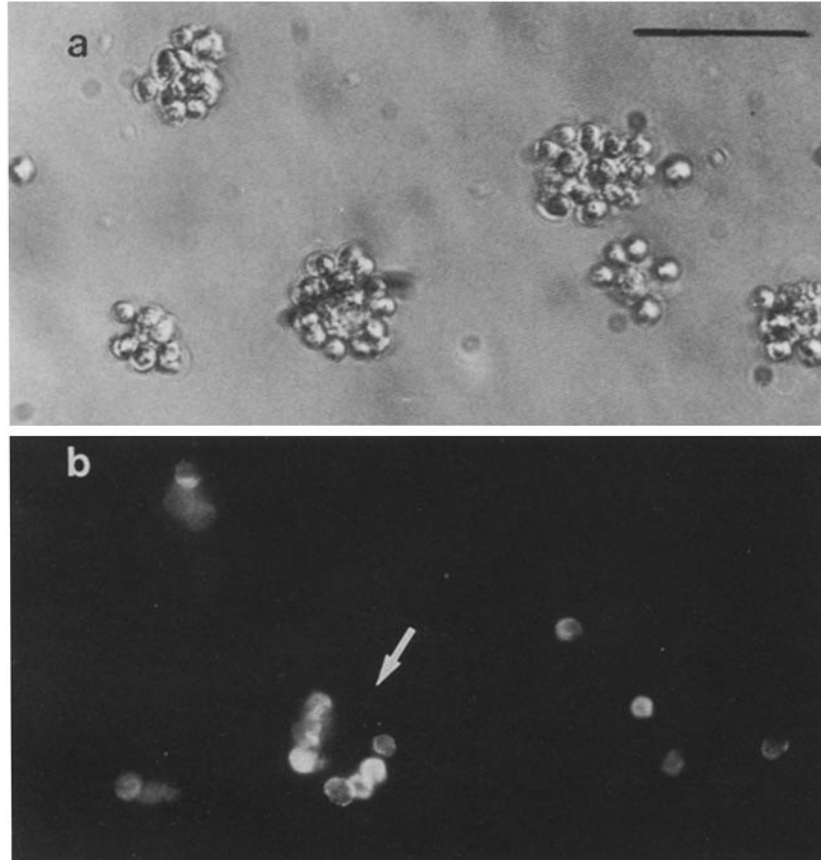
FIGURE 4.   Segregation of Thy-1.1 donor cells into individual lymphostromal cell complexes during steady-state nonradiation chimerism (10% Thy-1.1 and 90% Thy-1.2 cells), isolated 42 d after reconstitution (see Exp. II, Table III). (a and b) Mϕ-ROS, (c–e) DC-ROS, (f–h) TNC. Note the tendency of donor cells to form contiguous clusters within individual complexes. Cells in b, d, and g stained with anti-Thy-1.1–TRITC; e with anti-I-A–FITC; h with anti-Thy-1.2–FITC mAbs (Bar = 30 μm).

This result is not to be expected if the associations occurred randomly during isolation.

With regard to the colonization of the adult thymus as analyzed by tissue sections, the results provide the first analysis of traffic of T cells through the unperturbed postnatal thymus, and in addition confirm previous reports describing colonization of the irradiated thymus (28–30). In contrast to the colonization of the thymic rudiment by the earliest wave of T cell precursors at days 11–12 through migration of the capsule of the anlage (1, 2), prothymocytes seem to enter the vascularized postnatal thymus through capillaries at the cortical side of the cortex/medulla boundary. The pattern of distribution of Thy-1$^+$ donor cells during the early phase of seeding is reminiscent of the distribution of cells expressing interleukin 2 receptors in adult thymus, which has been defined as a marker for thymocyte precursors (31). This multifocal seeding and early proliferation of donor cells in the inner/mid-cortex contrasts with previous models
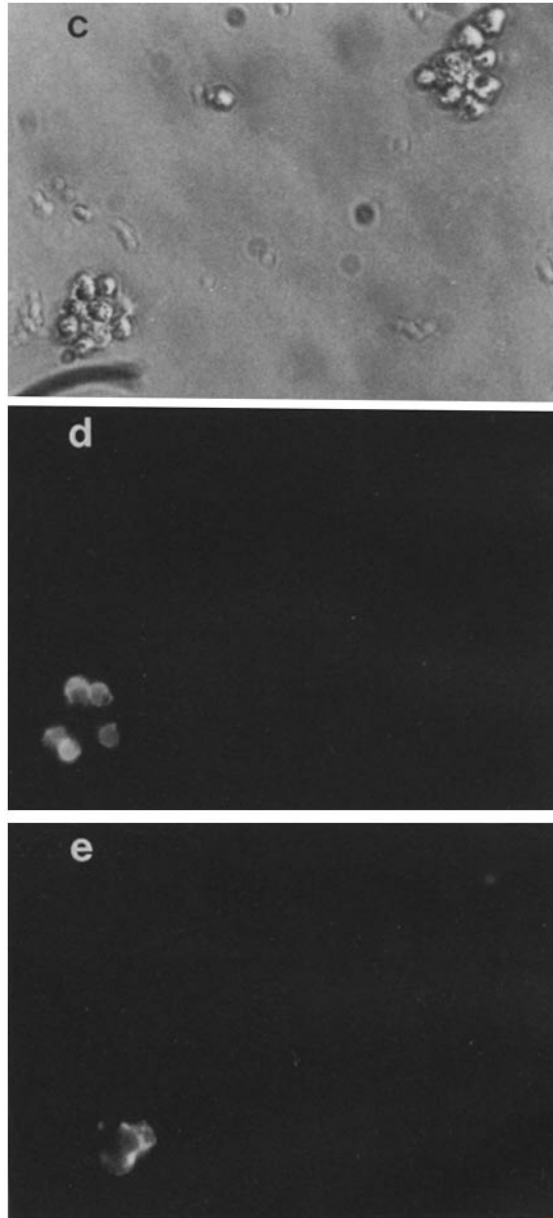
which suggested that the subcapsular zone represents the most immature generative compartment of the thymus (32). Using Thy-1 as a lineage marker, this analysis, however, fails to detect the localization and possible cell–cell interactions of most recent Thy-1$^-$ immigrants (30, 33). With this qualification in mind, the results suggest a pattern of thymopoiesis that proceeds multifocally from individual precursors in the mid-cortex (31).

Both in the depleted and the nondepleted thymus, repopulation of the medulla
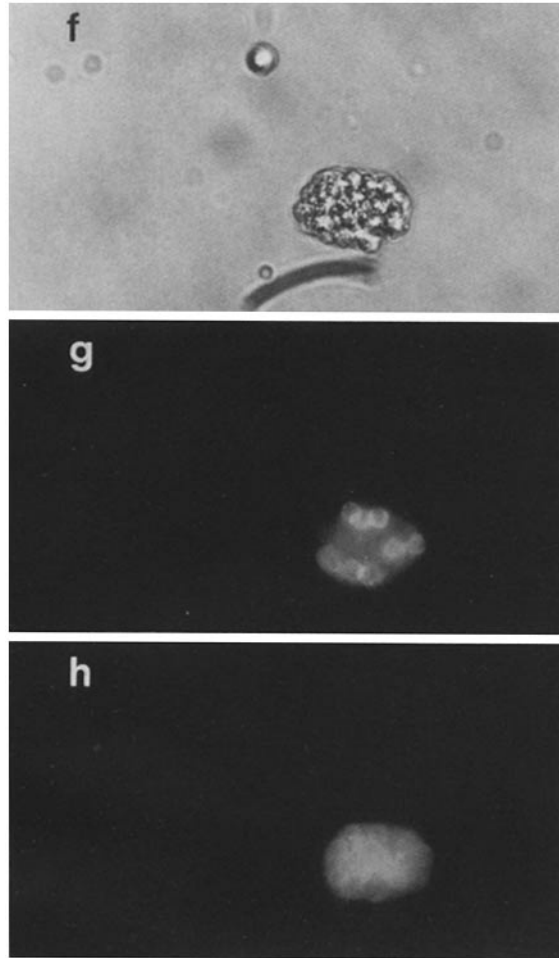
followed that of the cortex. It was particularly intriguing to notice that donor cells appeared first in the outer medulla in those areas of the thymus where repopulation of the adjacent cortex had proceeded farthest; an observation compatible with transmigration of a minority of cortical cells into the medulla after extensive generation of T cells in the adjacent cortex. Such a colinear cortex-medulla maturation sequence would also comply with results reported recently in other models. (10, 34, 35). Our data, however, do not exclude a separate recruitment of immigrating prothymocytes into the cortex and the medulla as suggested previously (36, 37). If this latter explanation is correct, the delayed seeding of the medulla would have to be precisely synchronized with regard to the preceding cortical phase.

The data show that postnatally injected bone marrow cells colonize the nondepleted thymus (there was no significant difference in number of thymocytes between injected and noninjected mice, data not shown). In the absence of a self-renewing intrathymic stem cell, this demonstrates the continuous recruit-

TABLE III

Seeding Frequencies and Clonal Expansion of T Cells Within T-ROS and TNC During Steady-state Nonradiation Chimerism

Cells used	Exp.	Thy-1.2 only	Thy-1.1 only	Mixed	Seeding frequency	Cell divisions
		%	%	%		
Whole thymocytes	I	91.7 ± 2.9	8.4 ± 2.7	0	/	/
	II	90.1 ± 2.4	9.9 ± 2.4	0	/	/
TNC	I	61 (15)	0	39 (85)	5.3	1.9
Complex	II	68 (12)	0	32 (88)	3.7	2.4
TNC	I	95.1	4.9	0	/	/
T cells	II	93.8	6.2	0	/	/
T-ROS	I	27 (24)	0	73 (76)	15.7	<0.1
Complex	II	18 (20)	0	82 (80)	16.2	<0.1
ROS	I	91.1	8.9	0	/	/
T cells	II	86.6	13.4	0	/	/
DC-ROS T cells	II	86.5	13.5	0	/	/

The frequencies of unselected thymocytes represent the mean of 12 (Exp. I) and 14 (Exp. II) thymic lobes of nonradiation chimeras analyzed individually 8 and 6 wk after injection, respectively. The frequencies of TNC and T-ROS are derived from pooled thymuses, 500 complexes were analyzed. An average number of 20 thymocytes per TNC and 15 per T-ROS was assumed. Numbers in parentheses refer to expected frequencies of Thy-1.2-only complexes, given random and independent interactions between thymocytes and stromal cells. For details of calculations see Material and Methods.

ment of prothymocytes from the bone marrow after birth, as suggested previously (26). This prethymic traffic is significant and nonclonogenic, since both lobes were seeded at similar frequencies and donor cell colonization proceeded evenly throughout the cortex via multifocal seeding. Rare entrance of donor cells would have resulted in the spreading of donor cells from few clones, and asynchronous repopulation. Such a pattern was recently reported in high-dose radiation chimeras (37). The depleted organ, however, may allow for a larger burst size of repopulating donor cell clones, whereas pre-T cells entering a nondepleted thymus may have a smaller clonal progeny. The clusters of four to six donor cells seen in nonirradiated thymuses in situ (data not shown) and within individual lymphostromal cell complexes 4–7 wk after injection (Fig. 4) may represent such clones. Interestingly, a similar clone size was derived from the segregation analysis for intra-TNC T cells (Table III). Other interpretations accounting for the appearance of donor cell clusters in situ, e.g., preferential sites of entry, or migration in cohorts, however, cannot be dismissed.

The kinetics indicate that I-A⁻ Mφ are involved at an intermediate stage of cortical thymopoiesis. Seeding of Mφ-associated thymocytes followed the repopulation of the precursor pool with a significant delay, but clearly preceded repopulation of cortical epithelial cell–associated T cells and unselected thymocytes. This hierarchy was observed in both chimeric models. Mφ–thymocyte

interactions are thus the first discernible cell–cell interactions of Thy-1$^+$ thymocytes during thymopoiesis. Mϕ-associated thymocytes coexpress the lineage markers T4/T8 and TCRs and are thus phenotypically more mature than precursor cells (21 and B. Kyewski, manuscript submitted for publication). The sequential association of individual small T cells, T cell blasts, and clusters with individual Mϕ (as well as TNC and DC) during repopulation would be predicted if T cells proliferated in situ in contact with stromal cells; alternatively, formation of donor cell clusters could have occurred before association, and T cells could have interacted en groupe rather than individually with stromal cells.

It should be emphasized that the different repopulation kinetics of Mϕ- and DC-associated thymocytes, and the different antigen-presentation capacity of thymic Mϕ and DC (24) clearly point to different roles of these two types of accessory cells in T cell differentiation. A similar functional distinction between Mϕ and DC has been described for the peripheral immune response (38). Multicellular Mϕ-clusters have recently been identified as sites of hematopoiesis in mouse bone marrow in situ (39 and B. Kyewski, unpublished data), and multicellular DC clusters have been identified as sites of primary B cell responses in vitro (40).

In the cortex, associations of donor thymocytes with I-A$^+$ epithelial cells (TNC) occurred significantly later than interactions with Mϕ. This result suggests that recognition of epithelial cells, as represented by TNC, is a relatively late event in cortical T cell differentiation. TNCs may represent the sites where a subset of thymocytes interact with cortical epithelial cells via TCRs (41). The relative late occurrence of these associations would comply with the necessity to first randomly generate a T cell repertoire out of which T cell clones with appropriate specificity could bind to epithelial cells. The time interval of ~3–4 d spanning the repopulation of the earliest DN thymocyte pool and the intra-TNC subset may be sufficient to generate such a repertoire in the adult thymus. A similar delay between incipient intrathymic lymphopoiesis (days 13–14) and the first seeding of TNC (day 17) is observed during ontogeny (24). Evidence for the generation of T cell diversity during this prenatal period by rearrangement and expression of TCR genes has been reported (8).

DC are known to be strictly confined to the medulla and corticomedullary junction (42, 43). The kinetics indicate that cortical epithelial cells and medullary DC are seeded at a similar time. Given the resolution limits of our assay (~1 d) this finding is compatible with a rapid migration of selected thymocytes from the cortex to the medulla. DC-associated thymocytes seem to represent recent immigrant cells in the medulla; the seeding of PNA^{low} thymocytes representing the majority of medullary T cells is ~2 d later than DC-associated thymocytes; donor cells are detectable within isolated DC-ROS earlier than within the medulla in corresponding sections. Thus, associations of T cells with those two types of stromal cells, which express class II MHC antigens, occur only after a certain lag period of intrathymic T cell differentiation. If recognition of I-A$^+$ stromal cells in situ is indicative of a diversified T cell repertoire, the kinetics suggest the presence of such a repertoire late during cortical but early during medullary differentiation.

The strict correlation of the different cell–cell interactions with intrathymic T

cell differentiation suggests that they are obligatory stages in this process. Hence, the actual number of stromal cell interaction sites at each step may be critically rate-limiting in T cell generation; such rate-limiting microenvironments have been recently postulated (44).

The results are compatible with the following model of intrathymic T cell differentiation, which makes the yet-unproven assumption of a colinear differentiation sequence among the various compartments. DN prothymocytes enter the thymus at multiple sites in the cortex. This immigration occurs at a significant rate even in the postnatal and young adult thymus. An intermediate and possibly antigen-nonspecific stage of cortical thymopoiesis is defined by associations with I-A⁻ macrophages. Specific recognition of MHC class I- and II–positive cortical epithelial cells marks a late event of the cortical phase. This late phase of cortical maturation is rapidly followed by the earliest stage of medullary differentiation defined by specific interaction of T cells with MHC class I- and II–positive DC. Cortical epithelial cells are invoked in selection of thymocytes with appropriate self-MHC specificity (14, 15), and the late occurrence of thymocyte–TNC interactions during the antigen-secluded (sterile) cortical phase would comply with such a role. Medullary DC on the other hand seem to be associated with induction of T cell tolerance (17–19). By inference, tolerance induction would occur at a very early stage of the antigen-exposed (nonsterile) phase of T cell differentiation in the medulla (24).

Summary

Seeding of distinct intrathymic microenvironments defined by direct thymocyte–stromal cell interactions was correlated with T cell development in situ using radiation and nonradiation chimeras of Thy-1.1/1.2 congenic mice. The results identify associations of thymocytes with I-A⁻ macrophages in the cortex as the earliest discernible cell–cell interactions during thymopoiesis. After a significant delay, this recognition stage is followed by concomitant interactions of T cells with I-A⁺ epithelial cells in the cortex and bone marrow–derived I-A⁺ dendritic cells in the medulla. All three types of T cell–stromal cell interactions occur after seeding of the intrathymic precursor cell subset and before development of mature medullary-type T cells. The seeding kinetics imply that recognition of cortical epithelial cells by thymocytes in situ represents a relatively late stage of cortical T cell development, whereas thymocyte–dendritic cell interactions denote a very early stage of T cell development in the medulla. The relative positioning of these cell–cell recognition stages during the course of T cell maturation pertains to a putative role of these microenvironments in selection and tolerization of the T cell repertoire.

References

1. LeDouarin, N. M., F. Dieterlein-Lievre, and P. D. Oliver. 1984. Ontogeny of primary lymphoid organs and lymphoid stem cells. *Am. J. Anat.* 170:261.
2. Champion, S., B. A. Imhof, P. Savagner, and J. P. Thiery. 1986. The embryonic thymus produces chemotactic peptides involved in the homing of hemopoietic precursors. *Cell.* 14:781.
3. Reichert, R., M. Gallatin, E. C. Butcher, and I. L. Weissman. 1984. A homing receptor bearing cortical thymocyte subset: Implication for thymus cell migration and the nature of cortisone-resistant thymocytes. *Cell.* 38:89.
4. Gallatin, M., T. P. St. John, M. Siegelman, R. Reichert, E. C. Butcher, and I. L. Weissman. 1986. Lymphocyte homing receptors. *Cell.* 44:673.
5. Haskins, K., J. Kappler, and P. Marrack. 1984. The major histocompatibility complex-restricted antigen receptor on T cells. *Annu. Rev. Immunol.* 2:51.
6. Roehm, N., L. Herron, J. Cambier, D. DiGuisto, K. Haskins, J. Kappler, and P. Marrack. 1984. The major histocompatibility complex–restricted antigen receptor on T cells: Distribution on thymus and peripheral T cells. *Cell.* 38:577.
7. Davis, M. M. 1985. Molecular genetics of T-cell receptor beta chain. *Annu. Rev. Immunol.* 3:537.
8. Born, W., J. Yague, E. Palmer, J. Kappler, and P. Marrack. 1985. Rearrangement of T-cell receptor beta-chain during T-cell development. *Proc. Natl. Acad. Sci. USA.* 82:2925.
9. Owen, M. J., E. J. Jenkinson, G. T. Williams, R. Kingston, and J. J. T. Owen. 1986. An investigation of T cell receptor rearrangement and expression in organ culture of normal embryonic thymus and Thy-1$^+$ cells of nude mice. *Eur. J. Immunol.* 16:875.
10. Kingston, R., E. J. Jenkinson, and J. J. T. Owen. 1985. A single stem cell can recolonize an embryonic thymus producing phenotypically distinct T-cell populations. *Nature (Lond.).* 317:811.
11. Williams, G. T., R. Kingston, M. J. Owen, E. J. Jenkinson, and J. J. T. Owen. 1986. A single micromanipulated stem cell gives rise to multiple T-cell receptor gene rearrangements in the thymus in vitro. *Nature (Lond.).* 324:63.
12. Scollay, R. 1983. Intrathymic events in the differentiation of T cells: a continuing enigma. *Immunol. Today.* 4:282.
13. Scollay, R. G., E. C. Butcher, and I. L. Weissman. 1980. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210.
14. Fink, P. J., and M. J. Bevan. 1978. H-2 antigen of the thymus determines lymphocyte specificity. *J. Exp. Med.* 148:766.
15. Zinkernagel, R. M., R. M. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of H-2 self recognition by T cells: evidence for dual recognition? *J. Exp. Med.* 147:882.
16. Steinman, R. M., and M. C. Nussenzweig. 1980. Dendritic cells. Features and functions. *Immunol. Rev.* 53:127.
17. Ready, A. R., E. J. Jenkinson, R. Kingston, and J. J. T. Owen. 1984. Successful transplantation across major histocompatibility barrier of deoxyguanosine-treated embryonic thymuses expressing class II antigens. *Nature (Lond.).* 310:231.
18. Lo, D., and J. Sprent. 1986. Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature (Lond.).* 319:672.
19. von Bohmer, H., and K. Hafen. 1986. Minor but not major histocompatibility antigens of thymic epithelium tolerize precursors of cytolytic T cells. *Nature (Lond.).* 320:626.
20. Wekerle, H., U.-P. Ketelsen, and M. Ernst. 1980. Thymic nurse cells. Lymphoepi-

thelial complexes in murine thymus: Morphological and serological characterization. *J. Exp. Med.* 151:925.

21. 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 murine thymus. *Proc. Natl. Acad. Sci. USA.* 79:5645.

22. Kyewski, B. A., and H. S. Kaplan. 1982. Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. *J. Immunol.* 128:2287.

23. Kyewski, B. A., M. Travis, and H. S. Kaplan. 1984. Intrathymic lymphopoiesis: stromal cell-associated proliferation of T cells is independent of lymphocyte genotype. *J. Immunol.* 133:1111.

24. Kyewski, B. A., C. G. Fathman, and R. V. Rouse. 1986. Intrathymic presentation of circulating non-MHC antigens by medullary dendritic cells. An antigen-dependent microenvironment for T cell differentiation. *J. Exp. Med.* 163:231.

25. Takada, A., Y. Takada, C. Huang, and J. Ambrus. 1969. Biphasic pattern of thymus regeneration after whole body irradiation. *J. Exp. Med.* 129:445.

26. Scollay, R., J. Smith, and V. Stauffer. 1986. Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol. Rev.* 91:129.

27. Brecher, G., J. D. Ansell, H. S. Micklem, J.-H. Tijo, and E. P. Cronkite. 1982. Special proliferative sites are not needed for seeding and proliferation of transfused bone marrow cells in normal syngeneic mice. *Proc. Natl. Acad. Sci. USA.* 79:5085.

28. Ceredig, R., and M. Schreyer. 1984. Immunohistological localization of host and donor-derived cells in the regenerating thymus of radiation bone marrow chimeras. *Thymus.* 6:15.

29. Hirokawa, K., T. Sado, S. Kubo, H. Kamisaku, K. Hitomi, and M. Utsuyama. 1985. Intrathymic T cell differentiation in radiation bone marrow chimeras and its role in T cell emigration to the spleen. An immunohistochemical study. *J. Immunol.* 134:3615.

30. Huiskamp, R., and W. van Ewijk. 1985. Repopulation of mouse thymus after sublethal fission neutron irradiation. I. Sequential appearance of thymocyte subpopulations. *J. Immunol.* 134:2161.

31. Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. MacDonald. 1985. Expression of interleukin-2 receptor as a differentiation marker on intrathymic stem cells. *Nature (Lond.).* 314:98.

32. Weissman, I. L. 1973. Thymus cell migration. Studies on the origin of cortisone-resistant thymus lymphocytes. *J. Exp. Med.* 137:504.

33. Trowbridge, I. S., J. Lesley, J. Trotter, and R. Hyman. 1985. Thymocyte subpopulation enriched for progenitors with an unrearranged T-cell receptor beta-chain gene. *Nature (Lond.).* 314:539.

34. Fowlkes, B. J., L. Edison, B. J. Mathieson, and T. M. Chused. 1985. Early lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J. Exp. Med.* 162:802.

35. Penit, C. 1986. In vivo thymocyte maturation, BUdR labeling of cycling thymocytes and phenotypic analysis of their progeny support the single lineage model. *J. Immunol.* 137:2115.

36. Jotereau, F. V., and N. M. Le Douarin. 1982. Demonstration of a cyclic renewal of the lymphocyte precursor cell in the quail thymus during embryonic and perinatal life. *J. Immunol.* 129:1869.

37. Ezine, S., I. L. Weissman, and R. V. Rouse. 1984. Bone marrow cells give rise to distinct cell clones within the thymus. *Nature (Lond.).* 309:629.

37a.Kyewski, B. A., F. Gomburg, and V. Schirrmacher. 1987. Phenotype of stromal cell–

associated thymocytes in situ is compatible with selection of the T cell repertoire at an "immature" stage of thymic T cell differentiation. *Eur. J. Immunol.* In press.

38. Steinman, R. M., B. Gutchinov, M. D. Witmer, and M. C. Nussenzweig. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157:613.

39. Crocker, P. R., and S. Gordon. 1985. Isolation and characterization of resident stromal macrophages and hematopoietic cell clusters from mouse bone marrow. *J. Exp. Med.* 162:993.

40. Inaba, K., M. D. Witmer, and R. M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858.

41. Farr, A. G., S. K. Anderson, P. Marrack, and J. Kappler. 1985. Expression of antigen specific MHC-restricted receptors by cortical and medullary thymocytes in situ. *Cell.* 43:543.

42. 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.

43. Guillemot, F. P., P. D. Oliver, B. M. Peault, and N. M. leDouarin. 1984. Cells expressing Ia antigens in the avian thymus. *J. Exp. Med.* 160:1803.

44. Goldschneider, I., K. L. Komschlies, and D. L. Greiner. 1986. Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J. Exp. Med.* 163:1.