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Immunology

Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor γδ

(thymectomy/thymic independence/T-cell ontogeny)

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ABSTRACT **The kinetics of postnatal intestinal colonization by T cells carrying γδ and αβ T-cell antigen receptors were studied in nude and normal mice by flow cytometry and immunohistology. Furthermore, γδ and αβ T-cell development was analyzed in lethally irradiated mice that were reconstituted by fetal liver precursors with or without a thymus. Our results establish that a major subpopulation of γδ intestinal intraepithelial lymphocytes is produced from uncommitted precursors at extrathymic sites. This work further shows that a small pool of T cells carrying αβ T-cell receptors can also differentiate extrathymically from CD3⁻ fetal liver precursors but with rates of production and peripheral expansion much reduced as compared with those observed in thymus-bearing animals.**

The thymus plays an essential role in the differentiation of T lymphocytes from precursors, as well as in the determination of mature T-cell repertoires. Thus, rearrangement of the receptor genes occurs in differentiating thymocytes, and antigens in the thymic environment shape T-cell repertoires (1–5). It has long been known that very few T cells are generated in athymic, nude animals (6, 7); these studies, however, analyzed peripheral lymphoid organs which harbor a very large excess of T lymphocytes bearing αβ T-cell receptors (8, 9). In contrast, recent observations have indicated that nude mice contain substantial numbers of γδ T cells (10, 11). The possible thymus-independent origin of γδ-subset cells could contribute to our understanding of the specific characteristics of this T-lymphocyte population, such as their selective localization in epithelia (12–17), the preferential variable region of T-cell receptor γ- or δ-chain gene utilization at different anatomic sites (17–23), and the very limited repertoires in some epithelia (17, 18, 22, 23).

We have, therefore, analyzed intestinal intraepithelial lymphocytes (i-IEL) of nude mice and found that γδ T cells with characteristics similar to those isolated from normal animals are abundant, in contrast to αβ T cells that are nearly completely absent. Given the early ontogenic appearance of γδ T cells and the possibility that a thymic rudiment exists transiently in the nude embryo (6), we have proceeded to study the kinetics of postnatal intestinal colonization by γδ and αβ cells, as well as T-lymphocyte development in thymectomized animals reconstituted with fetal liver cells after lethal irradiation. The results are consistent with an extrathymic origin for a major subpopulation of γδ i-IEL.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c euthymic and nude mice were from our breeding colony at Institut Pasteur and a gift from Motoya Katsuki of Tokai University, Kanagawa, Japan. C57BL/6 Ba (Thy-1.1) were from the animal facilities of Centre National de la Recherche Scientifique, Villejuif, France.

Monoclonal Antibodies. The following monoclonal antibodies (mAbs) were used: mAb 3A10, anti-Cδ (9); mAb H57-597, anti-Cβ (8); mAb 145-2C11, anti-CD3 (24); mAb 30H12, anti-Thy-1.2; mAb 19XE5, anti-Thy-1.1; mAb GK 1.5, anti-CD4 (25); mAb 53.6.72, anti-CD8 (26); mAb 44.22.1, anti-V_β6 (27); mAb F23.1, anti-V_β8 (28); and mAb RR3-15, anti-V_β11 (29).

Thymectomy, Irradiation, and Fetal Liver Reconstitution. C57BL/6 mice were thymectomized or sham-operated at the age of 8 weeks. One and one-half months later, they were irradiated with 850 rads (a dose previously shown to be lethal; 1 rad = 0.01 Gy) and reconstituted by an i.v. injection of five million fetal liver cells (15–16 days old) from C57BL/6 Ba (Thy-1.1) mice. No $CD3^+$ T cells could be detected in this inoculum by flow cytometry among 17,000 cells examined (< 0.01%). In addition, cells isolated by gating for positive events turned out to be autofluorescent cells present in equal numbers in non-CD3 mAb-labeled controls.

Cell Preparations. Spleens and mesenteric lymph nodes were removed in balanced salt solution without phenol red/3% fetal calf serum/0.015 M sodium azide (fluorescence medium). IEL cell suspensions were prepared as described (30) and according to standard procedures (31–33). In short, after being flushed extensively to eliminate the lumen content, small intestines were separated from Peyer's patches, longitudinally opened, and cut into pieces of 1–2 cm. Fragments were then gently shaken in five changes of Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution and incubated with gentle stirring (100 rpm) for 7–10 min at room temperature in 25 ml of Ca^{2+}- and Mg^{2+}-free Hanks' solution/5 mM EDTA/dithiothreitol at 70 μg/ml. Fragments were allowed to sediment for 5 min on ice after which the supernatant, containing cells, was recovered. IEL cells were washed three times and passed through nylon mesh and siliconized glass wool columns to remove cell aggregates and dead cells. No further purification step, such as Percoll gradients, was used. At this stage cell viability was scored in trypan blue, and fluorescence stainings were done.

Immunofluorescence and Flowcytometric Analysis. Immunofluorescence staining and flowcytometric methods have been described (30, 34). Briefly, 1–1.5 × 10^6 cells from individual mice were incubated in microtiter plates for 20 min on ice with biotin- or fluorescein isothiocyanate-labeled mAbs and subsequently counter-stained with streptavidin/phycoerythrin (Becton Dickinson). Flow cytometric analysis was done in a FACScan analyzer (Becton Dickinson). Dead cells were eliminated by staining with propidium iodine. To

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Abbreviations: i-IEL, intestinal intraepithelial lymphocytes; mAb, monoclonal antibody.

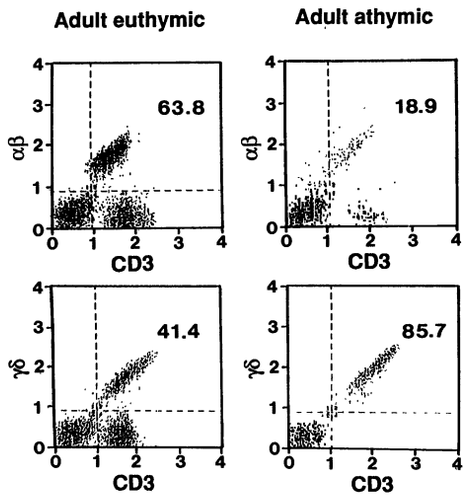

FIG. 1. FACS analysis of i-IEL from 6-week-old mice. Values in each plot are percentages of $\alpha\beta^+$ or $\gamma\delta^+$ cells among total $CD3^+$ cells. Fluorescence intensity is shown in logarithmic (to base 10) scale.

determine the absolute numbers of lymphocytes per intestine, the parameters of forward scatter and side scatter were used.

Immunohistology Procedures. Immunohistochemistry by light microscopy was done as described in detail (35).

RESULTS

Analysis of i-IEL Composition in Nude and Normal Animals. Fig. 1 compares the distribution of $\gamma\delta$ and $\alpha\beta$ T lymphocytes among i-IEL isolated from age-matched (6-week-old) normal (euthymic) and nude (athymic) mice. Although both cell types were well represented in i-IEL of normal mice, nude mice contained mostly $\gamma\delta$ T cells, with $\alpha\beta$ T cells comprising between 7% and 19% of total $CD3^+$ i-IEL. This result indicates that $\gamma\delta$ i-IEL and $\alpha\beta$ i-IEL differ in their relative degree of dependence on the thymus for their generation. As shown in Table 1, 6-week-old nude mice contained 50- to 100-fold less $\alpha\beta$ i-IEL than age-matched normal mice but only 2- to 5-fold less $\gamma\delta$ i-IEL. Thus, a substantial proportion of $\gamma\delta$ i-IEL seems to be generated and/or expanded without a fully developed thymus. As reported (22) $\alpha\beta$ T cells were virtually undetectable in the spleen of 6-week-old nude mice (Table 1). In contrast, the number of splenic $\gamma\delta$ T cells is much less diminished in nude mice relative to normal mice (Table 1).

The $\gamma\delta$ i-IEL population from nude mice was compared with that from normal mice for the expression of some cell-surface molecules. As shown in Fig. 2 *A* and *C* the density of the $\gamma\delta$ T-cell receptor–CD3 complex as measured by staining intensity was the same for the two cell populations. However, i-IEL from nude mice failed to express Thy-1 surface molecules, whereas in normal mice, one- to two-thirds of these cells were $Thy\text{-}1^+$ (Fig. 2*B*). Although this marker has been postulated to be an activation marker for $\gamma\delta$ i-IEL (36), as recently discussed (11), the virtual absence of $Thy\text{-}1^+$ $\gamma\delta$ T cells in nude mice suggests that Thy-1 expression is acquired during intrathymic differentiation. On the other hand, neither CD8 expression (Fig. 2*D*) nor cell size (Fig. 2*E*) seemed to differ significantly between the $\gamma\delta$ i-IEL populations from the two types of mice. Immunohistological analysis of intestinal preparations from nude mice confirmed that $\gamma\delta$ T cells were located intraepithelially similarly to normal mice (Fig. 3 and ref. 11).

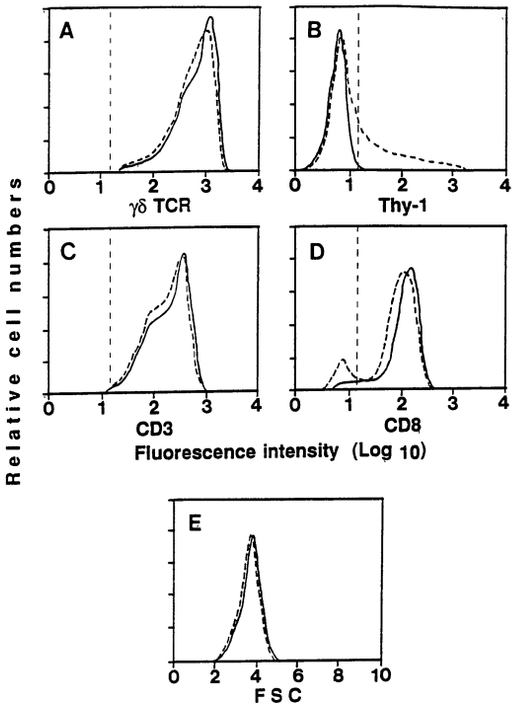

FIG. 2. Phenotypes of $\gamma\delta$ T cells from intestines of nude and normal mice. Data concern the $\gamma\delta$ subset only, analyzed by gating for cells stained by anti-$\gamma\delta$ mAb. Solid and dotted lines show the data from nude and normal mice, respectively. Vertical dotted lines indicate the upper limits of autofluorescence of cells. Fluorescence intensity is shown in logarithmic (to base 10) scale. Forward scatter (FSC) is shown in linear scale.

Postnatal Development of $\gamma\delta$ i-IEL in Normal and Nude Mice. The above results could indicate either that $\gamma\delta$ i-IEL are generated at extrathymic sites or that they are produced by some putative transitory thymic function restricted to the early embryonic development of nude mice (6). According to the latter hypothesis, $\alpha\beta$ T cells are generated at relatively late ontogenic periods when such thymic rudiments would no longer be functional. Also by this hypothesis we might expect to detect $\gamma\delta$ i-IEL early in development. We therefore analyzed the postnatal development of i-IEL composition. Using standard techniques to prepare i-IEL, we could detect only very low numbers of $CD3^+$ lymphocytes up to day 16 after birth (Table 2). At this time, virtually no $CD3^+$ i-IEL could be detected in nude mice, whereas low numbers of these cells, 66% of which expressed $\gamma\delta$ TCR, were seen in normal mice (Table 2). At day 20 $\gamma\delta$ i-IEL could also be detected in nude mice, although at 5-fold lower levels than in normal mice. This ratio of $\gamma\delta$ i-IEL in nude versus normal mice remained the same throughout adult life. It is possible

Table 1. Numbers of $\gamma\delta$ and $\alpha\beta$ i-IEL in normal and nude mice in i-IEL and spleen of adult mice

Mice	Number of cells per organ					
	i-IEL ($\times 10^{-5}$)			Spleen ($\times 10^{-6}$)		
	$CD3^+$	$\gamma\delta$	$\alpha\beta$	$CD3^+$	$\gamma\delta$	$\alpha\beta$
Normal	220 ± 29	51 ± 7	176 ± 41	28 ± 1	1.3 ± 0.6	29 ± 4
Nude	18.5 ± 0.7	17 ± 1	2 ± 1	<1.9 ± 0.4	0.2 ± 0.1	<1.6 ± 0.3

Numbers are means and SDs of three 6-week-old mice per group.

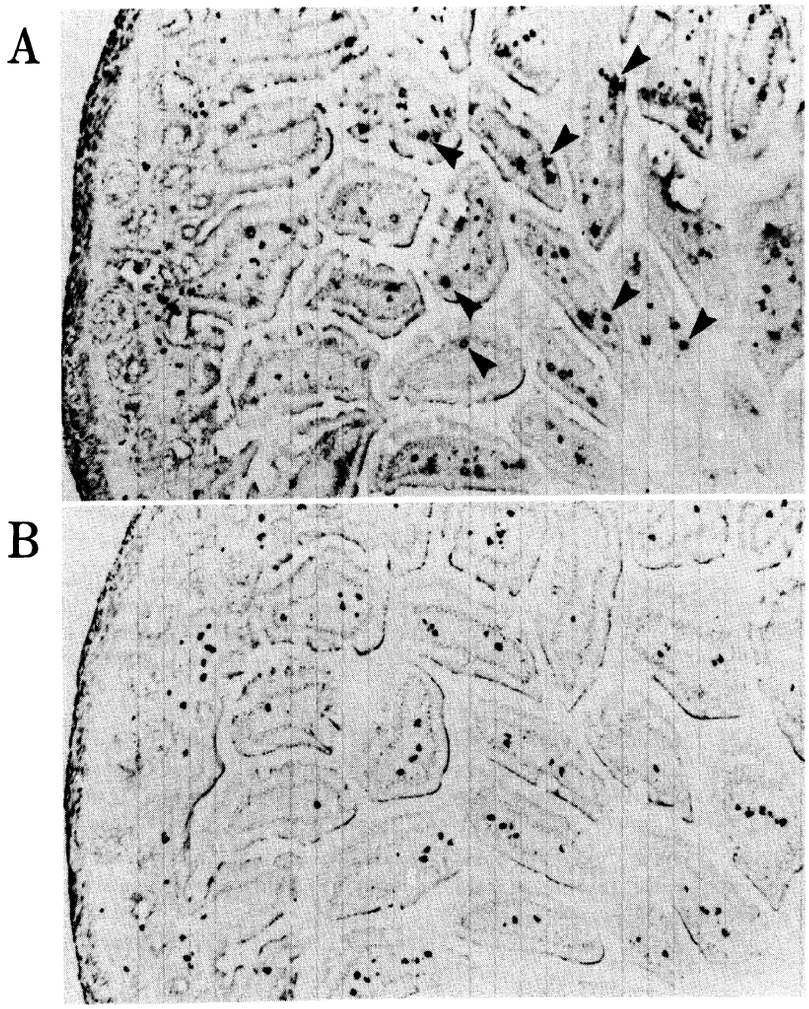

FIG. 3. γδ T-cell distribution in the gut mucosa from nude mice. (*A*) Anti-γδ mAb (3A10) staining. (*B*) Primary antibody-negative control. Arrows indicate representative γδ T cells.

that the colonization of *in situ* development of γδ cells in the intestine occurs earlier than this type of analysis indicates. In nude mice, αβ i-IEL were undetectable until 3 weeks after birth and subsequently remained one to two orders of magnitude lower in abundance than αβ i-IEL in normal mice (Table 2). These results support the notion that a majority of γδ i-IEL are generated after birth and make it unlikely that the presence of γδ i-IEL in nude mice is due to transitory thymic function early in embryonic life.

Generation of γδ i-IEL from Fetal Liver Precursors in Thymectomized Mice. One could argue that γδ T cells, which are formed in a putative thymic rudiment present in nude mice for a short period in early development, accumulate elsewhere and colonize the intestine only after birth. According to this scheme, the putative local expansion of γδ T cells would be thymus-independent, but the initial generation of a few competent receptor-bearing progenitors would not be. To assess this possibility, we have compared the regeneration of γδ and αβ i-IEL in adult C57BL/6 mice that were either thymectomized or sham-operated, then lethally irradiated, and reconstituted with 15-day fetal liver cells from B6.Thy-1.1 congenic donors. At various times after reconstitution, recipient mice were sacrificed, and the number of γδ T cells in the intestinal epithelium bearing the Thy-1.1 marker was determined. Given the fact that many γδ i-IEL are Thy-1$^-$, their total numbers were also determined. In addition, the reconstitution of lymph node αβ T cells in the two groups of mice was followed by using an anti-CD4 mAb as well as a mixture of three anti-variable region β-chain mAbs [note that γδ T cells are mostly CD4$^-$ (ref. 9)]. All mice sacrificed had their cervicothoracic region dissected and examined for remnants that would indicate incomplete thymectomies. One case where these were detected was discarded from analysis, although it showed a staining pattern identical to completely thymectomized mice. As shown below, however, an excellent internal control for the effectiveness of thymectomy is provided by the absence of αβ cells in the same mice where γδ cells are detected.

Table 2. Postnatal development of i-IEL

Days	Mice	i-IEL (× 10^{-4})*		
		CD3$^+$	γδ	αβ
16	Normal	8.2 ± 3.8	5.3 ± 2.7	3.0 ± 1.1
	Nude	1.2 ± 0.2	ND	ND
20	Normal	92 ± 24	66 ± 23	27 ± 0
	Nude	25 ± 5	12 ± 3	ND
23	Normal	274 ± 72	139 ± 43	136 ± 23
	Nude	20 ± 1.5	16 ± 1.0	3.5 ± 0.5
42	Normal	2203 ± 293	510 ± 70	1760 ± 410
	Nude	185 ± 5.0	165 ± 5.0	20 ± 10
48	Normal	1079 ± 458	722 ± 281	357 ± 177
	Nude	135 ± 54	124 ± 52	11 ± 1.3

Each point represents the mean of two to three mice per group.
*Detection limit was 10,000 cells. ND, not done.

The results from this series of experiments are shown in Figs. 4 and 5. At 5 weeks after irradiation and reconstitution (Fig. 4), thymectomized and sham-operated recipients differed markedly in the proportions of CD4$^+$ cells in the lymph nodes (3–4% in *A* and *B* vs. 15–17% in *C* and *D*), and only the

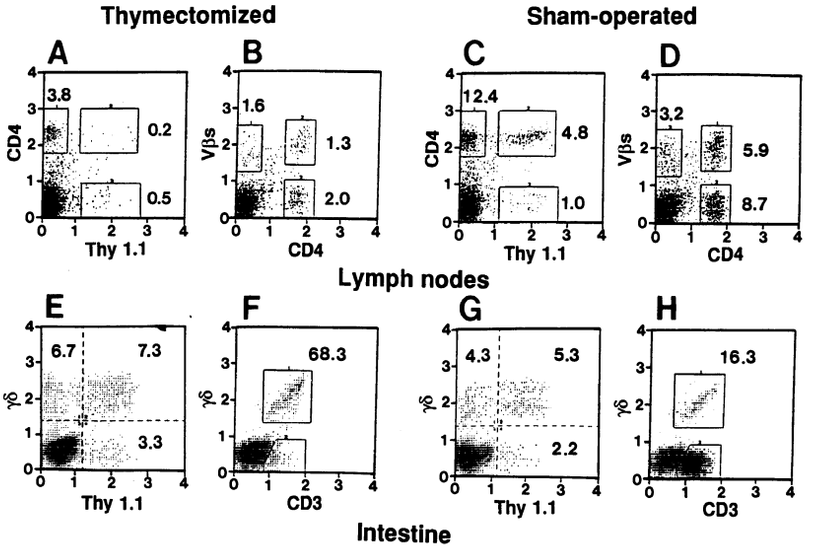

FIG. 4. FACS analysis of lymph node cells (*A–D*) and i-IEL (*E–H*) from thymectomized (*A*, *B*, *E*, and *F*) and sham-operated (*C*, *D*, *G*, and *H*), irradiated C57BL/6 mice reconstituted with B6.Thy1.1 fetal liver cells 5 weeks after reconstitution. Values are proportions (%) of total lymphoid cells, with the exception of the γδ vs. CD3 plots, where indicated values represent percentages of total CD3$^+$ cells. Variable region β-chains (V_βs) are a pool of V_β6, V_β8, and V_β11. Relative fluorescence intensity, both ordinate and abscissa, is shown in logarithmic (to base 10) scale.

sham-operated mice contained Thy-1.1$^+$ CD4$^+$ lymph node cells differentiated from the donor fetal liver precursors (0.2% in *A* vs. 4.8% in *C*). Similar differences were also apparent in the proportions of lymph node cells stained with a mixture of anti-variable region β-chain (V_β) antibodies (2.9% in *B* vs. 9.1% in *D*). In contrast, the thymectomized mice were not deficient with respect to total γδ i-IEL (14% in Fig. 4*E* vs. 9.6% in *G*), or Thy-1.1$^+$ γδ i-IEL of donor origin (7.3% in *E* vs. 5.3% in *G*). The proportion of γδ T cells among CD3$^+$ i-IEL was higher in thymectomized (68.3%, Fig. 4*F*) than in sham-operated recipients (16.3%, *H*) because the former mice were deficient in αβ i-IEL, whereas the latter mice contained a normal number of αβ i-IEL (data not shown).

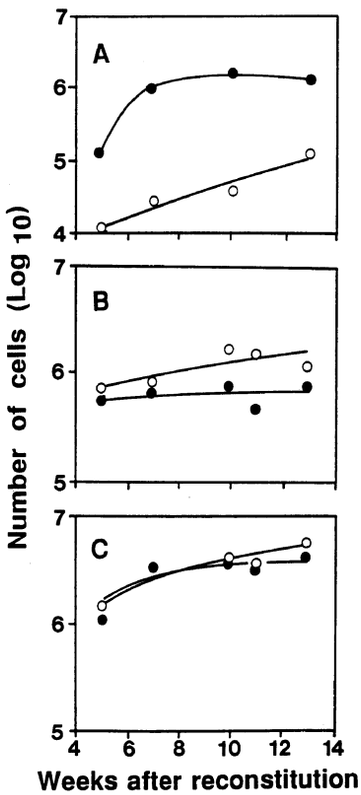

FIG. 5. Kinetics of reconstitution of mesenteric lymph node T cells (*A*), Thy1.1$^+$ (*B*), and total (*C*) γδ i-IEL of thymectomized (○) and sham-operated (●), irradiated C57BL/6 recipient mice reconstituted with B6.Thy-1.1 fetal liver cells. Total γδ T cells were enumerated from double-staining data obtained by using anti-γδ and anti-CD3 mAbs. The two types of animals were independently analyzed for each group at each time point. (*C*) At 7 weeks open circle overlaps with corresponding closed circle.

At 7 weeks after reconstitution, very few Thy1-1$^+$ CD4$^+$ T cells (which are mostly αβ T cells) were recovered from lymph nodes of thymectomized recipients, whereas in sham-operated recipients reconstitution of CD4$^+$ cells was virtually complete (Fig. 5*A*). In thymectomized mice the number of Thy-1.1$^+$ CD4$^+$ T cells continued to increase slowly in lymph nodes, but at 13 weeks it was still one order of magnitude less than in sham-operated mice (Fig. 5*A*). In contrast, there was virtually no difference in the kinetics of reconstitution of either Thy-1.1$^+$ γδ i-IEL or total γδ i-IEL between thymectomized and sham-operated recipients (Fig. 5 *B* and *C*).

These results demonstrate that injected receptor-negative cells from fetal liver can differentiate into γδ i-IEL in the absence of a thymus. Furthermore, because there was no significant difference in the total numbers of such lymphocytes recovered from sham-operated, control, and thymectomized recipient mice at any point after reconstitution, these results suggest that thymus-dependent γδ T cells do not contribute significantly to intestinal colonization in normal mice. In addition, a small but significant proportion of αβ T cells as measured by the anti-CD4 mAb can differentiate from CD3$^-$ immature fetal liver precursors in the absence of a thymus. Interestingly, the rate of expansion of these cells differs from that of thymus-derived αβ T cells (Fig. 5*A*).

DISCUSSION

The occurrence of a substantial number of i-IEL γδ T cells in athymic nude mice cannot be explained by transitory thymic function early in embryonic development because, as shown here, day-15 fetal liver precursors differentiate and produce γδ i-IEL in thymectomized adult hosts. Thus, the present observations establish that a major subpopulation of γδ i-IEL is produced from uncommitted precursors at extrathymic sites. The expression of the Thy-1 marker by γδ i-IEL of donor origin in thymectomized recipients but not by nude γδ i-IEL T cells indicates that, although lack of Thy-1 could

indicate thymus independence of i-IEL cells (11), environmental conditions in the irradiated recipient can lead to its expression. Interestingly, while appearing early after reconstitution, Thy-1$^+$ $\gamma\delta$ i-IEL do not seem to expand with time in contrast to Thy-1$^-$ $\gamma\delta$ i-IEL (compare in Fig. 5 *B* and *C*). After 10 weeks of reconstitution, the ratio of Thy-1$^+$ to Thy-1$^-$ $\gamma\delta$ i-IEL is decreased. Because fetal liver cells produce equivalent numbers of $\gamma\delta$ i-IEL in thymectomized and thymus-bearing hosts, it seems that $\gamma\delta$ T cells of thymic origin contribute little to the i-IEL population of normal mice. The reconstitution experiments also show that $\alpha\beta$ i-IEL, in contrast to $\gamma\delta$ i-IEL, require a thymus to be produced in normal numbers. The extrathymic production of $\gamma\delta$ i-IEL shows that rearrangement and expression of T-cell receptor genes can be activated outside the thymus. It would be interesting, therefore, to identify the extrathymic precursor cells and the anatomic sites where rearrangement occurs.

The fact that intestinal colonization by $\gamma\delta$ T cells takes place mostly at weaning could be related to the drastic modification in the intestinal content that takes place at that point. Colonization by commensal bacteria, interruption of maternal transmission of immunoglobulins, as well as the intake of a diversified diet, all occur concomitantly with $\gamma\delta$ colonization. Which of these factors, if any, have a crucial effect on such localization remains to be determined. However, that bacterial colonization has little impact on i-IEL $\gamma\delta$ composition while it has a marked influence on the numbers of $\alpha\beta$ i-IEL is already established (30).

Given that $\gamma\delta$ i-IEL are produced outside the thymus, it follows that the initial repertoire selection, be it positive or negative, cannot occur in this organ. While there is no evidence for negative selection of self-reactive cells among $\gamma\delta$ i-IEL, there is an indication of positive selection as usage of one variable region γ-chain gene segment, $V_\gamma 7$, is predominant (16, 19). Such putative positive extrathymic selection has also been invoked recently for other $\gamma\delta$ T-cell subsets (37).

The results presented here reinforce previous observations by other laboratories on the extrathymic origin of a small pool of $\alpha\beta$ T lymphocytes (38–40). It has been established that $\alpha\beta$ T-cell numbers increase with age in nude mice and that the deletion of some variable region β-chain families that normally occurs in the thymus does not take place among these cells that are generated extrathymically (41, 42). The slow accumulation of $\alpha\beta$ T cells in the peripheral organs of thymectomized mice shown here suggests that the expansion rate of these T cells is significantly lower than that of $\alpha\beta$ CD4$^+$ T cells produced in normal mice. As suggested before (43, 44), the thymus appears to play an important role in generating cells capable of extensive peripheral expansion.

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