Two Gut Intraepithelial CD8$^+$ Lymphocyte Populations with Different T Cell Receptors: A Role for the Gut Epithelium in T Cell Differentiation

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Summary

Mouse gut intraepithelial lymphocytes (IEL) consist mainly (90%) of two populations of CD8$^+$ T cells. One bears heterodimeric α/β CD8 chains (Lyt-2$^+$, Lyt-3$^+$), a T cell receptor (TCR) made of α/β chains, and is Thy-1$^+$; it represents the progeny of T blasts elicited in Peyer's patches by antigenic stimulation. The other bears homodimeric α/α CD8$^+$ chains, contains no β chain mRNA, and is mostly Thy-1$^-$ and TCR-γ/δ^+ or -α/β^+; it is thymo-independent and does not require antigenic stimulation, as shown by its presence: (*a*) in nude and scid mice; (*b*) in irradiated and thymectomized mice repopulated by T-depleted bone marrow cells bearing an identifiable marker; (*c*) in thymectomized mice treated by injections of monoclonal anti-CD8 antibody, which lead to total depletion of peripheral CD8$^+$ T lymphocytes; and (*d*) in germ-free mice and in suckling mice. In young nude mice, α/α CD8 chains, CD3-TCR complexes, and TCR mRNAs (first γ/δ) are found on IEL, while they are not detectable on or in peripheral or circulating lymphocytes or bone marrow cells. IEL, in contrast to mature T cells, contain mRNA for the RAG protein, which is required for the rearrangement of TCR and Ig genes. We propose that the gut epithelium (an endoderm derivative, as the thymic epithelium) has an inductive property, attracting progenitors of bone marrow origin, and triggering their TCR rearrangement and α/α CD8 chains expression, thus giving rise to a T cell population that appears to belong to the same lineage as γ/δ thymocytes and to recognize an antigenic repertoire different from that of α/β CD8$^+$ IEL.

Mouse gut mucosal lymphocytes, especially intraepithelial lymphocytes (IEL),[1] have an unusual surface phenotype and an incompletely understood function (1; and reviewed in reference 2). IEL are predominantly CD8$^+$ T lymphocytes, but, in contrast to peripheral or circulating CD8$^+$ T cells, which are all Thy-1$^+$, a subset of the CD8$^+$ IEL present in nude mice is Thy-1$^-$ (2–4). It has been reported that IEL bear TCRs predominantly made of γ/δ chains (4–9). Since it is well established that the vast majority of peripheral T lymphocytes bears TCRs made of α/β chains, this raises the possibility that IEL have migrated directly from the thymus to the gut. On the other hand, previous experiments have led to the conclusion that the majority of Thy-1$^+$ IEL are the progeny of dividing precursors arising in Peyer's patches (PP) under antigenic stimulation. These blasts follow a special circuit, reaching the blood via the thoracic duct (TD) lymph to colonize the mucosa of the whole gut because of their peculiar gut-homing property (1, 2, 10, 11). All these observations seem difficult to reconcile with the evidence that IEL bear mostly TCR-γ/δ and may come directly from the thymus.

The present experiments show that CD8$^+$ IEL consist in fact of two ontogenically distinct populations, one bearing CD8 molecules made of two different polypeptide chains (Lyt-2 or α; and Lyt-3 or β chains), and the other bearing CD8 molecules lacking β chains, a phenotype previously observed in gut IEL by Parrott et al. (12); these two populations will be named heterodimeric α/β CD8$^+$ and homodimeric α CD8$^+$ (since it is likely that their α chains are expressed as homodimers [13]). The first population is thymodependent, TCR-α/β^+, and in all likelihood represents the PP-derived Thy-1$^+$ IEL previously described. The second population does not require a thymus to appear, probably derives

[1] *Abbreviations used in this paper:* DN, double negative; IEL, intraepithelial lymphocytes; PP, Peyer's patches; TD, thoracic duct.

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from cells having migrated directly from the bone marrow, and bears TCR with γ/δ or α/β chains, depending upon the age and other conditions of the mice. On the basis of observations made on thymus-deprived mice and on the presence of mRNA for the recombination activating gene 1 or RAG-1, (14, 15) in IEL, it is proposed that: (*a*) the gut epithelial environment plays an attractive and a differentiation-inducing role on the appearance of the α/α $CD8^+$ cell population and of its CD3 TCR complexes; and (*b*) this population has ontogenic similarities with the thymus γ/δ cell lineage.

Materials and Methods

Animals and In Vivo Treatment. C3HeB, C3H-DBA_2, C57Bl/6 mice were raised in our animal house, and C57Bl-Bg^j Bg^j (Beige) mice and CB17 *scid/scid* mice (SCID) were kindly provided by Dr. J. C. Guenet, Pasteur Institute, Paris, France. Nude mice (Swiss or BALB/c *nu/nu* mice) were obtained from Iffa Credo or CNRS, Orléans, France, and germ-free mice were a kind gift of C. Moreau (INRA, CNRZ, Jouy-en-Josas, France). GVHRs were elicited in lethally irradiated mice as previously described (11). Mice reconstituted with Beige bone marrow were prepared as follows: 8-wk-old C57Bl/6 mice were thymectomized, and 15 d later, they were 9 Gy irradiated and injected with 5×10^6 cells from bone marrow of Beige mice treated with anti-Thy-1 and rabbit complement (Cedarlane Laboratories, Hornby, Canada). For in vivo depletion of $CD8^+$ lymphocytes, thymectomized mice were injected twice at 1-wk intervals with 1 mg of H35-17 mab (IgG2b) (gift of M. Pierres, Marseille-Lumigny, France); the injected mice were studied 1 mo after the last injection as described (16).

Preparation of Cell Suspensions and Separation of Cell Subsets. IEL were prepared as previously described (1) with a mechanical procedure (scraping and passing through a glass wool column) and were obtained as a pure suspension (as attested by the absence of B cells and epithelial cells). In the case of nude mice, however, IEL preparations were always contaminated by epithelial cells and B lymphocytes for unknown reasons. To prepare $Thy\text{-}1^-$ IEL, IEL were treated with anti-Thy IgM mab (AT83) (gift of F. Fitch, University of Chicago) and rabbit complement. For the preparation of IEL deprived of $CD4^+$ lymphocytes, cells were treated with anti-CD4 mab (GK1.5) (gift of F. Fitch), then with mouse anti-rat κ, chain mab (gift of H. Bazin, University of Louvain, Belgium) and complement.

Lymphocytes from TD lymph were obtained as described (17). In adult mice, lymph was collected for 20 h ($\sim 5 \times 10^7$ lymphocytes recovered) and in young mice (25 d; 15-g weight) for 10 h only, because of the difficulty of keeping these small mice alive ($\sim 4 \times 10^6$ lymphocytes recovered). Bone marrow cells enriched in lymphoid precursors and depleted in most cells of the erythroid and myeloid lineage were prepared as described (18). Blood lymphocytes were isolated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). Peripheral lymph node cells deprived of B cells were obtained by passage through nylon wool followed by panning on rabbit anti-mouse Ig-coated plates (affinity-purified antibody kindly provided by S. Izui, University of Geneva).

Immunofluorescent Studies and Cytological Procedures. The following mabs were used: (*a*) for detection of CD3 and TCR, 145-2C11 hamster mab (19), which detects epitopes on the ϵ subunit of CD3, H57-597 hamster mab (kind gift of R. Kubo and P. Marrack, University of Colorado Health Science Center, Denver, CO), which reacts with all murine TCR-α/β (20); (*b*) for the detection of various T cell related antigens, TB107, and Thy-1 mab; GK1-5; anti-CD4 mab; H35-17-2 (called here H35), anti-CD8 mab, which stains the CD8 α/β heterodimer (Lyt-2 Lyt-3 complex) (21; see also present results); H59-101.7 (called here H59), which stains all CD8 α chains (Lyt-2) irrespective of their association with the CD8 β chains (Lyt-3) (21); 53-5.8 (22), which stains the CD8 β chain; RA3-2C2, which detects CD45RA, the high molecular weight form of the T 200 (Ly-5) antigen (23); for flow cytometry analysis and double staining, FITC labeled or biotinylated (revealed with streptavidin-coupled phycoerythrin) mabs were used (kind gift of B. Rocha, INSERM, Paris). For cytological studies, combined with radioautography in the case of cells incubated in vitro with [^{3}H] TdR (10), labeling with anti-CD3 and anti-α/β mab was revealed with a rabbit anti-hamster Ig rhodamin-labeled $F(ab')_2$ fraction (The Jackson Laboratory, Bar Harbor, ME), and the staining was increased by the use of a rhodamin-labeled sheep anti-rabbit Ig. The percentage of stained cells detected on the same cell preparations by cytofluorometry or microscopy (in smears) was very close. For double staining of TCR-α/β or CD3 antigen and other T cell antigens, capping of rhodamin-labeled anti-TCR or anti-CD3 antibody was first induced by 37°C incubation without N_3Na for 30 min; the cells were then incubated in presence of N_3Na, with anti-CD8, CD4, or Thy-1 rat mabs (revealed by biotinylated mouse anti-rat IgG [The Jackson Laboratory] and FITC avidin). When, as a control, anti-TCR-α/β mab was omitted, <1% of FITC-labeled cells showed an artifactual rhodamin cap; B cells showed strongly stained caps, because of the crossreactivity between hamster and mouse Ig. Autoradiography was performed with Ilford K5 (Ilford Ltd., Ilford, Essex, England).

Northern Blot and Polymerase Chain Reaction Analyses. RNA was prepared by the guanidium isothiocyanate method and transfered to Biodyne membranes, and all techniques, except when mentioned otherwise, were performed as previously described (24). 5–10 μg of total RNA per track was loaded, except when mentioned otherwise. The efficiency of transfer and the homogeneity of the amount of ribosomal RNA present in each track was verified by methylene blue staining of the filters before hybridization. The following DNA probes were used: for TCR Cα, a 500-bp EcoRI fragment derived from a TCR α cDNA (probe 1 in reference 25); for TCR Cβ, a 3-kb HindIII fragment derived from cosmid 2.3 w7 (probe 4 in reference 26); for TCR Cγ1, a 1-kb EcoRI + AvaI fragment derived from the 8/10-2 γ 1.1 TCR Cγ cDNA (27) (detects Cγ1, Cγ2, and Cγ3 isotypes); for TCR Cγ4, a 450-bp AvaII fragment derived from the 5/10-13 γ 1.2 TCR γ cDNA (27); and for Cδ, a 300-bp EcoRI-MboII fragment derived from cosmid 51.1w7 (probe 16 in 25); this last fragment was also subcloned in a plasmid pSp 65, to label cmRNA. As CD8 α chains (Lyt-3) probe, a 1.1-kb PstI fragment from the p Ly-3-23.1 Lyt-3 cDNA (21) was used. All these probes were labeled by random priming. For PCR, the following primers were used: (*a*) for RAG-1, 5′ primer, 5′-GTCTCCAGTAGTTCCAGA (276–294); 3′ primer: 5′-CTAGCCTGAGTTCTCTTG (841–859), giving a 580-bp fragment (14); (*b*) for mouse β actin, 5′ primer: 5′-AACGAGCGCTTCCGCTGTCC (753–773); 3′ primer: 5′-AATCTTGATCTTCATGGTGC (964–984), giving a 231-bp fragment for cDNA and a 1.1-kb fragment for genomic DNA (28); (*c*) for mouse CD3 γ chains, 5′ primer: 5′-TCAACGCACTGTAGCCCA; 3′ primer: 5′-TTCACACATTCTGTAATACAC, giving a 265-bp fragment (29). These primers were used to amplify genomic DNA for the RAG gene segment, which was introduced in a plasmid pSp 65, and to amplify the cDNA resulting from the reverse transcription (using poly(dT) or random priming) of 1–5 μg of total cell RNA. 30 cycles of amplification

were used (94°C for 50 s, 55° for 30 s, 72°C for 2–5 min), and part of the reaction mixture was separated on 6% polyacrylamide or 2% agarose gels and stained with ethidium bromide or transferred to nitrocellulose and hybridized to ^{32}P-labeled RAG cmRNA.

Results

Gut IEL Contain Two Populations of CD8$^+$ T Lymphocytes: Heterodimeric α/β CD8$^+$ Cells, Bearing TCR-α/β and Homodimeric α CD8$^+$ Cells, Bearing TCR-α/β or -γ/δ. In adult normal mice, ~90% of IEL were stained by the mab H59, which recognizes an epitope on CD8 α (Lyt-2) chains; only 30%, however, were stained by the mab H35, which binds α or β (Lyt-3) chains only when associated in heterodimers (21) (Table 1). The existence of two CD8$^+$ IEL subpopulations, α/β CD8$^+$ and α only CD8$^+$ (presumably in homodimeric α/α form [13]), was confirmed by double immunofluorescence (using H59 and H35 mabs) (Fig. 1) and by using the mab 53-5.8, which recognizes the β chains only (see legend of Table 1). Double immunofluorescence with an anti-Thy-1 antibody showed that all α/β CD8$^+$ IEL (and all CD4$^+$, ~10% of total IEL) were Thy-1$^+$, but that only a fraction of the H59$^+$ IEL, i.e., of all CD8$^+$ IEL, were Thy-1$^+$ (not shown). This was confirmed by treatment of IEL with anti-Thy-1 in the presence of complement. After such treatment, the remaining viable cells were virtually all α/α CD8$^+$ IEL (Table 1). These cells, in contrast to total IEL, indeed did not contain Lyt-3 (β) chain mRNA (Fig. 2 *D*). About 95% of IEL bore the CD3 molecule and ~70% bore TCR-α/β (Table 1 and Fig. 3). By double immunofluorescence with an anti TCR-α/β antibody, all α/β CD8$^+$ (and all CD4$^+$), but only about half of the α/α CD8$^+$ (H59$^+$) IEL, were TCR-α/β^+ (as can also be calculated from Table 1). To determine whether the remaining α/α CD8$^+$ IEL bore TCR-γ/δ, the presence of mRNAs coding for TCR α, β, γ, and δ chains in various IEL populations was studied by Northern blots, since no mab allowing the detection of TCR-γ/δ by immunofluorescence was available. All four classes of chains were detected in total IEL (Fig. 2 *A*, lane *T*, in which only Cα and Cγ4 chains are shown as representative examples) and in IEL depleted in Thy1$^+$ or CD4$^+$ cells (Fig. 2 *A*, lanes θ^- and 4^-). Thus, the α/α CD8$^+$ IEL that were not TCR-α/β^+ must be those which are known to bear TCR-γ/δ (5, 7)[2]. In contrast, in TD cells, only α and β TCR chains mRNAs were detectable (Fig. 2 *A*, compare lanes *IEL* with lanes *TDL*). Previous findings have shown that the precursors of Thy-1$^+$ IEL are antigen-stimulated blasts, arising in PP under antigenic stimulation and circulating in the TD (1). By double immunofluorescence and autoradiographies on smears of TD cells (see Materials and Methods),

[2] After the submission of this manuscript, an anti-TCR-δ mAb (kind gift of L. Lefrançois) has become available, and confirmed that most IEL that were not TCR-α/β^+ were TCR-δ^+ (see Table 1).

Table 1. *Gut IEL Surface Phenotypes in Various Conditions*

	Recovery per mouse	CD8$^+$ H35^{+*} (α/β CD8$^+$)	CD8$^+$ H59^{+*} (all CD8$^+$)	CD4$^+$	Thy-1$^+$	CD3$^+$	TCR-α/β^+	TCR-γ/δ‖‖
	$\times 10^6$				%			
Normal adult mice‡								
Total IEL	7.5 ± 4.8	33 ± 10.8	89.4 ± 2.4	9.8 ± 4.7	50.4 ± 14.2	95.1 ± 4.2	71.8 ± 8.5	27
Thy-1$^-$ cells (anti-Thy-1 + C)		<0.1	92	<0.1	<0.1	93	56	
Nude mice§	1 ± 0.3	<1	83.5 ± 14.5	<1	6 ± 4	36.5 ± 17	0	53
SCID mice‖	0.25	3.5	50	1	9	3	0	ND
Suckling mice¶	1.5 ± 0.6	5 ± 0.1	79 ± 5.5	2.4 ± 0.5	11 ± 4	89 ± 3	12 ± 1	81
Germ-free mice**	0.6	2	65	4	24.5	82	14§§	ND
Mice with GVHR‡‡	5	94	95	5	>99	>99	>99	0

Fluorescent lymphocytes were numbered either by flow cytometry or by microscopic analysis.

* H35. stains CD8 only when α chains are associated to β chains (α/β CD8$^+$), and H59 stains all α chains bearing cells (all CD8$^+$). The mab 53-5.8, which recognizes the β chains stains the same percentage of IEL as the H35 mab.

‡ 7-wk- to 10-mo-old (C3H × DBA)F$_1$ mice. IgA plasma cells, 50 ± 2/villus unit in lamina propria on tissue sections. SD were calculated from results of 7–15 experiments.

§ 7–11-wk-old mice. Results are identical in BALB/c and Swiss *nu/nu* mice. SD calculated on four to nine experiments.

‖ 7-wk-old mice (pooled cells from two mice).

¶ 20–25-d old mice. IgA plasma cells, 3.6 ± 1.5/villus unit. SD calculated on four experiments. The paucity of IgA plasma cells (compare to normal mice, and germ-free mice) probably reflects the very weak antigenic stimulation of the gut.

** Two 8-mo-old C3H mice. IgA plasma cells, 6.5/villus unit.

‡‡ Lethally irradiated (C3H × DBA)F$_1$ mice killed 6 d after injection of 8 × 10^6 C3H lymph node lymphocytes. Results of a representative experiment with <1% of persistent host cells.

§§ In the lymph nodes of these germ-free mice, all CD3$^+$ cells were also TCR-α/β^+, in contrast to the IEL.

‖‖ Results from a single experiment performed with an anti-TCR-δ mAb (GL3, a gift of L. Lefrancois, the Upjohn Company, Kalamazoo, MI). See footnote 2.

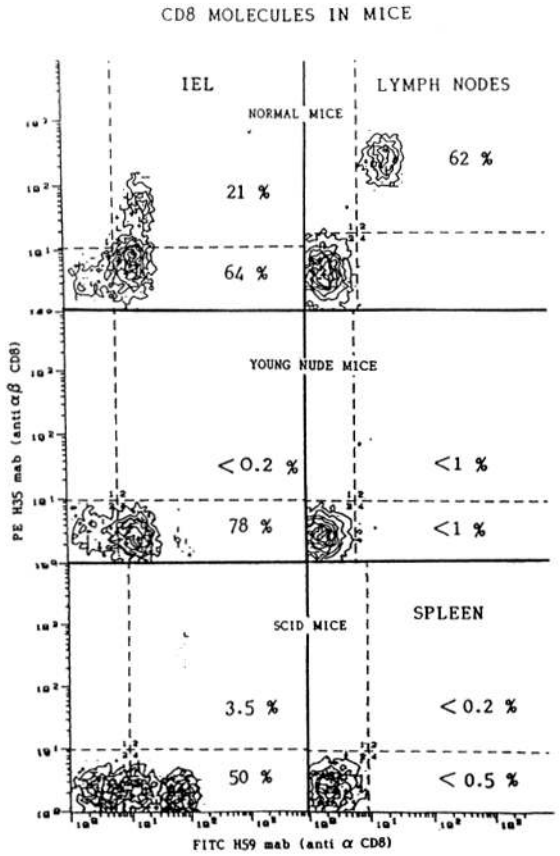

Figure 1. Double immunofluorescence analysis of IEL and peripheral lymphocytes in normal mice, in young nude mice, and in SCID mice for CD8 molecules. In normal mice (*top*) ~75% of the $CD8^+$ IEL bear only homodimeric α CD8 chains while all $CD8^+$ lymph node lymphocytes bear both α and β chains. In nude mice (*middle*), the majority of IEL are also $CD8^+$ and bear only α chains; in lymph nodes, $CD8^+$ lymphocytes are hardly detectable (lymph nodes are nylon wool passaged). In SCID mice (*bottom*), IEL bear high amount of CD8 molecules with α/α chains and no $CD8^+$ cells are found in the spleen.

nearly all lymphocytes and 98% of [^{3}H]TdR-labeled blasts were found to be Thy-1^+, α/β $CD8^+$ or $CD4^+$, TCR-α/β^+, suggesting that precursors of α/α $CD8^+$ IEL do not circulate in TD lymph (or do not bear this phenotype). Finally, using double immunofluorescence with anti-CD45 RA mab, an antigen present on virgin T cells (23), the $CD4^+$ and the α/β $CD8^+$ IEL populations were CD45 RA^-, which is in agreement with the fact that they are all the progeny of antigen-stimulated cells; in contrast, about 1/3 of the α/α $CD8^+$ cells were CD45 RA^+ (not shown).

Nude Mice and SCID Mice Have Homodimeric α $CD8^+$ IEL, which Are Also the Almost Exclusive $CD8^+$ IEL Population in Germ-free Mice and in Young Mice Shortly before Weaning. Nude mice (2 mo old) had decreased amounts of IEL, consisting mostly of strongly positive homodimeric α $CD8^+$ cells, with a smaller fraction of $CD3^+$ cells, very few Thy-1^+, and no TCR-α/β^+ cells (Fig. 1 and 4 *A*; Table 1); heterodimeric α/β $CD8^+$ and $CD4^+$ IEL were totally lacking (Table 1). Mice with the *scid/scid* mutation, which have no functional B and T lymphocytes, probably because they lack a functional enzyme required for Ig and TCR genes rearrangements (30), had no CD3 and α/α or α/β $CD8^+$ cells in their spleen. Nevertheless, they had IEL in small amounts. These cells were nearly all $CD3^-$, but 50% bore high amounts of homodimeric α CD8 molecules (Fig 1 and Table 1). Germ-free mice and young mice shortly before weaning, whose antigenic stimulation in the gut is very weak (Table 1), had also decreased amounts of IEL; strikingly, almost all their $CD8^+$ IEL were homodimeric α $CD8^+$. In these mice, in contrast to nude mice, $CD3^+$ IEL exceeded in percentage $CD8^+$ plus $CD4^+$ IEL, indicating that double-negative $CD4^-$ $CD8^-$ IEL (Table 1) were more numerous than in normal mice (in which a small percentage of these cells was observed: data not shown). Furthermore, $CD3^+$ IEL also markedly exceeded in percentage TCR-α/β^+ IEL (Table 1 and Fig. 3); since γ/δ chains mRNAs were found in IEL of suckling mice (not shown), most of the $CD3^+$ IEL appear to be TCR-γ/δ^+ cells. In TD of nude mice and of suckling mice, α/α $CD8^+$ cells were not seen, further indicating that precursors of IEL of this phenotype do not circulate in TD lymph (or do not bear this phenotype).

Experiments Using Thymectomized and/or Irradiated Mice Are Compatible with a Bone Marrow Origin of Homodimeric α $CD8^+$ IEL, and a Thymic Origin of Heterodimeric α/β $CD8^+$, TCR-α/β^+ IEL. Thymectomized adult mice, treated in vivo by injections of H35 anti-CD8 mab (anti-CD8 α/β chains) (16) showed, 1 mo later, <1% heterodimeric α/β $CD8^+$ cells in the spleen, mesenteric, and peripheral lymph nodes; homodimeric α $CD8^+$ cells, not detected in these organs in normal conditions, were not seen even after this α/β $CD8^+$ cell depletion. In contrast, IEL contained virtually only homodimeric α $CD8^+$ cells (3.5% α/β and 87% α/α $CD8^+$, with 96% $CD3^+$ and 60% TCR-α/β^+ cells, average of two closely comparable experiments; controls that were only thymectomized had a normal IEL surface phenotypic distribution). These results show that the homodimeric α and heterodimeric α/β $CD8^+$ IEL are independent populations and confirm that, in contrast to IEL, virtually all peripheral $CD8^+$ cells are α/β^+.

In thymectomized and lethally irradiated C57 mice reconstituted with T-depleted bone marrow cells of beige mice and killed a few weeks later, no heterodimeric α/β $CD8^+$ (<1%) nor $CD4^+$ IEL of beige origin (which can be easily identified on IEL smears because of their giant granules [1]) were found; in contrast, homodimeric α $CD8^+$ cells were present in high percentage (70.5%) among beige IEL, exceeding, as was observed with nude mice IEL (Table 1), that of $CD3^+$ cells (38.5%) (mean of four experiments, in which several hundreds of beige IEL were analyzed on smears) (Fig. 5).

In lethally irradiated F_1 mice injected with parental peripheral T lymphocytes, which develop an acute GVHR resulting in an infiltration of the gut by IEL of donor origin (11), almost all the IEL were heterodimeric α/β $CD8^+$ cells (Table 1); in these IEL, which are the progeny of blasts stimulated in the PP (1), RNA analysis showed the presence of

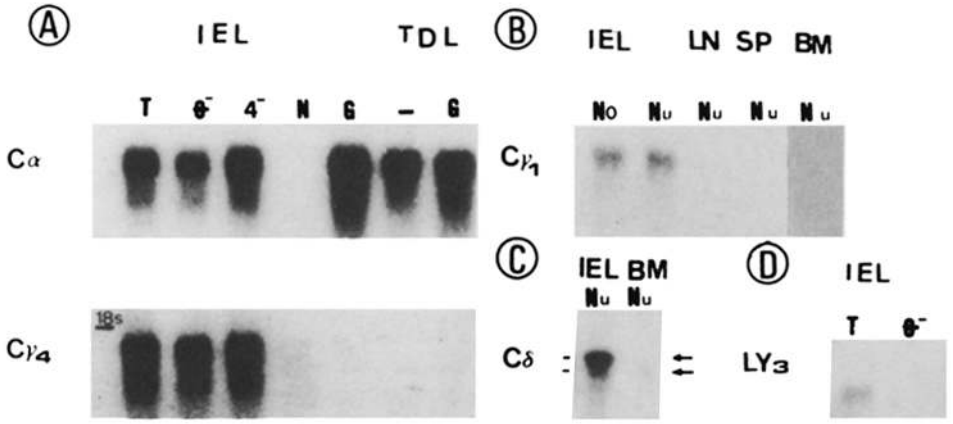

Figure 2. Northern blot analysis of RNAs from various lymphocyte preparations for the detection of TCR α, γ, or δ chains, or Lyt-3 (CD8 α) chains mRNA. (*A*) Analysis of IEL or TDL from normal or nude mice or mice with GVHR: *T*, θ^-, and 4^- refer to total IEL, anti-Thy-1 + C, or anti-CD4 + C–treated IEL respectively, and *N* and *G* to total IEL obtained from nude mice or mice with GVHR, respectively. TDL: – and *G* come, respectively, from normal mice or mice with GVHR. The results obtained with the Cβ probe and with the Cγ1 or the Cδ probes were similar to that obtained respectively with the Cα and Cγ4 probes. Note the existence of a very weak band of TCR Cγ4 chain mRNA in the IEL of nude mice. It should be noted that nude IEL preparations are contaminated by epithelial cells (see Materials and Methods) making precise quantitative comparisons more difficult. (*B*) Analysis of IEL from normal (*No*) or nude (*Nu*) mice, of nylon wool–filtered lymphocytes from lymph nodes (*LN*) or spleen (*SP*), or of selected (see Materials and Methods) bone marrow cells (*BM*) of nude mice. The specificity of the labeled probes used is indicated. Exposure time: 1 d for No IEL, 10 d for Nu IEL (in which epithelial contamination dilutes IEL RNA), LN, and SP, and 20 d for Nu BM (from another experiment, the longer exposure time resulting in higher background). (*C*) Analysis of IEL and total bone marrow cells of nude mice. RNA was hybridized with a ^{32}P-labeled cmRNA δ probe. IEL mRNA migrates as a 2-kD band, and in bone marrow, a faint band of 1.7 kD is detected. (*D*) Total (*T*) IEL RNA shows with the Lyt-3 DNA probe a band of ~1.3 kb corresponding to Lyt-3 mRNA, which is totally absent in Thy-1 + C–treated IEL (θ^-).

only α and β, but not γ and δ chain mRNAs (Fig. 2 *A*, compare total IEL, *T*; and GVHR IEL, *G*); TD blasts, the precursors of these IEL (1), also contained as expected only α and β chain mRNAs (Fig. 2 *A*, lane *TDL, G*).

Comparison of IEL and Other Lymphoid Populations in Nude Mice Suggests that the Gut Environment Plays a Differentiation Inducing Role in the Appearance of α CD8 Chains and TCR on Precursors of Bone Marrow Origin. In 2-mo-old nude mice, in which ~80% of IEL were homodimeric α CD8$^+$ (Table 1 and Fig. 1) and 30–40% CD3$^+$ cells (Fig. 4 *A*), as mentioned above, the following cell populations were also examined for CD8 α chains (Fig. 1 and not shown) and the CD3 complex (Fig. 4), which were not found on a significant percentage of cells: (*a*) spleen and lymph nodes cells (after nylon wool filtration to potentially enrich them in T lymphocytes [Fig. 4, *B* and *C*]); (*b*) bone marrow cells, either as total cell suspension or after fractionation by a procedure selecting lymphocytes and their precursors (18, 31) (Fig. 4 *D*); in the same fraction from bone marrow cells of normal mice, there was ~6% of CD3$^+$ cells; (*c*) blood lymphocytes (compare cells from nude mice in Fig. 4 *E* and normal mice in Fig. 4 *F*); (*d*) TD lymphocytes, as mentioned above. The CD3$^+$ IEL of these 2-mo-old nude mice probably all bore TCR-γ/δ, since no TCR-α/β$^+$ cells were seen (Table 1), while γ and δ TCR chain mRNAs were found (Fig. 2 *B*). In contrast, no TCR γ or δ chain mRNA was found in their spleen, lymph node, or bone marrow cells (total as well as fractionated as described above) (Fig. 2 *B*). With bone marrow cells, using a more sensitive cmRNA probe for δ chains, a faint band migrating slightly faster than IEL δ chain in RNA (1.7 vs. 2.0 kD), was detected (Fig. 2 *C*); it corresponds to the size of incomplete (V-D-J) transcripts observed by others (4, 32). In older (5 mo) nude mice, CD3$^+$ cells were also found in peripheral lymphoid organs, where a small percentage of TCR-α/β cells was detectable (Fig. 4, *G* and *H*). Thus, in nude mice, the first cell-bearing CD8 homodimeric α chains and the CD3-TCR complex appear among IEL. This suggests that the first TCR rearrangements are expressed among IEL.

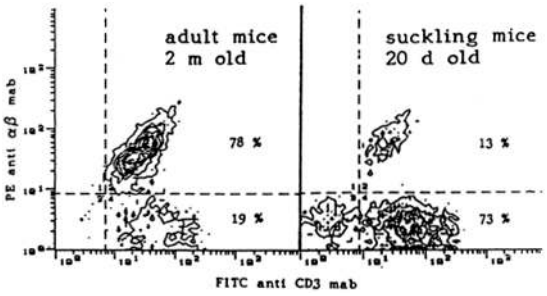

Figure 3. Double immunofluorescence analysis of IEL in normal adult and suckling mice for CD3 and TCR-α/β. The ratio of TCR-α/β$^+$ IEL among CD3$^+$ IEL in adult mice (*left*) and suckling mice (*right*) is strikingly different.

Gut IEL, but Not Peripheral T Lymphocytes, Contain mRNA for the RAG-1 Protein. The RAG-1 protein is necessary for rearrangements of TCR and Ig genes (14, 15). The presence of RAG-1 mRNA was explored by PCR in IEL and, as controls, in thymus and bone marrow cells, where it should be detectable, and in peripheral lymph node cells and TD lymphocytes, where it should not, since they contain mostly mature lymphocytes (14). RAG-1 mRNA was detected in thymus, bone marrow, and total IEL from adult mice (Fig. 6 *A*, *a* and *b*), but not in TD lymphocytes (Fig. 6 *A*, *b*). However, RAG-1 mRNA was also detected in peripheral lymph nodes. Since it is not known if RAG-1 is also involved in the rearrangements occurring during Ig class switch, which

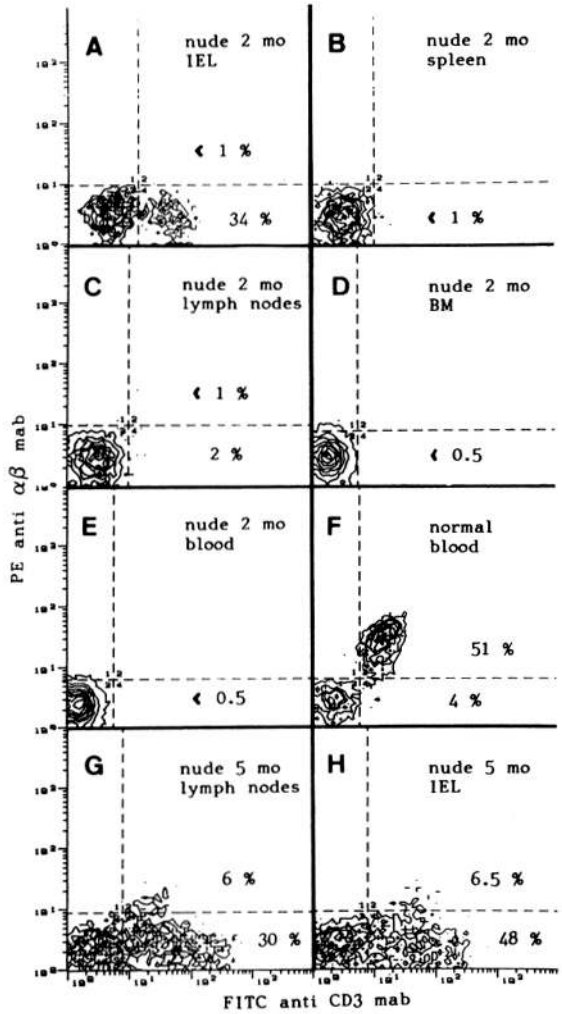

Figure 4. Double immunofluorescence analysis of various lymphoid population in 2-mo and 5-mo-old BALB/c nude mice stained for CD3 and TCR-α/β. In the 2-mo-old mouse, only IEL contain $CD3^+$ cells; in the 5-mo-old mouse (*G* and *H*), $CD3^+$ cells are found in IEL and lymph nodes (as well as in the spleen), and a small fraction of them bear TCR-α/β chains. Spleen and lymph nodes cells are nylon passaged lymphocytes (recovery ~15%). Bone marrow lymphocytes were enriched on sucrose gradient. For further details, see Results.

takes place in B blasts, in particular in germinal centers, PCR experiments were then performed in IEL and in LN cells depleted in B cells. RAG-1 mRNA was detected in B-depleted IEL, but was consistently undetectable in B cell–depleted LN cells (Fig. 6 *A*, *a*). To compare the amounts of RAG-1 mRNA in thymus, bone marrow, and IEL RNA, Northern blots of poly(A) RNA from these three sources were hybridized with a RAG cmRNA probe: RAG-1 mRNA was detectable in very large amounts in thymus RNA, comparatively in very small amounts in bone marrow RNA, and was not detectable in these conditions in IEL RNA (Fig. 6 *B*). An interpretation of these quantitative differences will be discussed below.

Discussion

The salient findings of this work are as follows: (*a*) mouse gut IEL, which are in vast majority $CD8^+$, contain two subpopulations with different CD8 phenotypes: one bears heterodimeric α/β CD8 molecules, as is also observed on all thymocytes (whether $CD4^+$ $CD8^+$ or $CD4^-$ $CD8^+$), and peripheral CD8 T lymphocytes in adult mice (33); the other bears only CD8 α chains, probably in a homodimeric form, and does not contain CD8 β chain mRNA; (*b*) these two $CD8^+$ gut IEL subpopulations belong to two distinct T cell lineages, most notably different in their thymic dependency and TCR type and repertoire; (*c*) emergence in the gut epithelium of homodimeric α $CD8^+$ IEL does not require the prior expression by precursors of this population of TCR and $CD8^+$ molecules; (*d*) gut IEL contain mRNA for the RAG-1 protein, which is necessary for TCR gene rearrangements. On the basis of these findings, it is proposed that the gut epithelium plays both an attractive and inductive role in the appearance of the homodimeric α $CD8^+$ IEL population, which may recognize antigens of a different nature than the thymic-derived IEL.

The heterodimeric α/β $CD8^+$ gut IEL subpopulation is $Thy\text{-}1^+$, thymodependent, as shown by its absence in nude mice, and bears CD3 and TCR-α/β complex. Its TCR Vβ chain repertoire indicates that it has been submitted to a process of negative selection in the thymus (see below). In these respects, this population is indistinguishable from all peripheral $CD8^+$ T lymphocytes; it appears to be the progeny of mature peripheral T cells having undergone antigenic stimulation in the Peyer's patches (1), and, after passage through the thoracic duct and the blood, having seeded the whole length of the gut to become IEL (1, 10, 34) ($CD4^+$ TD blasts of the same origin exist, but they have a lower gut homing property and $CD4^+$ cells are but a small minority among IEL [2, 11]). The relationship between gut antigenic stimulation and the heterodimeric $CD8^+$ IEL subpopulation

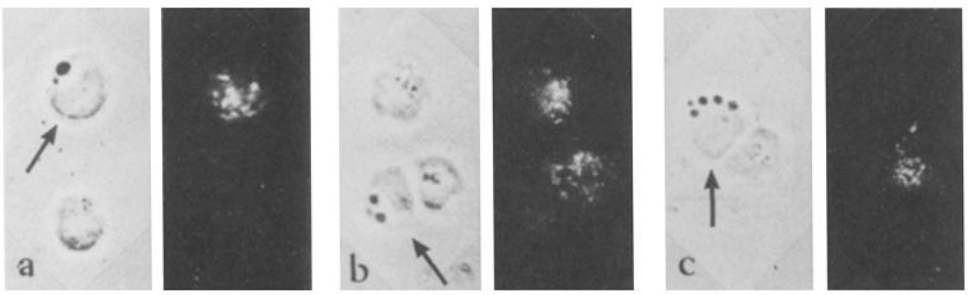

Figure 5. IEL isolated from thymectomized irradiated C57Bl/6 mice reconstituted with BM from beige mice. IEL from beige origin, identified because of their large granules (*arrows*), are stained with anti CD3: (*a*) a strong staining, (*b*) a weak staining, and (*c*) and unstained cell. Note that gut lymphocytes are specially radioresistant and that numerous host gut lymphocytes (detectable by their small granules) persist in lethally irradiated mice.

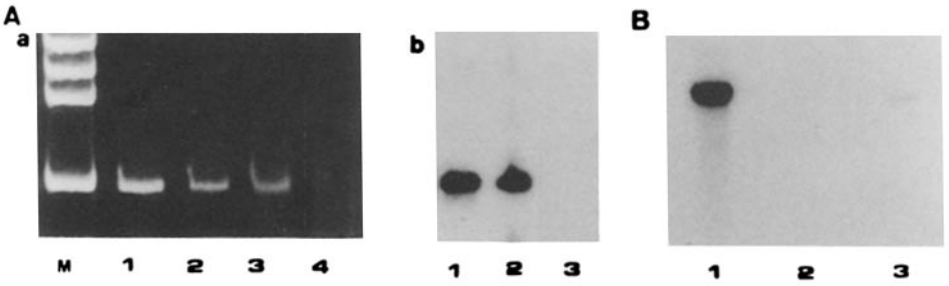

Figure 6. RAG-1 mRNA detection. (*A*) PCR analysis of reverse transcribed RNA (*a*) ethidium bromide staining after amplification of cDNA of thymus (lane *1*), bone marrow (lane *2*), IEL depleted in B cells (lane *3*), LN cells depleted in B cells (lane *4*); *M*, markers of size; (*b*) hybridization with a ^{32}P RAG-1 cmRNA probe, after Southern blotting of the DNA fragments amplified from thymus (lane *1*), IEL (lane *2*), and thoracic duct lymph (lane *3*). The RNA extracted from *a* and *b* are from different experiments. All reverse transcriptions and PCR amplifications in *a* and *b* have been performed simultaneously. Amplification were performed in parallel with actin primers or primers for the γ chains of CD3, to ascertain the absence of genomic DNA, or the efficiency of the reverse transcription in the samples explored (see Materials and Methods). (*B*) Northern blot analysis of poly(A) RNA obtained from 70 μg of thymus total RNA (lane *1*), or from 150 μg of bone marrow (lane *3*) or IEL (lane *2*) total RNA, hybridized with ^{32}P-labeled RAG cmRNA probe (see Materials and Methods); exposure times: thymus; 15 h, bone marrow and IEL, 5 d. The size of the bands seen on lanes *1* and *2* is ~6.6 kD. Scanning of autoradiogram exposed for similar length of times indicate >100× (not determinable) higher intensity in the thymus vs. bone marrow band.

is further stressed by its virtual absence in germ-free mice and in young mice before weaning, and by the fact that it does not bear the CD45 RA antigen, a marker of virgin T cells (23).

The homodimeric α $CD8^+$ subpopulation, which does not need antigenic stimulation to appear since it is present in germ-free and suckling mice, belongs to a different, $Thy\text{-}1^+$ or $Thy\text{-}1^-$, T cell lineage, as shown by the following evidence: (*a*) it is not altered by a treatment that leads to the almost complete and persistent depletion of the $CD8^+$ peripheral T lymphocytes and heterodimeric α/β $CD8^+$ gut IEL (injections of anti-CD8 β chains mAb in adult thymectomized mice); (*b*) it does not need to differentiate within the thymus, as shown by its presence in nude mice and in thymectomized and lethally irradiated mice reconstituted with T-depleted bone marrow cells; (*c*) its TCR type can be either γ/δ, appearing first, before weaning, or α/β, appearing later, in adult life; (*d*) its TCR-α/β chains repertoire does not show the restriction, due to a process of negative thymic selection, which is found in other peripheral T cells. In mice bearing the minor lymphocyte stimulatory antigen $Mls\text{-}1^a$, peripheral T lymphocytes are markedly depleted in cells bearing the Vβ8.1 and Vβ6 segments of the TCR β chains, as the result of clonal deletion of these $Mls\text{-}1^a$ reacting cells within the thymus (35, 36); the same depletion is observed in the heterodimeric α/β $CD8^+$ gut IEL subpopulation, in complete contrast to the homodimeric α $CD8^+$ cells with TCR of the α/β variety, which contain a high percentage of cells bearing these Vβ segments (36a).

As mentioned, a fraction of the homodimeric α $CD8^+$ gut IEL bear TCR-γ/δ. Comparison of its TCR repertoire with that of other TCR-γ/δ^+ populations (which are all $CD8^-$ and $CD4^-$) also adds important information concerning the peculiarity of this gut T cell lineage. While TCR-γ/δ^+ gut IEL appear to differ from the small populations of TCR-γ/δ^+ thymocytes and peripheral T lymphocytes by the preferential use of Vγ7 segments, they have nevertheless also an extended repertoire because of high junctional diversity (8, 37). This is in complete contrast with other intraepithelial T lymphocytes, which are exclusively TCR-γ/δ^+, namely those of the skin (dendritic epithelial cells or DEC) and of the mucosa of the female genital tract (uterus and vagina), which have a highly restricted repertoire: DEC bear only Vγ5 and Vδ1 chains without junctional diversity, as does the "first wave" of thymocytes appearing in fetal life (38), and IEL of the female genital tract only Vγ6 and Vδ1 chains, as does the "second wave" of fetal thymocytes (39, 40); all available evidence indicates that these two IEL populations stem from the fetal thymus, and this has even been conclusively proven for DEC (41).

The observations summarized above show that the homodimeric α $CD8^+$ gut IEL do not need any thymic contribution to appear and belong to a different T cell differentiation pathway than: (*a*) the heterodimeric α/β $CD8^+$ gut IEL; and (*b*) the $CD8^-$ IEL of skin and other mucosae of ectodermal or mesodermal origin. What could be the ontogenic pathway of this thymoindependent population of gut IEL? We propose that the gut epithelium, an endoderm derivative as the thymic epithelium, has the property of attracting circulating progenitors of bone marrow origin and of inducing the expression by these cells of CD8 α chains and of the CD3-TCR complex, through successive steps of differentiation. The thymic epithelium has been shown to secrete peptides chemotactic for a "pre-T" subpopulation of bone marrow cells, which subsequently undergoes TCR gene rearrangement (42). We propose that the gut epithelium (which also strikingly attracts mast cell precursors of bone marrow origin, as does the thymus [34]) has a somewhat comparable property. Further evidence supporting this hypothesis will be reviewed now.

The attractive and inductive ability of the gut epithelium, leading to its infiltration by circulating bone marrow–derived precursors of the thymo-independent gut IEL population, is suggested by the fact that, in normal or mutant mice, cells with the features of this population can be found only in the gut epithelium itself: (*a*) in normal mice, young or adult, homodimeric α $CD8^+$ T cells are not detectable in any other location, including the thymus (13); (*b*) in young nude mice, it is also in the gut epithelium that the first homodimeric α $CD8^+$ cells bearing CD3 molecules and containing mature TCR-γ/δ mRNA appear; (*c*) still more strikingly,

in SCID mice, gut IEL are present with normal cytological features (intracytoplasmic granules: see below): ~50% of these TCR-defective IEL bear CD8 α homodimeric chains (Table 1; 43); cells with this phenotype cannot be observed elsewhere. This last observation conclusively shows that prior TCR or CD8 homodimeric α chains expression is not required for the intraepithelial migration of gut IEL precursors, and is best explained by an unaltered homing in the gut epithelium of bone marrow precursors undergoing all differentiation steps compatible with their genetic defect. This lack of influence of TCR on gut intraepithelial homing by precursors may explain why, in transgenic mice with TCR γ and δ rearranged transgenes, accumulation of gut IEL, which express only the transgenes, is not influenced by their nature (9). It should be noted along this line that in germ-free or young suckling mice, ~10% of the IEL are neither $CD3^+$ nor α/α $CD8^+$ (Table 1), and could represent recently attracted, not yet differentiated precursors. In human gut IEL also, cells with the phenotype of pre T cells (44) are observed, i.e., $CD7^+$, $CD4^-$, $CD8^-$, and $CD3^-$, without markers of NK cells (45).

Induction by the gut epithelium microenvironment of TCR gene rearrangements in the recently attracted precursors of the homodimeric α $CD8^+$ IEL requires that these precursors are able to express the enzymatic machinery necessary for gene rearrangement. Productive TCR-α/β and- γ/δ genes rearrangements have been observed to occur in bone marrow precursor cells after a few days of culture in a variety of stimulatory supernatants, in the absence of epithelial cells of any origin (46–48). Thus, all that is necessary for bone marrow precursors to rearrange TCR genes appears to be an appropriate microenvironment, which may favor the expression of the rearranging enzymes. It was therefore of high interest to observe that IEL, but not mature peripheral T lymphocytes, contain mRNA for the RAG-1 protein, which is required for TCR and Ig gene rearrangements (14, 15). This detection required amplification by the polymerase chain reaction and could not be achieved by hybridization on Northern blots of IEL RNA, contrary to what was observed with thymus RNA, and with bone marrow RNA (which contain RNA or pre B cells). That RAG mRNA is found in much larger amounts in the thymus than IEL (and bone marrow cells) probably reflects the cellular composition and ontogenic role of this organ, in which the TCR recombination activity may last through a certain time span of TCR-α/β^+ T cell maturation, which occurs in a vast majority of thymocytes and the $CD4^+$ $CD8^+$ cell population (for review, see reference 49). In contrast, among IEL, only very few cells may, at the explored time (total IEL from adult mice), have unrearranged TCR and require the RAG-1 protein. In any event, the presence of RAG mRNA in IEL, although it does not prove that all TCR gene rearrangements occur in gut microenvironment (we have observed in the bone marrow of young nude mice a very small amount of incomplete [VDJ] δ chains mRNA [Fig. 2 *C*]), is compatible with the hypothesis of TCR gene rearrangements occurring in the gut. That some degree of TCR gene rearrangement occurs in the bone marrow in some precursors of thymocytes has not been ruled out either. In the thymus, however, a large scale process of expansion, rearrangements and selection takes place for most thymocytes, while there is no evidence that gut thymo-independent IEL undergo massive expansion and selection.

What could be the ontogenic lineage, among T cells, of the homodimeric α $CD8^+$ gut IEL subpopulation, bearing either TCR-γ/δ or -α/β? We suggest that it is comparable to the $CD4^-$ $CD8^-$ or double-negative (DN) $CD3^+$ subpopulation of the thymus, and that differentiation along this lineage can also occur in the gut epithelium. This small thymic subpopulation is not submitted to the MHC-linked repertoire selection process occurring among the very large $CD4^+$ $CD8^+$ double-positive subpopulation, which give rise to the mature TCR-α/β^+ peripheral T cells; in adult mice, it is the source of the TCR-γ/δ^+ cells found in the blood and peripheral lymphoid organs, and is sometimes referred to as the "γ/δ lineage", although it also contains TCR-α/β^+ DN thymocytes, which appear later in thymic development (for review, see reference 49). Emergence of TCR-α/β^+ DN thymocytes might reflect some degree of "leakiness" in the γ/δ lineage (49); it has indeed been observed that these TCR-α/β^+ DN thymocytes contain full-length γ chains mRNA (50). An essential observation relating gut homodimeric α $CD8^+$ IEL, whether TCR-γ/δ or -α/β^+, to an ontogenic pathway of the γ/δ lineage, is that, when stimulated in vitro by a lectin, DN thymocytes express homodimeric α CD8 chains (13), as is also the case for activated γ/δ cells from peripheral lymphoid tissues (51). Thus, it seems likely that this CD8 phenotype is a marker of activation of cells of the TCR-γ/δ lineage. It might be noted in this respect that in the uninfected gut of germ-free or suckling mice, ~10% of the $CD3^+$ IEL lack CD8 α chains, and thus are DN. In contrast, in normal adult mice, these cells are very rare, and the homodimeric α $CD8^+$ IEL are present in larger amounts, containing an increasing percentage of TCR-α/β-bearing cells. This may reflect a condition of enhanced local activation, accompanied by an increased "leakiness" of the γ/δ lineage as mentioned above. Accumulation, after weaning, of thymodependent $CD4^+$ and α/β $CD8^+$ cells among gut IEL and lamina propria lymphocytes is likely to influence, through the release of lymphokines and acceleration of the epithelium renewal kinetics (11), the expansion and activation of the thymo-independent IEL.

Finally, all gut IEL, whatever their origin, show at least two other characteristic features of differentiation, which thus also appears to be induced by the local microenvironment. These cells bear a new differentiation antigen, present on mouse, rat, or human IEL (52–54), that appears to be an activation molecule (45, 55). Most importantly, IEL show cytotoxic activities (6), and IEL of all surface phenotypes contain intracytoplasmic granules, identical to those observed in clones of cytotoxic T cells or in NK cells, detectable in up to 75% of the cells (1). These granules contain serine esterases of the granzyme family and perforin (in preparation), suggesting that the gut epithelial environment induces in IEL the differentiation of a strong cytotoxic machinery. It thus appears that a major role of gut IEL is to destroy altered epithelial cells. In this context, what is the specialized role of

the homodimeric α CD8$^+$ IEL, assuming that the thymo-dependent heterodimeric α/β CD8$^+$ IEL certainly recognize their targets through MHC class I molecules? Their TCR-γ/δ may recognize some putative peculiar antigens presented by "nonclassical", tissue-specific, class I MHC molecules (56), perhaps explaining the predominance of the Vγ7 segment in their Vγ repertoire (56). Their TCR-α/β may recognize some "superantigens", such as bacterial enterotoxins (57), presented in a nonclassical way on the classical MHC class II molecules (58) that are found in low amounts on normal gut epithelial cells (11) or such as antigens that might be bound to the CD_1 molecules so strikingly expressed on the surface of enterocytes (59); this would explain why their Vβ repertoire contains, contrarily to thymo-dependent T cells, such a high percentage of Vβ families deleted in the thymus.

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