Developmental Changes in Intraepithelial T Lymphocytes and NK Cells in the Small Intestine of Neonatal Rats

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ABSTRACT

The main objective of this study was to characterize developmental changes in small intestinal intraepithelial lymphocyte (IEL) subpopulations during the suckling period, thus contributing to the understanding of the development of diffuse gut-associated lymphoid tissue (GALT) and to the identification of early mechanisms that protect the neonate from the first contact with diet and gut microbial antigens. The study was performed by double labeling and flow cytometry in IEL isolated from the proximal and distal small intestine of 1- to 21-d-old Lewis rats. During the suckling period, intraepithelial natural killer (NK) cells changed from a typical systemic phenotype, CD8+, to a specific intestinal phenotype, CD8-. Analysis of CD8+ IEL revealed a progressive increase in the relative number of CD8+ IEL co-expressing TCR $\alpha\beta$, cells associated with acquired immunity, whereas the percentage of CD8+ cells expressing the NK receptor, *i.e.* cells committed to innate immunity, decreased. At weaning, IEL maturity was still not achieved, as revealed by a phenotypic pattern that differed from that of adult rats. Thus, late after weaning, the regulatory CD8+CD4+

T IEL population appeared and the NK population declined. In summary, the intestinal intraepithelial compartment undergoes changes in its lymphocyte composition associated with the first ingestion of food. These changes are focused on a relatively high proportion of NK cells during the suckling period, and after weaning, an expansion of the regulatory CD8+CD4+ T cells. (*Pediatr Res* 58: 885–891, 2005)

Abbreviations

DN, double negative (CD8–CD4–)
DP, double positive (CD8+CD4+)
dSI, distal small intestine
GALT, gut-associated lymphoid tissue
IE (L), intraepithelial (lymphocytes)
LPL, lamina propria lymphocytes
NK, natural killer
pSI, proximal small intestine

GALT is the largest immunologic organ in the body, with anatomical, phenotypic, and functional features that distinguish it from the systemic immune system and other peripheral lymphoid tissues (1,2). It is also one of the major sites of immunologic challenge. During the early postnatal period, newborns are exposed to a multitude of microbial and nutritional antigens, which challenge the development and complete maturation of both intestinal and systemic immune systems (3,4).

GALT contains organized lymphoid structures and diffusely distributed cell populations. Organized GALT comprises Peyer's patches, isolated follicles, and the mesenteric lymph

nodes. Diffuse GALT includes IEL and LPL (5). IEL are specialized immune cells whose precise function is not completely understood. They may contribute to cell-mediated mucosal defense with unusual antigen-recognition specificity, surveillance of epithelial cell integrity, and to the pathogenesis of a variety of diseases (6–8). IEL, like LPL, consist of effector and memory lymphocytes that are generated from antigen-stimulated cells in locations such as Peyer's patches. The progeny of these cells migrate systemically and ultimately populate distant mucosal sites (9).

The characterization of intestinal IEL has revealed a phenotypic and functional heterogeneity that is only comparable to that of the thymus; they express markers that are absent or only rarely found in systemic peripheral counterparts. The phenotypic composition of IEL appears to be influenced by both genetic and environmental factors (8). Most IEL are CD8+, and include cells bearing T cell receptors (TCR) composed of $\gamma\delta$ and especially $\alpha\beta$ chains (10,11). In addition to the peripheral classic phenotypes [CD8+CD4– and CD8–CD4+], some IEL exhibit the CD8+CD4+ (DP) and the CD8–CD4– (DN)

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phenotypes (12,13). Moreover, IEL include a high proportion of NK lymphocytes, which may constitute the first line of defense of GALT. NK cells in the rat IE compartment can display an NKR-P1A+ CD3- CD2- DN phenotype (8,14,15), and spontaneously secrete IL-4 and/or interferon- γ (15), thus defining specific intestinal IE characteristics.

The intestinal epithelium may be unique in its ability to function as a key site for the thymus-independent maturation of a substantial population of T cells (1,16). The presence in CD8+ T cells of the homodimer CD8 $\alpha\alpha$ instead of CD8 $\alpha\beta$ identifies extrathymically derived IEL (17,18). In addition to the presence of particular lymphocyte populations in intestinal epithelium, small and large intestinal IEL differ in their phenotype in some species and site-specific differences such as apical *versus* basal and proximal *versus* distal small intestine have also been described (8,19,20).

Little is known about the phenotype of IEL from birth to the end of the suckling period; thus, the main objective of this study was to characterize developmental changes in the phenotypic composition of rat small intestinal (proximal and distal regions) IEL during suckling. This will contribute to the understanding of diffuse GALT development and to the identification of early mechanisms that protect the neonate from the first contact with diet and gut microbial antigens.

METHODS

Animals. Pregnant Lewis rats (G14) and 10-wk-old male Lewis rats (used as reference adults) were obtained from Harlan (Barcelona, Spain). Pregnant rats were housed in individual cages in controlled conditions of temperature and humidity in a 12:12 light:dark cycle. They were monitored daily until delivery and allowed to deliver naturally. The day of birth was identified as d 1 of life. Litters were unified to eight pups per lactating mother, with free access to the nipples and rat chow. Reference adult rats were housed four per cage and kept in the same other conditions as pregnant rats. Adult rats were fed with commercial rat chow and water *ad libitum*. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation of the University of Barcelona, and procedures were approved by that committee.

Small intestine extraction. Adult rats and pups aged 1, 3, 5, 7, 11, 14 and 21 d (suckling period) were killed by humanitarian methods. The abdomen was opened and the entire small intestine, from the pyloric sphincter to the ileocecal junction, was flushed *in situ* with cold saline solution to remove the contents. Thereafter, the small intestine was removed and dissected from the mesentery. Intestine was divided into two equal length portions, designated as pSI and dSI. The intestines from 1- to 4-d-old rats were cut into 5-mm pieces, whereas those from 5- to 14-d-old rats were opened lengthwise before cutting, and those from 15- to 21-d-old or adult rats were everted and cut.

Isolation of IEL. Intestinal IEL suspensions were obtained from one animal or four animals depending on their postnatal age. In rats aged 1–21 d, intestines were pooled and processed four at a time in the same tube, whereas in adult rats, intestinal tissues were processed individually. From the intestinal samples, IEL and epithelial cells were removed by subsequent incubations, at 37°C in a shaker, once with DTT (5 mM, 20 min, Sigma Chemical Co., St. Louis, MO) in Hanks' balanced salt solution (HBSS) without calcium and magnesium (BioWhittaker, Verviers, Belgium), twice with EDTA (5 mM, 30 min, Pan-reac, Barcelona, Spain) in HBSS, and finally with RPMI 1640 (30 min, BioWhittaker). All culture media were supplemented with 5% fetal bovine serum (FBS, BioWhittaker). After each incubation, released cells were decanted from the tissue pieces and centrifuged. Pellets from each step were resuspended in RPMI 1640 with 5% FBS and collected. The resulting cell suspension containing IEL and epithelial cells from all steps was then subjected to IEL purification.

Purification of IEL. To discard epithelial cells, cell suspensions were subsequently passed throughout a glass wool column (0.145 g, Merck, Darmstadt, Germany) placed in a 10-mL syringe. The column was then washed twice with 5% FBS RPMI 1640 medium. The collected passing volume was centrifuged and cells were resuspended in 44% Percoll (Amersham Bio-

sciences, Uppsala, Sweden). Each cell suspension was overlaid on 67.5% Percoll and, after centrifugation (600 g, 30 min, room temperature), viable lymphocytes were recovered from the interface. Cell number and viability were determined after staining dead cells with ethidium bromide (Sigma Chemical Co.) and live cells with acridine orange (Sigma Chemical Co.).

Immunofluorescence staining and flow cytometric analysis. An immunofluorescence technique was used to stain 2×10^5 IEL. MAb directly conjugated to FITC or phycoerythrin (PE) were applied. Mouse anti-rat MAb used in this study include anti-CD3 (1-F4), anti-CD4 (OX-35), anti-CD5 (OX-19), anti-CD8 α (OX-8), anti-CD25 (OX-39), anti-CD44 (OX-50), anti-CD45 (OX-1), anti-CD45RC (OX-22), anti-CD71 (OX-26), anti-TCR $\alpha\beta$ (R73), anti-TCR $\gamma\delta$ (V65), and anti-NKR-P1A (10/78), all from BD PharMingen (San Diego, CA); anti-CD90 (OX-7), and anti-CD45RA (OX-33) from Caltag Laboratories (Burlingame, CA) and anti-CD8 β (3.41) from Serotec (Oxford, UK). Cell surface double-staining was performed as follows. Cells were incubated with a mixture of saturating concentrations of FITC- and PE-conjugated MAb in PBS containing 2% FBS and 0.1% Na $_2$ S $_2$ O $_8$ (Merck) at 4°C in the dark for 20–30 min. Stained cells were washed with PBS, fixed with 0.5% p-formaldehyde (Merck), and stored at 4°C in the dark until analysis by flow cytometry. For each sample, a negative control staining using an isotype-matched MAb was included. Analyses were performed with an Epics XL flow cytometer (Beckman Coulter Inc., Fullerton, CA). All samples were analyzed considering the same gate, which included 100% of cells labeled with anti-CD45 MAb from adult and 21-d-old rats. Phenotypical results were expressed as the percentage of positive cells with respect to the total number of gated lymphocytes or with respect to a particular IEL subset (NK or CD8+ cells). Anti-CD8 α MAb was used to stain total CD8+ cells (both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IEL population), and the presence or absence of this molecule is expressed in the text and figures as CD8+ and CD8- cells, respectively.

Statistical analysis. Statistical analysis was performed by conventional ANOVA. For each dependent variable (e.g. CD8+ cells), we considered as independent variables the age of animals (d 1–21) and the intestinal IEL location (pSI or dSI). When age or location variables had a significant effect on the dependent variable, *post hoc* comparisons (LSD test) were performed. Moreover, differences between adult and 21-d-old animals or between pSI and dSI in adult rats were analyzed by means of the Mann-Whitney *U* test. All analyses were performed using the Statistica program (StatSoft, Tulsa, OK). Significant differences were accepted at $p < 0.05$.

RESULTS

Recovery of IEL from small intestine. The number of lymphocytes recovered from the small intestinal IE compartment increased in an age-dependent manner (Fig. 1a; $p < 0.001$). At birth, intestinal IEL were present in very low numbers ($\sim 1 \times 10^5$ in total small intestine; Fig. 1a) and cells displaying flow cytometric characteristics of lymphocytes were almost absent (Fig. 1b). On d 3, the typical cluster of lymphocytes in forward scatter (FSC)/side scatter (SSC) cytograms was already apparent (Fig. 1c), and, thereafter, a dramatic increase in the IEL number was observed (Fig. 1, a and d). At the end of the suckling period, adult IEL numbers were not achieved ($\sim 2 \times 10^6$ on d 21 *versus* 3.5×10^6 in adult; $p < 0.05$).

In addition, on the day of birth, only 40% and 30% of the gated cells showed CD45 positive staining in pSI and dSI, respectively (Fig. 2a), revealing that these young cells with FSC/SSC lymphocyte characteristics did not yet express the typical surface leukocyte marker. On d 3, CD45 was already present in 85% of gated cells obtained from pSI, and in 90–95% from d 5 to adulthood. In dSI, the proportion of CD45+ cells inside the gate was significantly lower than in pSI on d 3–7 ($p < 0.05$), and did not achieve the adult percentage until d 14 (Fig. 2a).

The percentage of CD3+ IEL cells in gated lymphocytes was already higher than 60% in both pSI and dSI on d 1 (Fig. 2b), showing that the expression of this T-cell-specific molecule occurs earlier than that of CD45. The proportion of CD3+

Figure 1. Recovery of IEL from the small intestine during the suckling period and in adult rats. (a) Number of IEL recovered from total intestinal tissue. Data were calculated from the total cell yield by microscopy and corrected for the percentage of cells with lymphocyte FSS/SSC morphology determined by flow-cytometric analysis. Each individual value corresponds to the total number of cells present in the whole intestine (proximal and distal portions) of one adult animal or calculated data obtained from four samples processed together. Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Statistical differences of d-21 data vs those of adult rats are shown. (b–d) IEL FSC/SSC cytograms obtained from representative samples corresponding to (b) the day of birth, (c) 3-d-old rats, (d) adult rats. Typical lymphocyte cluster is located in region marked R1.

Figure 2. Percentage of CD45+ and CD3+ cells in IEL obtained from the small intestine during the suckling period and in adult rats. (a) CD45+ cells and (b) CD3+ cells expressed with respect to the total number of gated IEL in pSI (black) and dSI (white). Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Each value was obtained from one adult rat intestine ($n = 8$) or four suckling rat intestines processed together ($n = 16$ for each data point). * $p < 0.05$ dSI vs pSI.

IEL decreased from d 1 to d 3, but this drop did not represent a decrease in the total number of these cells in the intestine. On the contrary, due to total IEL age-dependent increase in the small intestine (from 1.32×10^5 IEL/intestine on d 1 to $\sim 2.85 \times 10^5$ IEL/intestine on d 3, Fig. 1a), the absolute number of CD3+ IEL on d 3 was higher than that on d 1 ($\sim 1.5 \times 10^5$ CD3+ IEL/intestine versus $\sim 0.91 \times 10^5$ CD3+ IEL/intestine). The decrease in CD3+ IEL proportion on d 3 is due to a higher relative increase in CD3– cells, such as NK cells (discussed later). From d 3 and throughout the suckling period,

the proportion of CD3+ IEL continued to rise progressively both in pSI and dSI ($p < 0.001$).

The presence of B lymphocytes within the population of gated cells was analyzed to ascertain whether IEL suspensions were contaminated with LPL. The percentage of B cells determined by CD45RA staining was lower than 2% throughout the studied period (data not shown).

CD8/CD4 IEL subpopulations during postnatal life. Analysis of data obtained from CD8/CD4 double staining on gated lymphocytes revealed four subpopulations with a different pattern of development in each case. The percentage of CD8+CD4– IEL in both pSI and dSI increased markedly during the first days of life, achieving values by d 3 that did not differ significantly from those of adult rats (Fig. 3a). Thus, the predominance of CD8+ IEL described in adults was also observed in suckling rats. However, the CD8+CD4– IEL proportion in dSI was significantly higher than that in pSI during the later suckling period ($p < 0.05$ in 21-d-old rats), showing clear regional differences in the main effector population during this young period of life.

Throughout the suckling period, only a few IEL, if any, were CD8+CD4+ ($<3\%$, Fig. 3b). In contrast, the DP phenotype was present in at least 18% of intestinal IEL from adult animals without significant differences between pSI and dSI, showing that DP IEL underwent a late postweaning expansion.

A high proportion of DN IEL ($\sim 80\%$) was seen on the day of birth (Fig. 3c). Thereafter, this proportion remained at around 30% of IEL, which was higher than that found in adult animals ($p < 0.001$). Moreover, the DN IEL proportion in dSI was significantly lower than that of pSI during the later suckling period ($p < 0.05$ in 21-d-old rats), showing regional differences complementary to those found in the CD8+CD4–

Figure 3. Percentage of CD8+CD4–, DP, DN, and CD8–CD4+ cells in IEL during the suckling period and in adult rats. (a) CD8+CD4– cells, (b) CD8+CD4+ cells, (c) CD8–CD4– cells, and (d) CD8–CD4+ cells compared with the total number of gated IEL in pSI (black) and dSI (white). Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Each value was obtained from one adult rat intestine ($n = 8$) or four suckling rat intestines processed together ($n = 16$ for each data point). Statistical differences of d 21 data vs those of adult rat are shown for each intestinal portion. * $p < 0.05$ dSI vs pSI.

IEL subset. The DN IEL population may include either T cells in the process of maturation or NK cells lacking co-expression of CD8 (discussed later).

The percentage of CD8⁺CD4⁺ IEL was very low, and similar in both intestinal portions during the first and second postnatal weeks (Fig. 3d); this level increased up to 5% during the third week ($p < 0.05$). At weaning, the percentage of CD4⁺CD8⁺ IEL reached the adult level in pSI (6%), but not in dSI, where it was lower than that found in adults (11%; $p < 0.05$). Thus, in adult rats, the proportion of CD4⁺CD8⁺ cells in IEL was higher in dSI than in pSI ($p < 0.05$).

Changes in IE NK cell phenotype in neonatal rats. A characteristic time-course pattern of IE NK cell proportion was found during the first weeks of life. The proportion of NK cells in the IE compartment was higher throughout the suckling period than that found in adult rats ($p < 0.05$ and $p < 0.01$ d 21 versus adult; Fig. 4a). From d 1 to d 3, a dramatic increase in IE NK cell number was found (from $\sim 2.1 \times 10^5$ NK cells/intestine to $\sim 13.4 \times 10^5$ NK cells/intestine). The rise was particularly high in pSI, as shown in NK cell proportions (Fig. 4a) and the proportion in pSI was double that of dSI on d 3–7 ($p < 0.01$). The strong NK cell percentage increase in both intestine fragments induced a decrease in the proportion of other IEL subpopulations, such as CD3⁺ cells (see above).

In addition, the phenotype of IE NK cells also depended on the age. During the first 2 wk of life, with the exception of the day of birth, most IE NK cells expressed CD8 (Fig. 4, b and c),

Figure 4. Developmental time-course of IE NK cell phenotypes during the suckling period and in adult rats. (a) NKR-P1A⁺ cells with respect to the total number of gated lymphocytes, and (b) NKR-P1A⁺CD8⁻ cells with respect to total NK cells, in pSI (black) and dSI (white). Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Each value was obtained from one adult rat intestine ($n = 8$) or four suckling rat intestines processed together ($n = 16$ for each data point). Representative cytograms of the frequency distribution of fluorescence intensity obtained by NKR-P1A/CD8 MAb double labeling of IEL from (c) newborn rats (5-d old) and (d) an adult rat. Marked region includes NK cells showing distinct phenotype depending on the age. Statistical differences of d-21 data vs those of adult rat are shown for each intestinal portion. * $p < 0.05$ dSI vs pSI.

whereas the complementary percentage of the NKR-P1A⁺CD8⁻ subset increased over the suckling period ($p < 0.001$; Fig. 4b). At weaning, the proportion of NK IEL that were NKR-P1A⁺CD8⁻ was higher in pSI than in dSI ($p < 0.001$) and did not differ significantly from that of adult rats in either of the regions (Fig. 4b). Thus, the NKR-P1A⁺CD8⁻ subset represented the main NK cell population on the day of weaning and in adult rats (Fig. 4d).

Changes in CD8⁺ IEL subpopulations during the suckling period. The time course of appearance of several CD8⁺ IEL subsets was established using double-labeling techniques. Results are expressed as the percentage of the considered phenotype with respect to the CD8⁺ IEL population (Figs. 5 and 6).

Figure 5 summarizes the relative changes in CD8⁺ IEL expressing the mutually exclusive receptors NKR-P1A, TCR $\alpha\beta$, and TCR $\gamma\delta$. As can be seen in both intestinal portions, the pattern clearly varied with age ($p < 0.001$), and distinct distributions were observed during the first 3 wk of life that also differed from the adult pattern. About 80% of CD8⁺ IEL from adult rats expressed TCR $\alpha\beta$, 11–17% expressed TCR $\gamma\delta$, and only 3–6% expressed NKR-P1A. The proportion of cells bearing the antigen receptor TCR $\alpha\beta$ in CD8⁺ IEL was low on d 1 but increased progressively until d 11 in both intestinal fragments. Although after the first week the TCR $\alpha\beta$ ⁺ became the principal phenotype observed in CD8⁺ IEL, at the end of the suckling period its proportion was still lower than that of adult animals ($p < 0.05$). Throughout the study period, TCR $\gamma\delta$ ⁺ lymphocytes from either of the fragments did not represent more than 15% of CD8⁺ lymphocytes in the total IEL population. Although the proportion of TCR $\gamma\delta$ ⁺CD8⁺ IEL was relatively high in the first few days of life, at weaning this proportion did not differ significantly from that of adult rats. The proportion of NK cells in CD8⁺ IEL decreased from d 3 until d 21 in both pSI and dSI, without reaching adult values ($p < 0.05$). Thus, it can be concluded that throughout suckling, the proportion of TCR $\alpha\beta$ ⁺CD8⁺ IEL increase with age at the expense of NKR-P1A⁺CD8⁺ IEL, both in pSI and dSI.

Two CD8 phenotypes can be established in IEL: CD8 $\alpha\alpha$ and CD8 $\alpha\beta$. Their proportion showed some fluctuations without

Figure 5. Proportions of excluding CD8⁺ IEL populations during the suckling period and in adult rats. Proportion of CD8⁺NKR-P1A⁺, CD8⁺TCR $\alpha\beta$ ⁺, and CD8⁺TCR $\gamma\delta$ ⁺ cells with respect to total CD8⁺ lymphocytes in pSI and dSI, respectively. TCR $\alpha\beta$: black circles and column; TCR $\gamma\delta$: triangles and hatched columns; NK: circles and column. Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Each value was obtained from one adult rat intestine ($n = 8$) or four suckling rat intestines processed together ($n = 16$ for each data point). Statistical differences of d 21 data vs those of adult rat are shown for each intestinal portion.

Figure 6. Developmental time-course of various CD8⁺ IEL phenotypes during the suckling period and in adult rats. Percentage of (a) CD8 $\alpha\alpha$ ⁺ (solid line) and CD8 $\alpha\beta$ ⁺ (dashed line) cells, (b) CD8⁺CD3⁺ cells, (c) CD8⁺CD5⁺ cells, (d) CD8⁺CD90[−] cells, and (e) CD8⁺CD44^{high} cells with respect to CD8⁺ IEL in pSI (black) and dSI (white). Each data point corresponds to the mean $\pm$ SEM of four values (d 1–21) or eight values (adult). Each value was obtained from one adult rat intestine ($n = 8$) or four suckling rat intestines processed together ($n = 16$ for each data point). Statistical differences of d-21 data vs those of adult rat are shown for each intestinal portion. * $p < 0.05$ dSI vs pSI.

significant changes, during the suckling period, and between pSI and dSI (Fig. 6a). Moreover, the percentages did not differ from those of adult animals (Fig. 6a). The CD8 $\alpha\alpha$ phenotype always dominated over CD8 $\alpha\beta$, and in adult rats the proportion of CD8 $\alpha\alpha$ was two and three times higher than CD8 $\alpha\beta$ in dSI and pSI, respectively.

On the day of birth, more than half of CD8⁺ IEL co-expressed CD3, both in pSI and dSI (57% and 90%, respectively; Fig. 6b). In both fragments, the CD3⁺ proportion gradually increased from d 3 onwards during the suckling period ($p < 0.001$). On d 21, and in adulthood, more than 95% of CD8⁺ IEL expressed CD3.

In contrast to CD3, the thymic developmental marker CD5 was only co-expressed in a minor population of CD8⁺ IEL, in either pSI or dSI (Fig. 6c). From d 3, and during the suckling period, the percentage of CD5⁺ in the CD8⁺ population of IEL varied, but was always lower than 27%. On d 21, it was about 12% in both fragments and significantly lower than that of adult rats ($p < 0.05$). This increase in CD5⁺ phenotype of CD8⁺ cells may be associated with a postweaning expansion of CD8⁺CD4⁺ IEL.

Thy-1 (CD90) usually disappears in peripheral T cells during the early stages of cell maturation (21). Accordingly, analysis of CD90/CD8 double-labeling in IEL during suckling (Fig. 6d) revealed that CD8⁺ IEL co-expressed CD90 only on the day of birth (about 30%), but thereafter, including in adult life, almost 100% of CD8⁺ IEL were CD90[−], in both pSI and dSI.

CD44 is a receptor that participates in T-cell adhesion within the intestinal mucosa and allows the differentiation between memory (CD44^{high}) and naive (CD44^{low}) cells. In adult rats, there was high expression of CD44, but in distinct proportions of CD8⁺ IEL from pSI and dSI (42% and 60%, respectively,

$p < 0.05$; Fig. 6e). During the suckling period, the proportion of CD44^{high} in CD8⁺ IEL was similar to that of adult rats (Fig. 6e).

Although IEL are activated lymphocytes, our studies on suckling and also in adult animals indicate that only a few CD8⁺ IEL, if any, coexpress the activation molecules CD25 (IL-2 receptor), CD45RC (signal transduction), or CD71 (transferrin receptor) (data not shown).

DISCUSSION

The present study addresses the developmental changes in intraepithelial lymphocyte composition undergone in rat small intestine from the day of birth and throughout the suckling period. Moreover, results were compared with those found in adult rats. Almost all the major IEL subsets identified in adults were already present in suckling rats, but with specific proportions at different time points during this period.

Although GALT structures develop during fetal life, their activation does not occur until shortly after birth (22). During the suckling period, luminal factors begin to stimulate immune cells present in the gut compartment, and this challenge is increased by solid diet ingestion that in rats begins spontaneously during the late suckling period (23). In humans, although intestinal IEL are present during gestation, stimulation by luminal factors clearly determines their number, which increases postnatally up to 10-fold by 1–2 year of age (24). Our results agree with this IEL postnatal expansion. Analysis of the IEL population from rat small intestine during the first days of life suggests that the lymphocytes colonize the gut epithelium mostly during the first few postnatal days, and the majority of those precocious lymphocytes present at birth express CD3, but not CD45 on their surface. The proportion of IEL expressing CD3 also increased throughout the suckling period, which is consistent with studies performed in mice (25).

NK cells were relatively abundant in the gut IE compartment of newborn rats, and at weaning the percentage was still higher than that present in adult life. These results agree with those of Tice (26), who found higher NK activity in the IEL population of neonate and weanling rats than in adults, and Todd *et al.* (15), who described ~50% of NKR-P1A⁺ cells in the IEL in weanling rats and ~20% in adult animals. Two subsets of NK cells were found in the intestinal IE compartment: NKR-P1A⁺CD8⁺ and NKR-P1A⁺CD8[−] cells. The latter group has not been described in other lymphoid tissues, like spleen and peripheral blood (15,27,28). The age-dependent expansion of CD8[−]NK cells may represent a distinct functional role or a maturational state. In this context, in small intestine, cells co-expressing CD8 and the NK receptor, as in the systemic compartment, may be essential at birth and during the first days of life and may reflect their peripheral origin. The prevalence of NKR-P1A⁺CD8⁺ IEL declined with age, and the main NK cell population in adult rats lacked CD8, this phenotype becoming the mature mucosal subset in rat small intestine.

The relatively high proportion of TCR $\gamma\delta$ +CD8⁺ IEL during the first days of life found here is consistent with the fact that $\gamma\delta$ T cells emerged earlier than $\alpha\beta$ T cells in terms of both phylogeny and ontogeny (29). Moreover, our results from flow

cytometry agree with histologic studies showing that TCR $\gamma\delta$ + cells are present in the gut epithelium of newborn rats (8,30). In addition to their cytolytic activity, TCR $\gamma\delta$ + IEL may mediate specific functions at each developmental stage. Thus, they seem to be required for the development of mucosal B cells (31,32) and may secrete cytokines and growth factors that influence epithelial cell proliferation and differentiation (33). These roles may explain the relatively high proportion of TCR $\gamma\delta$ +CD8+ IEL during the first days of life. Considering results relating to both NKR-P1A+CD8+ and TCR $\gamma\delta$ +CD8+ IEL subpopulations, it can be postulated that these cells constitute the first line of defense in the gut mucosa of newborn rats when acquired immunity is not fully developed.

The proportion of TCR $\alpha\beta$ -expressing CD8+ IEL increased progressively from the day of birth, and became the predominant population at the end of the first week of life. Although our results do not agree on the day of TCR $\alpha\beta$ +CD8+ IEL appearance, they nevertheless confirm the data reported by Helgeland *et al.* (30) regarding the age-dependent increase in this population during the perinatal period. Therefore, our results are consistent with those indicating that the most early TCR $\alpha\beta$ + IEL are recruited before the conventional luminal microflora is established after weaning (8). In mice, a similar age-dependent increase in TCR $\alpha\beta$ + IEL has been described (3), although other studies point to a decrease during the first 3 wk of life (25). The relative increase in CD8+ IEL expressing the antigenic receptor TCR $\alpha\beta$ at the expense of lymphocytes involved in the nonspecific immune response (*i.e.* NK cells) during suckling, may be the result of the specific antigenic stimulation that occurs after the first food intake.

In contrast to peripheral CD8+ T cells, CD8+ IEL can express this molecule as either $\alpha\alpha$ or $\alpha\beta$ chains. The former is commonly described as type b IEL (16), or as a thymus-independent IEL marker, and corresponds to a nonconventionally selected lymphocyte population that develops in the gut microenvironment and expresses CD8 $\alpha\alpha$, alone or in combination with CD4, and an oligoclonal TCR repertoire (2,34). The CD8 $\alpha\alpha$ phenotype has been described in intestinal TCR $\gamma\delta$ + and TCR $\alpha\beta$ + IEL (33,35) and NK cells (36). Our results show that at any given time, the CD8 $\alpha\alpha$ phenotype is relatively more common than the CD8 $\alpha\beta$ phenotype, as reported for adult rats (8). These results suggest that the IE colonization of CD8 $\alpha\beta$ + cells classically selected in the thymus occurs in parallel to the expansion of CD8 $\alpha\alpha$ + cells. In this study, variations in the total proportion of CD8 $\alpha\alpha$ + or CD8 $\alpha\beta$ + IEL were not observed during suckling, probably due to the reciprocal changes in CD8+TCR $\alpha\beta$ + and CD8+NK IEL.

CD5+ cells only represent a small proportion of CD8+ IEL during the suckling period. Consequently, unlike peripheral lymphocytes, a substantial number of TCR $\alpha\beta$ +CD8+ IEL lack CD5 expression, as reported elsewhere (3). The lack of CD5 expression is associated with thymus-independent development (37) and, thus, these CD5- cells may correspond to CD8 $\alpha\alpha$ + IEL. Our results relating to CD90 (Thy-1) expression by CD8+ IEL agree with previous studies performed in mice by Klein (38), who showed that the Thy-1 molecule is

absent in almost 70% of IEL. Moreover, in our study, a significant proportion of IEL expressing activation molecules like CD25, CD45RC, and CD71 were not found in either suckling or adult rats.

Among the IEL subsets considered here, CD8+CD4+ T cells constitute a unique phenotype that was hardly expressed during the suckling period and clearly expanded after weaning. A similar behavior has been described by Takimoto *et al.* (12), who also found an age-related increase in these cells in 4-, 10- and 30-wk-old rats from various genetic backgrounds, and by Yamada *et al.* (39), who reported a considerable number of CD8+CD4+ IEL in aged Lewis rats. This DP subset may be involved in intestinal T-cell maturation and development with functions in both innate and adaptive immunity (40,41) and important regulatory functions (39). Although their origin remains to be elucidated in the gut epithelial environment, it has been postulated that either CD8 $\alpha\alpha$ IEL become DP IEL by acquisition of the CD4 molecule (40) or that mature post-thymic CD4+ IEL may be induced to express CD8 in the intestinal compartment (41). The low percentage of the CD4+CD8- IEL subpopulation found here in suckling rats agrees with the latter hypothesis. Thus, the CD8 $\alpha\alpha$ molecule may be acquired by peripheral CD4+ lymphocytes that only migrate to the intestine late after weaning. In conclusion, the later appearance of these CD8+CD4+ cells suggests that their physiologic regulatory role is not required during the early stages of life.

Finally, as we differentiated between the two halves of the rat small intestine, we were able to determine whether IEL development was regionally specialized during the suckling period, as described in adult animals (8,19). Several differences between intestinal regions were found during the first week. In particular, proximal intestinal epithelia presented a higher percentage of total NK cells, and a lower proportion of CD3+CD8+ and TCR $\alpha\beta$ +CD8+ IEL subpopulations than distal intestine. At weaning, a relatively high proportion of DN cells, which may represent IE NK CD8- cells, were also found in pSI. This distribution is suggestive of certain regional differences during suckling, consisting of a relatively high innate immunity in the proximal small intestine. These regional phenotypic variations during suckling may depend on more abundant microbiota in dSI than in pSI, differences in luminal food-derived antigen distribution, or a different intrinsic ability of each intestinal region.

In summary, in the IE compartment, the lymphocyte subpopulations composition underwent changes associated with the first ingestion of food and the establishment of the gut microbiota. However, changes still follow when the gut is exposed to new antigens from microbiota or solid diet. Modifications associated with the suckling period mainly come from an interesting behavior of IE NK cells, which changed from a typical systemic phenotype (NKR-P1A+CD8+) to a characteristic intestinal phenotype (NKR-P1A+CD8-). Moreover, there was a progressive relative rise in acquired immunity associated to the TCR $\alpha\beta$ +CD8+ IEL subpopulation during the first two-thirds of the suckling period. After weaning, IEL underwent regulatory CD8+CD4+ subset expansion. The developmental pattern described here by means of changes in IEL

composition in the small intestine can be used as a reference with which to evaluate the effect of drugs, prebiotics, or dietary supplements on effector mucosal immunity during the suckling period.

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