Appearance and Maturation of T-Cell Subsets During Rat Thymus Ontogeny

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In previous papers, we have described the ontogenetical development of thymic stromal-cell components (epithelium, macrophages, dendritic cells) of Wistar rats. Here, we correlate those results with the maturation of rat T-cell precursors along the fetal and postnatal life. First T-cell precursors, which colonize the thymus anlage around days 13-14 of gestation, largely express CD45, CD43, CD53, and Thy 1 cell markers, and in a lesser proportion the OX22 antigen. Rat CD3-CD4-CD8- thymocytes present in the earliest stages of gestation could be subdivided in three major cell subpopulations according to the CD44 and CD25 expression: CD44-/+CD25- → CD44+CD25+ → CD44+CD25+. On fetal days 17-18, a certain proportion of CD4-CD8- cells weakly express the TcR/β chain, in correlation with the appearance of the first immature CD4-CD8+ thymocytes. This cell subpopulation, in progress to the CD4+CD8+ stage, upregulates CD8α before the CD8β chain, expresses the CD53 antigen, and exhibits a high proliferative rate. First mature thymocytes arising from the DP (CD4+CD8+) cells appear on fetal days 20-21. Then, the CD4+CD8+ cell ratio is ≤1 changing to adult values (2-3) just after birth. Also, the percentage of VβTcR repertoire covered in adult thymus is reached during the postnatal period, being lower during the fetal life. Finally, in correlation with the beginning of thymocyte emigration to the periphery a new wave of T-cell maturation apparently occurs in the perinatal rat thymus.

Keywords: Ontogeny, rat thymus, T-cell development

Classification Categories
Abbreviations used:
DN, CD4-CD8- DP, CD4+CD8+SP, CD4-CD8+/CD4+CD8-F, fetalP, postnatalAD, adult

INTRODUCTION

The development of the thymus gland is governed by mutual influences between the cell components of thymic stroma and lymphoid-cell progenitors that colonize the organ early during ontogeny. The rat thymic primordium is colonized around fetal day 14 by cell progenitors, the phenotype of which has not been clearly established although the expression on them of different cell markers has been reported...
(Ritter et al., 1978; Paterson and Williams, 1987; 
Kampinga and Aspinall, 1990; Crook and Hunt, 
1996). Moreover, the existence, as reported in mice 
(Godfrey et al., 1993), of cell subpopulations defined 
by the expression of CD44 and CD25 in the DN-cell 
compartment of rats is controversial. CD44, a 
molecule involved in the homing of cell precursors to 
mouse thymus has not been described in the rat 
thythic progenitors and the CD25 expression reported 
early in ontogeny throughout thymic parenchyma 
(Habu et al., 1985; Brocke et al., 1987) and, after 
birth, in the thymic medulla (Habu et al., 1985; 
Brocke et al., 1987; Kampinga and Aspinall, 1990) 
have not been associated with DN thymocytes, 
although IL-2-induced proliferation of rat DN cells 
has recently been reported (Gotlieb et al., 1993).

The transition from DN-cell compartment to DP 
cell in Wistar rats is marked by the expression of the 
CD8 molecule, which allows an intermediate highly 
proliferative CD8^+CD4^- population (Paterson and 
Williams, 1987), which in adult rats seems to weakly 
express the TcRαβ (Hünig et al., 1989a, 1989b). On 
the other hand, the appearance of mature SP 
(CD4^-CD8^+ and CD4^-CD8^-) thymocytes around 
birth involves changes in the expression of some cell 
markers, such as CD45R (Kampinga and Aspinall, 
1990) and Thy 1 (Hosseinzadeh and Goldschneider, 
1993), which could be related to the emigration of 
first T lymphocytes to the periphery. Two other 
relevant events occur in the rat thymus during the 
perinatal period. As previously reported in mice, there 
is an important increase of thymic cellularity that 
could be associated with in situ increased proliferation 
(Ceredig, 1990; Lawetzky et al., 1991) and/or 
with the arrival at the organ of a second wave of T-
cell precursors (Penit and Vasseur, 1989). In addition, 
the CD4/CD8 cell ratio gradually changes during 
perinatal life to the adult condition. In mice, this 
phenomenon has been correlated with a distinct 
capability of the embryonic and adult T-cell pre-
cursors to differentiate (Adkins, 1991) and with the 
existence of different mechanisms of thymic selection 
in each period due to the presence or absence of TdT 
activity in adult or fetal precursor cells, respectively 
(Shortman and Wu, 1996).

Despite this available evidence, a systematic analy-
sis of the ontogenetical maturation of both the 
lymphoid and nonlymphoid cell populations of rat 
thyms is, to our knowledge, lacking, although 
Kampinga and Aspinall (1990) carried out an immu-
nohistochemical study on rat thymus ontogeny and 
Hünig et al. (1989a) analyzed by flow cytometry the 
expression of some rat T-cell markers, including 
CD2, TcRαβ, and CD25 (IL-2Rα chain) in the last 
stages of fetal life. Previously, we studied the 
ontogeny of rat thymic stromal-cell components, 
including epithelial cells, dendritic cells, and macro-
phages (Vicente et al., 1994, 1995, 1996). In the 
present study, we combine both immunohistochem-
istry and flow cytometry to analyze the appearance 
and differentiation of distinct rat T-cell subsets and 
the influence of the thymic nonlymphoid cell micro-
environments on these processes.

RESULTS

Evolution of Thymic-Cell Numbers During 
Ontogeny

Between days 15-21 of embryonic life, the cell 
number exponentially increased in the thymus of 
Wistar rats. At birth, it remained constant, and from 
day 2 of postnatal life onwards, the rat thymus gland 
underwent a new exponential growth to reach the 
adult condition (Table I). On the other hand, evolution 
of the thymic-cell numbers correlated well with the 
frequency of cycling cells in each studied stage. 
Between fetal days 16-19, there was a high percentage 
of cells in S + G2 + M phases, decreasing rapidly at 
day 21. Around birth, the percentage of cycling cells 
increased transiently, decreasing again to adult values 
by the second week of postnatal life (Table I).

The Earliest Thymocytes

As previously reported (Vicente et al., 1996), the first 
T-cell precursors reached the rat thymus anlage at 
days 13-14 of gestation. These progenitor cells 
expressed CD45, CD53, and CD43 antigens (Figure 
1a). On days 15-16, flow cytometry analysis showed
TABLE I  Evolution of Thymus Size and Cell Proliferation During Embryonic and Postnatal Wistar Rat Development

<table>
<thead>
<tr>
<th>Days</th>
<th>Thymic cellularity ($\times 10^{-6}$)</th>
<th>% of cells in $S + G2 + M$ phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>15F</td>
<td>$0.006 \pm 5 \times 10^{-4}$</td>
<td>$32 \pm 3.0$</td>
</tr>
<tr>
<td>16F</td>
<td>$0.033 \pm 0.002$</td>
<td>$33 \pm 2.4$</td>
</tr>
<tr>
<td>17F</td>
<td>$0.21 \pm 0.01$</td>
<td>$44 \pm 4.4$</td>
</tr>
<tr>
<td>18F</td>
<td>$1.29 \pm 0.1$</td>
<td>$52 \pm 3.7$</td>
</tr>
<tr>
<td>19F</td>
<td>$3.51 \pm 0.3$</td>
<td>$44 \pm 4.4$</td>
</tr>
<tr>
<td>20F</td>
<td>$6.16 \pm 0.9$</td>
<td>$21 \pm 3.1$</td>
</tr>
<tr>
<td>21F</td>
<td>$7.47 \pm 1.1$</td>
<td>$12 \pm 2.1$</td>
</tr>
<tr>
<td>22F</td>
<td>$7.44 \pm 0.9$</td>
<td>$22 \pm 3.1$</td>
</tr>
<tr>
<td>1P</td>
<td>$7.55 \pm 1.3$</td>
<td>$23 \pm 3.3$</td>
</tr>
<tr>
<td>2P</td>
<td>$8.25 \pm 1.2$</td>
<td>$21 \pm 4.5$</td>
</tr>
<tr>
<td>3P</td>
<td>$13.2 \pm 2.5$</td>
<td>$19 \pm 3.9$</td>
</tr>
<tr>
<td>7P</td>
<td>$60 \pm 10$</td>
<td>$13 \pm 2.1$</td>
</tr>
<tr>
<td>AD</td>
<td>$850 \pm 47$</td>
<td>$10 \pm 2.3$</td>
</tr>
</tbody>
</table>

Note: Mean of six independent determinations ± SEM. Five to ten pooled thymuses before birth.
Abbreviations: F = fetal; P = postnatal; AD = adult.

FIGURE 1  Phenotype of the earliest thymocytes occurring in the rat thymic primordium. (a) Expression of different antigens (CD45, CD45R, CD53, and Thy 1) on 15 (solid profile) and 17- (line profile) day-old fetal rat thymocytes. (b) Thymocytes from 15-day-old fetal rats were triple-labeled for CD4 versus CD8 and CD3 cell markers.

that most thymocytes corresponded to CD4$^-$CD8$^-$CD3$^-$, triple-negative (TN) cells (Figure 1b), strongly expressing Thy 1 antigen (60-65%) (Figure 1a). In addition, the mAb OX22, which identifies high-molecular-weight isoforms of CD45, reacted with a significant population of thymocytes (20-25%) on fetal days 15-16 (Figure 1a). Because neither B cells nor mature thymocytes, known to express the OX22 epitope, were noticed in the rat thymus until fetal day 20 (data not shown), these early OX22$^+$ cells probably correspond to T-cell precursors.

In the following developmental stages, the percentage of both CD45$^+$ and CD43$^+$ cells remained constant, although the level of expression of both antigens varied (Figure 1a). On the contrary, the proportion of CD53$^+$ and OX22$^+$ cells decreased, whereas the frequency of Thy 1$^+$ cells remained unchanged on fetal days 17-18, although the proportion of the Thy 1$^+$ cells increased (Figure 1a). From
day 19 onwards, the pattern of Thy 1 expression was similar to that found in adult thymus, with most cells being Thy 1 hi.

**Interleukin-2 Receptor α Chain (IL-2Rα) Expression During Thymus Ontogeny**

The number of IL-2Rα chain (CD25)-positive cells reached maximal values in 16-day-old fetal thymus (Figure 2a), although the expression of this cell marker was already cytometrical and immunohisto- logically detectable on day 15 of embryonic life (Figures 2b and 2c). The proportion of IL-2R-positive cells gradually declined until day 20, peaking again in the perinatal period (Figure 2a).

On the other hand, the expression of CD25 and CD44 antigens allowed to define three major cell subpopulations in the 15- to 17-day-old fetal thymus: CD44^-/CD25-, CD44^+/CD25^-, CD44^+/CD25^- (Figure 2c). Furthermore, the CD25 expression on CD44^+CD25^- cells showed an important heterogeneity. The CD25^- cell subset predominating on days 15-16 rapidly diminished in parallel with the acquisition of the T-cell maturation associated molecules (Figure 2c). Moreover, 30-40% of total CD25^+ cells expressed neither Thy 1 nor CD2 antigens (Figure 2d).

**Acquisition of Cell Markers Associated with T-Cell Maturation**

From the first day studied (day 15), 20-30% of thymocytes expressed CD2 antigen. The numbers of CD2^+ cells rapidly increased (day 16: 60-70%) to

![Illustration](image-url)
reach adult values on days 17-18 (90-95%). On the contrary, the first CD5+ cells did not appear until day 18 (70-75%), increasing in the following days (80-85%), although at no stage studied was the number of CD5+ cells higher than that of CD2+ thymocytes.

Although the flow cytometry analysis did not reveal CD8+ thymocytes in the 16-day-old fetal thymus (Figure 3a), positive cells were able to be detected throughout the organ by immunohistochemistry (data not shown), demonstrating the cytoplasmic expression of this antigen. One day later, about 40-45% of total thymocytes already expressed a surface CD8 α chain, whereas a β chain was only detected in half of these cells (Figure 3b). In the following stages, most CD8+ cells (98%) expressed both α and β chains (data not shown). In addition, on fetal days 17-18, most immature CD4−CD8+ cells expressed the CD53 antigen (Figure 3c), and showed a high proliferative rate that accounted for the increased numbers of total cycling cells found in these developmental stages (Figure 3d). This immature CD4−CD8+ cell population preceded the appearance of CD4+CD8+ double-positive (DP) and mature CD4−CD8+/CD4+CD8− single-positive (SP) cells, which occurred on fetal days 18-19 and 20-21, respectively (Figure 3a).

Expression of TcRαβ on Thymocytes

The first TcRαβ-positive cells were immunohisto-logically identified scattered throughout the thymus on day 17, 1 day before (day 18) that TcRαβ surface expression was detected by flow cytometry (Table II).

FIGURE 3 (a) Evolution of rat thymocyte subpopulations defined according to CD4- and CD8-cell-marker expression. Thymocyte suspensions were prepared from embryonic, newborn, and adult thymuses, and analyzed for CD4 versus CD8 expression. Values represent the mean of over five independent tests. (b) CD8α and β-chain surface expression on the total thymocytes from 17-day-old embryonic rats. Note the existence of a numerically important CD8α+β− cell subpopulation. (c) Expression of CD8 versus CD53 on thymocytes from 18-day-old embryonic rats. Percentages of the different subpopulations are included in the right top corner. (d) Three-color flow cytometry analysis of the total thymocytes from 18-day-old embryonic rats. CD4 versus CD8 expression is represented in the dot plot. Percentage of the CD4−CD8+ gated cells in S and G2 + M phases of the cell cycle after propidium iodide staining.
Gradually, the percentage of TcRαβ/CD3hi cells increased with the first CD3/TcRαβhi cells appearing on days 19-20 (Table II). In the perinatal period, the proportion of TcRαβhi-expressing cells suddenly increased to further reach adult values by the beginning of the second week of postnatal life (Table II). This increase of mature thymocytes correlated well with a high incidence of dividing cells (25-30%) observed in the TcRαβhi-cell subset during the perinatal period. On the contrary, only 4-6% TcRαβhi cells proliferated in 2-week-old thymus. In addition, mature thymocytes quickly upregulated the expression of other surface antigens, including CD45R, CD43, CD53, CD5, and CD25 (data not shown).

A three-color flow cytometrical analysis was carried out to examine the distribution of TcRαβlo and TcRαβhi thymocytes among CD4- and CD8-defined subpopulations. In all stages studied, TcRαβlo cells largely corresponded to DP (50-60%) and CD4−CD8+ (30-45%) thymocytes. However, on fetal day 18, when the first TcRαβ+ cells were detected by flow cytometry, around 30-40% CD4+CD8− (DN) cells expressed low levels of T-cell receptor (Figure 4a). The proportion of DN TcRαβlo cells dropped drastically (2-7%) in the following days (Figure 4a). Furthermore, a DN-cell subset expressing intermediate levels of TcRαβ was identified from the second week of postnatal life, but not during the embryonic period (Figure 4a). On fetal days 19-20, TcRαβhi cells consisted of a significant proportion of DP (60-80%) and CD4+CD8− (20-30%) cells (Figure 4b). Only a few of these cells (12-15%) were, however, CD4+CD8− on day 20 (Figure 4b). In contrast, during the first week of postnatal life, the major percentage of TcRαβhi cells belonged to the CD4+CD8− cell subset (40-50%), as detected in the adult thymus (Figure 4b). Thus, 20-day-old fetal thymocytes showed a lower CD4:CD8 ratio (~0.43) than that achieved by thymocytes from 1-week-old neonatal rats (~1.5-3).

Further analysis of the TcRαβhi-cell subset using a limited panel of mAb specific to rat Vβ gene products, showed similar values for the different Vβ TcRαβhi cell subsets on fetal days 19-20 (Figure 5). In correlation with the increased numbers of total TcRαβhi-expressing cells that occurred around birth, there was an increase of TcRVβhi thymocytes, especially in the Vβ8.5- and Vβ8.2-cell subsets (Figure 5). Remarkably, in the following week of postnatal life, the proportion of both thymocyte subsets underwent a profound reduction, reaching adult values only on the second and third week, respectively. On the contrary, the Vβ10- and Vβ16-cell subpopulations remained practically unchanged from birth onwards, slightly increasing from the second week of postnatal life (Figure 5).

**Perinatal Increase of Immature Thymocytes**

Along with the high numbers of mature thymocytes occurring in the perinatal period, there was a transitional increase in both the absolute numbers and the proportion of CD4−CD8− cells (Figure 3a), which, unlike SP thymocytes, was not accompanied by an increased proliferative rate (always around 25-30% until the end of the first week of postnatal life). An increase in immature CD8+ thymocytes, as well as a notable reduction of DP and CD3/TcRαβhi cell subsets also occurred in this period (Figure 3a, Table II).
DISCUSSION

Rat thymus anlage is colonized by cell precursors around days 13-14 of gestation (Vicente et al., 1996). These cells largely expressed CD45, CD43, and CD53 antigens. A similar phenotype has recently been described in cell progenitors from early fetal rat liver (Crook and Hunt, 1996), suggesting that cells reaching the rat thymic rudiment could already express these cell markers. The expression of high-Mr isoform of the leukocyte common antigen (CD45) has been demonstrated in adult human (Deans et al., 1991), mouse (Goff et al., 1990), and rat (Law et al., 1989) DN cells, and precursor-cell activity largely reside in the CD45RA⁺ fraction of adult DN thymocytes (Law et al., 1989; Goff et al., 1990). On the contrary, the expression of high-Mr isoforms of CD45 on T-cell precursor during development is unclear. Whereas some authors (Law et al., 1989; Kampinga and Aspinall, 1990) were unable to identify OX22⁺ cells in the early rat thymic primordium, our flow cytometry analysis demonstrates, as in adult thymus, the presence of a CD45R⁺ (OX22-reacting cells) thymocyte subset on days 15-17 of gestation, which rapidly decreases in the following days in correspondence to T-cell maturation.

The arrival of cell progenitors into the rat thymic primordium (a homogeneous mass of primitive epithelial cells) results in the differentiation of several ultrastructurally distinct epithelial-cell types, the

FIGURE 4  (a) TcRαβ expression on DN rat thymocytes during fetal and postnatal development. DN cells were gated as indicated in the CD4 – CD8 dot plot. (b) Percentage of TcRαβ⁺ thymocytes either from 20-day-old embryonic rats or 3-day-old postnatal rats that express CD4 and/or CD8. TcRαβ⁺ was defined as indicated in the histogram. Two thousand TcRαβ⁺ gated events are shown.
expression on them of MHC class I and class II molecules, and the acquisition of first specific cortical- and medullary epithelial-cell markers (Vicente et al., 1996). Other authors have emphasized the relevance of cell-progenitor colonization for inducing growth and differentiation of the thymic epithelium (Haynes and Heinly, 1995). On the other hand, the interactions between developing epithelial cells and T-cell precursors presumably induce the proliferation of the latter, the appearance of a pre-TcR, the expression of CD8α and then CD8β chain, and, finally, the progression to the DP stage. Meanwhile, surface-cell markers strongly expressed on TN cells, such as CD43, CD53, CD45R, and CD25 are totally or partially lost.

The relevance of IL-2/IL-2R complex for T-cell maturation is a controversial issue. The IL-2Rα chain is detected in a high proportion of adult and fetal DN cells from humans, mice, and chickens (Ceredig et al., 1985; Toribio et al., 1989; Fedecka-Brunner et al., 1991; Kondo et al., 1993). However, previous works have failed to demonstrate IL-2Rα expression on adult and fetal rat DN thymocytes (Tackacs et al., 1988; Kampinga and Aspinall, 1990). On the contrary, and in agreement with our results, Brocke et al. (1987) immunohistochemically detected CD25+ cells in early embryonic rat thymus. These authors proposed, however, that they could be DP and/or mature SP cells, because the thymic lobes also expressed CD4 and CD8 molecules. In fact, the expression of these antigens at early stages of development is clearly cytoplasmic, as shown by our flow cytommetrical and immunohistochemical results, corresponding to late DN cells differentiating to the DP-cell compartment. Our data demonstrate, therefore, that the CD25 expression in rat follows an analogous kinetics and distribution to that described for embryonic mice (Ceredig et al., 1985) and chickens (Fedecka-Brunner et al., 1991), and indirectly suggest certain functional relevance of the IL-2/IL-2R complex during rat thymic ontogeny. In support, the addition of IL-2 or anti-CD25 mAbs to 16-day-old fetal rat thymus organ cultures stimulated or inhibited, respectively, the proliferation and differentiation of immature thymocytes (Varas et al., 1997b).

On the other hand, a significant proportion of early CD25+ thymocytes do not express either Thy 1 or CD2. As suggested by the current results and by those in mouse thymus (Mertsching and Ceredig, 1996), Thy 1−/CD2− progenitor cells colonize the thymic primordium and rapidly differentiate into a more mature Thy 1+ CD2+ cell population. Thus, this evidence is consistent with the idea of CD25 being expressed on T-cell precursors immediately after their arrival to the thymic primordium. Furthermore, the analysis of CD44 and CD25 expression on 15- to 17-day-old fetal rat thymus defined various CD4−CD8−CD3− triple-negative (TN) cell subpopulations, the sequence of maturation of the first cohort of fetal thymocytes being: CD44−CD25− → CD44+CD25− → CD44+CD25+. This developmental sequence has not been, to our knowledge, described in TN cells of adult rats and it is different from that reported in adult mice (Godfrey et al., 1993). This could suggest that fetal thymocytes may follow different kinetics of maturation than adult ones, as previously pointed out in fetal mice (Andjelic et al., 1993).

In mice, previous results have shown that TcRβ gene rearrangement is necessary for the progression from DN- to DP-cell compartment (Levelt and Eichmann, 1995). In agreement, using R.73 mAb to a constant determinant of the rat TcRαβ (Hünig et al., 1989b), we found an important proportion of R.73+
DN cells in early developmental stages of Wistar rat thymus, which decreases during ontogeny. According to the TcRaβ expression on this DN-cell subpopulation and since rat TcRα mRNA is only detectable from the onset of the CD4+CD8+ stage (Park and Hünig, 1995), we propose that the R.73+ DN-cell subpopulation could correspond to immature CD4+CD8− cells bearing a TcRβ chain, as also reported in adult and fetal immature mouse thymocytes (Groettrup et al., 1993; Wilson and MacDonald, 1995). Moreover, the mature DN-cell subset, which expresses intermediate levels of TcRαβ, does not appear in the embryonic thymus, being only detectable after birth (our own results, Fowlkes et al., 1987).

As in human thymus (Haynes and Heinly, 1995), CD8α chain is expressed earlier than CD8β chain in fetal rat thymus. In fact, rat CD4−CD8− cell subpopulations progress to the CD4+CD8− stage through an intermediate CD4−CD8+ cell that appears for the first time on day 17 of gestation. This cell subpopulation represents less than 2% of total thymocytes in adult rats (Paterson and Williams, 1987), whereas in fetal days 17-18 it, represents 40-50% of total thymic cells. Furthermore, in adult rats, immature CD4−CD8+ thymocytes can be distinguished from mature CD8+ cells by the lack of OX44 expression (Paterson and Williams, 1987). In contrast, in the fetal thymus, the loss of OX44 expression does not occur until the DP stage. The physiological relevance of this phenotypical difference is currently unknown. In addition, CD4−CD8+ cells show a high proliferative rate in any condition analyzed (our own results; Paterson and Williams, 1987; Penit and Vasseur, 1989) suggesting that this intermediate-cell subset significantly contributes to the exponential growth of early rat thymus.

Despite their high proliferative rate, CD4−CD8+ immature cells have an extremely limited lifespan, quickly progressing to DP cells, which, thus, on fetal days 19-20 constitute the major thymic cell subpopulation. Their appearance involves the fully maturation of thymic epithelial cells, the histological differentiation of a thymic cortex and the increase of both expression and number of MHC class I and class II positive cells (days 18-19) (Vicente et al., 1996). The upregulation of TcRaβ/CD3 complex in the DP cells and the strong expression of MHC molecules on both epithelial cells and dendritic cells (Vicente et al., 1994, 1996) allow the intrathymic thymocyte selection. Nonselected apoptotic cells are eliminated by thymic macrophages, which mature in the last days of fetal life (Vicente et al., 1995).

Positively selected CD4+CD8+ cells produce the first mature SP thymocytes that accumulate in the central area of the organ defining the thymic medulla around day 20 of gestation. The analysis of rat SP thymocytes in both perinatal and adult conditions showed important differences. In agreement with previous reports (Ceredig, 1990), we detected a high incidence of perinatal TcRaβ+ thymocytes in S + G2 + M phases, whereas adult mature thymocytes showed a low cycling activity. This increased intrathymic proliferation of mature thymocytes could play an important role in providing a sufficiently large number of T lymphocytes leaving the thymus to colonize the peripheral lymphoid organs during the perinatal period. Accordingly, in the neonatal mouse thymus, the proportion of thymic emigrants is higher than in the adult thymus (Weissman, 1967). Moreover, as demonstrated in mice (Modigliani et al., 1994), the low proliferative rate of peripheral T cells in the neonatal period implies that in those stages, the peripheral expansion of T lymphocytes is basically a consequence of the increased thymic-cell emigration. The nature of the signal(s) promoting the increased thymocyte proliferation in perinatal thymus is yet unknown. Ceredig and Waltzinger (1990) proposed the involvement of some cytokines in the process. In agreement, we have recently demonstrated that in rat fetal thymus organ cultures (FTOC) supplemented with either IL-2 or IL-7 for 24 hr at different times of culture, the highest responses of mature SP thymocytes occur in those days of culture equivalent to the in vivo perinatal stage (Varas et al., 1997a, 1997b).

In correlation with the beginning of thymocyte emigration to the periphery, a new wave of T-cell maturation occurs in the perinatal rat thymus, a fact previously described in mice (Penit and Vasseur, 1989). Since the percentage of DNA synthetizing DN
cells remained constant throughout ontogeny (our own results, Penit and Vasseur, 1989), and the proportion of early immature thymocytes increases around birth, a new wave of precursor cells could be colonizing the rat thymus during the perinatal period. Remarkably, at that time, we demonstrated enlarged perivascular spaces throughout the thymic parenchyma, implying an increased thymic vascular permeability (Vicente et al., 1995), which could favor the emigration of mature thymocytes as well as the arrival of new cell precursors. Periodic renewal of the intrathymic progenitor-cell subpopulation has been previously described during embryonic life in both mice and birds (Jotereau et al., 1987). Most probably, a dynamic equilibrium between homing-cell precursors and emerging mature cells occurs (Zuñiga-Pflücker and Lenardo, 1996).

Other events that occur around birth in rat thymus could be related to the proliferative activity of perinatal thymocytes. Our results demonstrate that the percentage of repertoire covered in adult thymus (24-28%) was reached in the postnatal period, whereas only 17-19% of repertoire was covered in 20-day-old embryos. Remarkably, there is a preferential expansion of specific TcRβ-cell subpopulations during the perinatal period. Thus, between days 20-21 of gestation, the numbers of total TcRαβ<sup>hi</sup> thymocytes increased 3.6 times, similar to V<sub>B10</sub>TcR<sub>hi</sub> cells (3.3 times), and V<sub>B8.5</sub>TcR<sub>hi</sub> thymocytes showed the greatest expansion (7.5 times). V<sub>B8.2</sub> and V<sub>B16</sub>TcR<sub>hi</sub>-cell subsets increased in a lesser extent (6.1 and 5.8 times, respectively). Presumably, the distinct expansion of these mature T-cell subsets is correlated with differential responses to cytokines, for which they express specific receptors, as previously pointed out in humans (He and Kabelitz, 1993). This selective response has been recently confirmed by us in either IL-2- or IL-7-treated rat FTOC (Varas et al., 1997a, 1997b).

On the other hand, as previously reported (Adkins, 1991), during fetal development, fewer mature CD4<sup>+</sup> than CD8<sup>+</sup> T cells are produced. In contrast, in both postnatal and adult thymus, the frequency of CD4<sup>+</sup> cells is higher. Adkins and Hamilton (1994) explained that the proportions of CD4<sup>+</sup> cells would be regulated by a combination of the developmental ages of the T-cell precursors and the thymic stromal environment. Accordingly, during ontogeny, a unique communication system could operate between fetal thymic stroma and fetal progenitors, which would generate the decreased CD4/CD8 ratio. Recently, Shortman and Wu (1996) have speculated that differential production of CD4<sup>+</sup> and CD8<sup>+</sup> T cells could reflect the presence or absence of TdT in adult or fetal precursor cells, respectively. Accordingly, adult TdT<sup>+</sup> progenitors could lead to more MHC class II-restricted receptors appropriate for CD4<sup>+</sup> lymphocytes. We propose, however, that initially, the transition from fetal to adult CD4/CD8 ratios could reflect a higher proliferative rate of CD4<sup>+</sup> thymocyte subpopulation, compared to mature CD8<sup>+</sup> cells, since the typically decreased CD4/CD8 ratio at days 20-21 of gestation changes as soon as by 1 day of postnatal life.

**MATERIALS AND METHODS**

**Animals**

Wistar rat thymus glands were sampled from day 15 of fetal life to 2 weeks after birth. Adult animals were also included in the study.

**Antibodies**

Reagents included either unconjugated, FITC- or PE-conjugated anti-CD45 (OX-1), anti-CD45R (OX-22), anti-CD43 (W3/13), anti-Thyl (OX-7), anti-CD53 (OX44), anti-CD4 (44-2.3), anti-CD5 (OX-19), anti-CD3 (G4.18), and anti-TcR<sub>b</sub> (R.73) (all from Pharmingen, San Diego, California). A panel of mAbs to rat TcR V<sub>B8.2</sub> (R.78), V<sub>B8.5</sub> (B73), V<sub>B10</sub> (G101), and V<sub>B16</sub> (His 42) gene products was kindly provided by Dr. T. Hünig (Würzburg University, Würzburg, Germany) and Dr. J. Kampinga (Groningen University, Groningen, The Netherlands).

**Cell Suspensions**

Thymi were aseptically removed from adults, neonates and 15- to 22-day-old fetuses, these latter using...
a stereoscopic microscope. Single-cell suspensions of thymocytes were prepared by pressing disrupted thymic lobes through a steel sieve and maintained on ice in PBS with 1% FCS before use. Cell viability was assessed by using Tripan-blue and cell number determined in duplicate using a hemocytometer.

**Cell-Surface Staining**

Phenotypic analysis of thymocytes recovered from different stages was performed as follows: $1 \times 10^5$ cells were incubated with saturating amounts of antibodies for 20 min at 4°C. Two-color immunofluorescence labeling was done by incubating with a mixture of FITC- and PE-conjugated mAbs. For three-color analysis, cells were sequentially exposed to (i) unconjugated anti-TcRαβ or anti-TcRVβ monoclonal antibodies, (ii) tricolor-conjugated F(ab’)2 fragment of goat anti-mouse IgG, (iii) normal mouse serum (1:100), and (iv) anti-CD8-FITC and anti-CD4-PE mAbs. Background fluorescence was determined using an isotype-matched control mAb. Stained cells were analyzed using a FACSscan (Becton Dickinson, San José, California). Dead cells were excluded from data acquisition on the basis of forward/side scatter and in two-color stainings, by staining with propidium iodide. The data were analyzed using PC-LYSIS software (Becton Dickinson).

**Cell-Cycle Analysis**

Cell-cycle analysis was carried out by using $1 \times 10^6$ cells fixed for 7 min in 70% ethanol at $-20^\circ$C, washed in Tris-HCl buffer to pH 6.0, incubated with a RNAse dilution (1mg/ml Tris-HCl) for 30 min at 37°C, washed in Tris-HCl buffer and resuspended in a solution of 0.05 mg/ml propidium iodide in PBS. In some cases, in order to assess the proliferative rate of various thymocyte subpopulations throughout ontogeny, cells were firstly stained with FITC-TcRαβ or FITC-CD8 and PE-CD4 according to the previously described protocol. Analysis was carried out in a FACSscan, using Cell Fit software (Becton Dickinson).

**Immunohistochemistry**

Thymic lobes were snap frozen in liquid nitrogen and stored at $-80^\circ$C until use. Five-micrometer-thick cryosections were fixed for 10 min in acetone and then incubated with different primary mAbs. Endogenous peroxidase was blocked with 1% H$_2$O$_2$ in methanol for 15 min. After washing in PBS, the histological sections were incubated with 1:40 solution of peroxidase-conjugated rabbit anti-mouse Ig in PBS (Dakopatts, Glostrup, Denmark) supplemented with 1:100 normal rat serum. After washing, the peroxidase reaction was developed with 0.05% 3,3’ dianinobenzidine (Sigma, St. Louis, MO) in PBS with 0.1% H$_2$O$_2$ for 10 min. Sections were counterstained with methylene blue. Preparations incubated without primary antibodies were used as negative controls.

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