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We studied the kinetics of maturation of B cell progenitors in the mouse embryo, from day 15 of development to birth, both in liver and bone marrow. The analysis of Ig heavy chain rearrangements at different time points of late fetal development shows that oligoclonal patterns of $V_{H}$-$D$-$J_{H}$ rearrangements are detected by day 15 in fetal liver. The pattern is polyclonal and diverse by day 17; however, 80% of the rearrangements are nonproductive. In bone marrow, the pattern of rearrangements is less diverse at birth, although the percentage of nonproductive rearrangements approaches adult bone marrow levels (35–40%). After day 17 in fetal liver, there is a sudden reversal in the percentage of nonproductive rearrangements that reaches 33% at day 19 (birth). Maturation of B cells, as measured by the fraction of surface Ig$^{+}$ in total B220$^{+}$ cells and the presence of N sequence additions in $V_{H}$-$D$-$J_{H}$ joints, occurs in the marrow before fetal liver. These results demonstrate that the lymphopoietic environment in fetal liver and bone marrow of animals at the same stage of development is functionally distinct. The Journal of Immunology, 1998, 160: 3274–3280.

B cell development occurs in the fetal liver during embryonic development and is subsequently maintained in the bone marrow of adult animals. In late mouse embryos, fetal liver and bone marrow coexist as major lymphopoietic organs. Ig gene rearrangement is an essential step in this process and has been extensively characterized using A-MuLV-transformed cell lines (1, 2) as well as in adult bone marrow (3, 4). During differentiation in the bone marrow, B cell precursors undergo $D$-$J_{H}$ and subsequently $V_{H}$-$D$-$J_{H}$ rearrangement. At this stage, the Ig heavy chain is expressed together with the surrogate light chains. Hematopoiesis can be detected in fetal liver as early as day 11 (5, 6), and proceeds until the first week after birth, while in the bone marrow, it becomes detectable around day 17 of gestation (7, 8) and continues in adult life.

We analyzed the kinetics of B cell differentiation in both locations, from embryos at day 15 of gestation to birth, by flow cytometry and PCR analysis of the Ig heavy chain rearrangements followed by cloning and sequencing the complementarity determining region 3 (CDR3) region. We show that the first B lymphocytes (surface Ig$^{+}$ (sIg$^{+}$)) are detectable in fetal liver and bone marrow at day 17 of development. B cells represent a higher proportion of the B220$^{+}$ compartment in bone marrow than in fetal liver. The analysis of heavy chain gene rearrangements from fetal liver cells shows a sudden shift from 80% of nonproductive rearrangements at day 17 of gestation to 30% at day 19 (birth time). Rearrangements isolated from bone marrow exhibit a higher proportion of N sequence additions when compared with those isolated from fetal liver. The accelerated maturation of B cells in bone marrow together with the intrinsic differences in the rearrangements observed in both sites allow us to propose that liver and marrow environment provide different selective constraints on B cell differentiation.

Materials and Methods

Animals and cell suspension preparations

C57BL/6 mice were purchased from Iffa-Credo (L’Arbresle, France). Experiments were performed with animals bred in specific pathogen-free barrier-free conditions in the Pasteur Institute’s animal facilities. Timed pregnancies were obtained by mating mice over 1 night. The following day was considered to be day 0 of pregnancy. Newborn refers to the day of birth (day 19). For cell suspension preparations, the uterus from pregnant female mice was isolated and extensively washed in medium. Embryos were then isolated and the absence of contaminating blood from the mother ascertained by the absence of sIg$^{+}$ cells, as detected by FACS staining at day 15, both in bone marrow and fetal liver. Direct LPS stimulation of fetal liver and bone marrow preparations at days 14 and 15 also shows the absence of detectable mature B lymphocytes. Cell suspensions were obtained as previously described (8).

Fluorescence surface staining and flow cytometry

Single cell suspensions were prepared from various organs and viable cells were determined using trypan blue exclusion. About 10$^5$ living cells per sample were stained as described (9) with phycoerythrin anti-B220 (PharMingen, San Diego, CA) and FITC anti-μ (Jackson ImmunoResearch, West Grove, PA). Dead cells were eliminated by propidium iodide exclusion and at least 5,000 events recorded per sample. Fluorescence was measured with FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) using Cell Quest software.

CDR3 length analysis with immunoscope

The repertoire analysis of Ig heavy chain has been described in detail elsewhere (10). In brief, PCR amplifications were performed with a sense primer specific for the J558 family and an antisense primer specific for the intron region 3’ of $J_{H}$ for DNA amplification (11). A run-off elongation with a fluorescent primer specific for $J_{H}$ was then performed on the amplification product, and the fluorescent PCR fragments covering the CDR3 region were separated on a sequencing gel run in an automated DNA sequencer (Applied Biosystems, Foster City, CA). Using software, the bands detected on the gel were converted into peaks whose length as well as intensity (in arbitrary units) were measured with FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) using Cell Quest software.

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4 Abbreviations used in this paper: CDR3, complementarity determining region 3; RF, reading frame; TdT, terminal deoxynucleotidyl transferase; sIg, surface Ig; N, nongermline-encoded.
are known. Each peak corresponds to one band and represents the multiple rearrangements with a particular CDR3 length. The pattern seen with any diverse population of cells (see adult bone marrow in Fig. 1) is that of a normal size distribution represented by the gaussian-shaped curve. Equivalent amounts of DNA were used in all experiments.

**Cloning and sequencing**

J558-JH4 amplification products were purified by electroelution, cloned into pCRII using the TA cloning kit (Invitrogen, San Diego, CA), and recombinant clones were sequenced using the T7 sequencing kit (Pharmacia, Uppsala, Sweden).

**FIGURE 1.** Flow cytometry analysis of fetal and newborn liver and marrow cells, and of adult bone marrow. Cells were stained with phycoerythrin anti-B220 (FL2) and FITC anti-\(\mu\) (FL1) and analyzed in a lymphocyte gate defined on FFC-SSC. Dead cells were eliminated by propidium iodide exclusion. The percentages of the B220\(^+\)\(\mu^-\) and B220\(^+\)\(\mu^+\) populations are indicated above each population, while the percentage of \(\mu^-\) cells in the B220\(^+\) population is indicated in the right of each plot. The percentage of cells in the B220\(^+\) compartment is indicated.
To avoid bias due to cloning, repeated sequences were not considered.

**Results**

**Analysis of B cell progenitors by flow cytometry**

The first major population of B cell progenitors that can be identified by phenotype corresponds to the pro- and pre-B lymphocytes, characterized by the expression of B220, the B cell-specific splice variant of the surface marker CD45. After successful rearrangement of both Ig chains, a complete IgM molecule can be produced and expressed on the cell surface. The B220\(^+\)\(\mu^+\) population thus encompasses the immature and mature B lymphocytes. To study the progression of the different maturation stages during the last part of fetal development, liver and bone marrow cells from day 15 to 18 embryos as well as from neonates (day 19) were analyzed by flow cytometry for the expression of B220 and surface \(\mu\) heavy chain. By flushing the four limbs, we collected about 2 \(\times\) 10\(^6\) cells in day 17 or 18 embryos, and 10 \(\times\) 10\(^6\) cells in neonates. For each stage of development, at least four embryos were studied individually. Variations in the percentages of B220\(^+\)\(\mu^+\) and B220\(^+\)\(\mu^-\) populations between individuals lead to a maximum SD of 20% in the ratio of these two populations. While day 15 fetal livers contained about 0.2% of B220\(^+\)\(\mu^-\) cells in all the studied embryos, the first sIg-bearing B lymphocytes were detectable in the liver at day 17, representing 0.4% of the B220\(^+\) population in the liver (Fig. 1). This result correlates with previous observations on fetal liver cells from BALB/c mice (12). Surprisingly, the B lymphocytes already represented 3% of the B220\(^+\) population in the marrow of day 17 embryos, suggesting that maturation of B lymphocytes in marrow precedes maturation in liver. Thereafter, the B cell compartment (B220\(^+\)\(\mu^-\)) developed rapidly, representing 2.6% of the B220\(^+\) population in liver cells from day 18 embryos and 6.8% in the marrow, and around 10% of the B220\(^+\) population in these two organs at day 19 (Fig. 1). In an adult bone marrow, the \(\mu^+\) population represents 27% of the B220\(^+\) cells, but it has been estimated that half of this B220\(^+\)\(\mu^+\) population corresponds to recirculating mature B lymphocytes (13). The ratio between pre-B and B cells is thus already close to the adult ratio in the newborn lymphoid organs.

**Ontogenic analysis of the Ig heavy chain rearrangements**

**Profiles of CDR3 length diversity.** The first DJ\(_H\) rearrangements in the fetal liver are detected around day 12, while the number of V\(_H\)DJ\(_H\) rearrangements increases mainly from day 15 on (11). We used the immunoscope technique to study the developing repertoire of Ig heavy chain rearrangements in fetal liver from day 15 embryos to newborns (10). In the mouse, several hundred V\(_H\) gene segments have been grouped in 13 families based on DNA sequence homologies (14–18). The J558 family encompasses about 100 genes and is the most represented in the adult mouse. In C57BL/6 mice, they represent 50% of the adult repertoire and are also the most representative family in fetal liver B cell precursors (19–21).

We have compared the profiles obtained after amplification of genomic DNA from fetal liver day 15 embryos to newborn mice. Amplification was performed using a primer specific for the J558 family with a primer specific for the sequences 3′ of J\(_{H4}\) (11), and run-off was done using a fluorescent primer specific for J\(_{H4}\). We performed the same experiments with the VHQ52 primer with similar results (data not shown). The study of genomic DNA allowed us to estimate the ratio of productive vs nonproductive rearrangements, allowing the distinction between intrinsic genetic events and cellular selection. Figure 2 shows a representative amplification of liver cells (day 17 to 19) compared with amplifications of neonate and adult bone marrow. Each peak of these profiles corresponds to a single band on a 1-bp discriminating polyacrylamide gel. Thus, these bands are able to correspond to one or several rearrangements sharing the same V\(_{H}\), the same J\(_{H}\), and the same CDR3 length. It has been shown that the relative intensities of the different peaks reflect the representation of the corresponding CDR3 length in the population before amplification (22, 23).

Random recombination of the different segments can lead to an Ig heavy chain in three different reading frames (RFs), of which only one is productive. Amplification from adult bone marrow DNA provides regular profiles where each peak is separated from the following by 3 bp (Fig. 2). It has been shown that, due to selection for functional heavy chains, 80% of V\(_{H}\)DJ\(_{H}\) rearrangements from adult bone marrow are productive (24, 25). The nonproductive rearrangements should be equally distributed between the three RFs, and thus the two out-of-frame peaks should represent less than 10% of the productive rearrangements, which has been shown to be the detection level in our technique (22, 23). On the contrary, the profiles obtained from day 17 fetal liver exhibit...
peaks separated by 1 bp (Fig. 2), reflecting rearrangements occurring in the absence of selection for productive rearrangements. The emergence of one band of three between day 17 and newborn corresponds to the increasing representation of in-frame rearrangements, and indicates that the ratio between productive and non-productive rearrangements increases rapidly from day 17 to birth (day 19). The irregular profiles obtained with newborn bone marrow indicate a less diverse pattern of rearrangements compared with fetal liver at the same age.

It has been shown that the insertion of nongermline-encoded (N) nucleotides at the junction of different gene segments is rare early in ontogeny due to the low activity of the terminal deoxyribonucleotide transferase (TdT) (26–30). When the amplification profiles obtained from fetal liver are compared with the profiles obtained from adult bone marrow, we can observe a shift of the profile to larger sizes. This observations indicates that the TdT activity in the adult increases the mean length of the CDR3 by three amino acids.

**Sequence analysis of heavy chain rearrangements expressing J558 and JH4.** To further analyze the heavy chain rearrangements, we cloned and sequenced J558-JH4 rearrangements from fetal liver at day 17, 18, and newborn and from newborn bone marrow. A summary of the numbers of sequences obtained and their characteristics is shown in Table I.

In accordance with the amplification profiles (Fig. 2), the percentage of nonproductive rearrangements decreases very rapidly in the fetal liver from day 17 to birth. Nonproductive rearrangements represent 81% at day 17, 54% at day 18, and 33% in the newborn. This result correlates with the rapid increase of the μ− cells in the B220+ population during the same period (Fig. 1). Sequences from the bone marrow of the same neonates contain 45% of nonproductive rearrangements. The difference in the percentage in fetal liver could be due either to different kinetics of maturation (in that case, it would indicate a delay in the bone marrow compared with fetal liver), or to differences in the proliferation of B cell progenitors with a productive rearrangement. The representation of the different D segments does not vary during these stages of active selection, and the overusage of RF1 and counterselection of RF2 can already be observed in the day 17 sequences (data not shown).

Figure 3 shows the CDR3 sequences obtained from newborns, liver, and bone marrow. Short homologies at or near coding sequence breakpoints have been proposed to mediate VμDJμ rearrangement and lead to the appearance of certain variable regions at increased frequency in fetal repertoires (27, 29, 31). This type of junction was found in 16 independent sequences from all the amplified samples. Interestingly, sequences from newborn bone marrow contained more N additions than sequences from newborn liver, both at the level of percentage of sequences with N additions (13% in liver and 30% in bone marrow), and of number of added nucleotides (an average of one per liver sequence and four per bone marrow sequence).

Figure 4 shows the total number of B220+ and sIg+ cells recovered from bone marrow and fetal liver in the last 3 days of gestation. The values for bone marrow represent cells recovered from the two hind limbs. The graph shows the progression of cells produced in both locations and the reduced contribution of bone marrow-derived cells for the total B cell compartment of newborn mice. It is interesting to notice that in bone marrow, B cell production is increased 10-fold from day 18 to 19. The low numbers of B220+ cells in bone marrow cell suspensions could explain the unrepeatable profiles of rearrangements obtained in Immunoscope analysis with bone marrow samples before birth (data not shown).

Taken together, these results show that 1) although the first VμDJμ rearrangements can be detected in fetal liver by days 14 to 15 of gestation, most rearrangements detected until birth are non-productive; 2) a rapid shift from cells expressing nonproductive to productive rearrangements is observed around birth; and 3) newborn bone marrow cells contain more N sequence additions than the newborn liver cells.

**Discussion**

Newly generated B cells express a primary repertoire that may reflect constraints of the Ig gene assembly process, and presumably of selection (32). To understand the mechanisms of establishment of the humoral immune system, we studied the ontogeny of Ig heavy chain as well as the kinetics of generation of mature B cells during late embryonic development.

The CDR3 of the mature Ig heavy chain region encompasses the D segment together with its junctional sequences to Vμ and Jμ and is thus diverse in size and sequence. This region has the highest variability in the Ig molecule (33), contributing to a large extent to the diversity found in the primary Ig repertoire in the mouse. Our study of the J558-JH4 CDR3 regions at different days of development corresponding to the detection of sIgM provides an insight into the development and selection processes of B cells in the neonates.

VμDJμ rearrangements are detectable in day 15 fetal liver, and their number increases rapidly thereafter (11). This is in accordance with the profiles we obtained using immunoScope, where day 15 fetal liver gave rise to sporadic and weak bands corresponding to J558-JH4 rearrangements on ImmunoScope profiles (data not shown), while day 17 fetal liver showed a normal distribution encompassing a large diversity of CDR3 sizes (Fig. 2). However, day 17 fetal liver contains 80% of nonproductive rearrangements. Within 2 days, this percentage drops drastically to 30% at birth, in correlation with the increasing percentage of B220−μ− cells. In a random set of VμDJμ rearrangements, around 70% are expected to be nonproductive (34). However, due to selection of cells bearing functional heavy chains, VμDJμ rearrangements from sIg-negative pre-B cells from the bone marrow are

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**Table I. Characteristics of J558-JH4 rearrangements at different time points of late embryonic development**

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of seq.</th>
<th>NP (%)</th>
<th>DFL (%)</th>
<th>Dsp (%)</th>
<th>DQ52 (%)</th>
<th>Mean Length of CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 17 fetal liver</td>
<td>42</td>
<td>34 (81)</td>
<td>9 (21)</td>
<td>26 (62)</td>
<td>4 (9)</td>
<td>35</td>
</tr>
<tr>
<td>Day 18 fetal liver</td>
<td>37</td>
<td>20 (54)</td>
<td>11 (30)</td>
<td>22 (59)</td>
<td>1 (3)</td>
<td>36</td>
</tr>
<tr>
<td>Newborn fetal liver</td>
<td>46</td>
<td>15 (33)</td>
<td>15 (33)</td>
<td>26 (56)</td>
<td>1 (2)</td>
<td>35</td>
</tr>
<tr>
<td>Newborn bone marrow</td>
<td>53</td>
<td>24 (45)</td>
<td>15 (28)</td>
<td>28 (53)</td>
<td>2 (4)</td>
<td>37</td>
</tr>
</tbody>
</table>

* Number of sequences: redundant sequences from the same embryo were not taken into account for the percentages.

* NP: nonproductive rearrangements.

* CDR3 length is given in amino acids according to Kabat et al. (32).
FIGURE 3. J558-J_{	ext{i}}4 sequences from newborn liver and bone marrow. The sample indicates the individual mouse from which the sequence was cloned.
FIGURE 4. Total numbers of B220<sup>+</sup> (■) and slg<sup>+</sup> (□, ●) cells found in bone marrow (○) and fetal liver (●) from day 17 of gestation until birth (day 19). The number of cells was calculated from the percentage of positive cells calculated by flow cytometry by the total number of nucleated cells recovered from each organ.

Generally around 80% productive (24, 25). A similar analysis done in a light chain rearrangements in fetal liver shows a shift of 33 to 46% of productive rearrangements from day 14 to 16 of gestation. The total number of κ-chain productive rearrangements at day 16 is around 2 x 10<sup>4</sup>, which could correspond to the actual number of B cells found as slg<sup>+</sup> by day 17 (35).

Our results, showing that a diverse repertoire is generated in fetal liver before any detectable selection for cells that undergo productive rearrangements, can be explained in two ways. First, we could envisage that before day 17 of gestation, the putative ligand(s) driving expansion of cells expressing heavy chain with the surrogate light chains is not present (36). This would thus be an absence of positive selection. Second, cells that undergo nonproductive rearrangements would not be eliminated from fetal liver until around birth. This absence of negative selection could account for the striking expansion of B cell precursors between days 12 and 16 of gestation. The third possibility is that most cells are synchronized in their development. Recently, the generation of hematopoietic progenitors in early embryos has been studied carefully. It was shown that hematopoietic progenitors originate in the paraaortic splanchnopleura/aorta-gonad-mesonephros regions (37–39). By day 10 of development, circulating hematopoietic progenitors can be detected in embryonic circulation, reaching a maximum by day 12 (8), and day 11 fetal liver cells have been shown to be capable of long-term reconstitution of irradiated recipients (39). If we assume that the colonization of fetal liver by stem cells is not an ongoing process but occurs predominantly between days 11 and 12, the results presented here suggest that the differentiation and selection processes between pre-B to B cells in the fetal liver are not progressive but occur in one synchronous wave. Similar conclusions were drawn from studies on the transition from dependence to independence of fetal liver B cell precursors on stromal cells (40). If correct, this hypothesis implies that a stem cell requires 7 days to differentiate into mature B cells in the fetal liver environment. While there is no direct evidence for the existence of a positively selecting ligand for pre-B cells in bone marrow and fetal liver, our third hypothesis seems more plausible. The observations that from day 15 to 17, the diversity of rearrangements and, later, the percentage of productive rearrangements and slg<sup>+</sup> B cells increase drastically is an argument in favor of a certain degree of synchronization in B cell precursors in fetal liver.

Day 17 fetal liver rearrangements are highly diverse when the sizes of CDR3 are probed (Fig. 2). Yet, day 17 fetal liver comprises 80% of nonproductive rearrangements, compared with 33% in newborn liver. In addition, we found a compelling difference in the presence of N additions between liver and bone marrow at the same age (newborn). As N additions reflect activation of the TdT, the observed differences could be a consequence of a difference in the expression of TdT between newborn liver and newborn bone marrow, or of a difference of kinetics of differentiation of a stem cell into mature B cells in both embryonic environments. This second possibility correlates with the more rapid appearance of μ<sup>-</sup> cells in the B220<sup>−</sup> population in bone marrow when compared with liver. The B220<sup>−</sup> μ<sup>-</sup> and B220<sup>−</sup> μ<sup>-</sup> compartments do not exhibit the same kinetics in fetal liver and bone marrow between days 17 and 19. It appears that while the B220<sup>−</sup> μ<sup>-</sup> cells proliferate extensively in liver, maturation to slg<sup>+</sup> cells is favored in the bone marrow in the absence of comparable expansion of the B220<sup>−</sup> μ<sup>-</sup> compartment. Thus, our results suggest that liver and marrow impose different environmental constraints in the differentiating B cell progenitors.

The observed differences in the maturation of B cells in the liver compared with bone marrow do not significantly affect the peripheral pool of cells in newborn animals. At this stage, more than 90% of the mature B cells are of liver origin. Bone marrow production of B cells becomes predominant after the second week of age.

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References


