B Lymphocyte Development in Rabbit: Progenitor B Cells and Waning of B Lymphopoiesis

Paul J. Jasper, Shi-Kang Zhai, Susan L. Kalis, Mae Kingzette and Katherine L. Knight

*J Immunol* 2003; 171:6372-6380; doi: 10.4049/jimmunol.171.12.6372
http://www.jimmunol.org/content/171/12/6372

**References**
This article cites 51 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/171/12/6372.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
B Lymphocyte Development in Rabbit: Progenitor B Cells and Waning of B Lymphopoiesis

Paul J. Jasper, Shi-Kang Zhai, Susan L. Kalis, Mae Kingzette, and Katherine L. Knight

In mammals that use gut-associated lymphoid tissues for expansion and somatic diversification of the B cell repertoire, B lymphopoiesis occurs early in ontogeny and does not appear to continue throughout life. In these species, including sheep, rabbit, and cattle, little is known about the pathway of B cell development and the time at which B lymphopoiesis wanes. We examined rabbit bone marrow by immunofluorescence with anti-CD79a and anti-μ and identified both proB and preB cells. The proB cells represent the vast majority of B-lineage cells in the bone marrow at birth and by incorporation of 5-bromo-2’-deoxyuridine, they appear to be a dynamic population. PreB cells reach maximum levels in the bone marrow at 3 wk of age, and B cells begin to accumulate at 7 wk of age. We cloned two VpreB and one Λ5 gene and demonstrated that they are expressed within B-lineage cells in bone marrow. VpreB and Λ5 coimmunoprecipitated with the μ-chain in lysates of 293T cells transfected with VpreB, Λ5, and μ, indicating that VpreB, Λ5, and μ-chains associate in a preB cell receptor-like complex. By 16 wk of age, essentially no proB or preB cells are found in bone marrow and by PCR amplification, B cell recombination excision circles were reduced 200-fold. By 18 mo of age, B cell recombination excision circles were reduced 500- to 1000-fold. We suggest that B cell development in the rabbit occurs primarily through the classical, or ordered, pathway, and show that B lymphopoiesis is reduced over 99% by 16 wk of age. The Journal of Immunology, 2003, 171: 6372–6380.
which cross-reacts with rabbit CD79a (BD Pharmingen clone HM47; San Diego, CA); PE mouse anti-rabbit MHC class II (clone 2C4) (25); FITC goat Fab anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove PA); streptavidin-PE (BD Pharmingen); and streptavidin–allophycocyanin (BD Pharmingen); FITC mouse anti-5-bromo-2′-deoxyuridine (BrdU) (eBioscience, San Diego CA).

**Immunofluorescence.** To detect proB and preB cells, bone marrow cells (1 × 10⁴) were fixed and permeabilized with Cytofix/Cytoperm (Promega, Madison, WI) and washed with Perm/Wash Buffer (Promega). Cells were stained with cross-reactive mouse anti-human CD79a/FITC goat anti-mouse IgG and biotinylated mouse anti-rabbit μ-chain/streptavidin-PE. Stained cells were analyzed at the FACS Core Facility (Loyola University Chicago Medical Center, Mayfield, IL) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). To distinguish surface μ⁺ and surface μ⁻ B-lineage cells, cells were first stained with mouse anti-rabbit μ-chain/FITC goat Fab anti-mouse IgG. Subsequently, cells were fixed and permeablized and then stained with biotinylated mouse anti-rabbit μ-chain/streptavidin-PE. Three-color immunofluorescent staining was performed first with mouse anti-rabbit μ-chain/FITC goat Fab anti-mouse IgG. Cells were then fixed and permeablized and stained with biotinylated mouse anti-human CD79a.

All flow cytometric data were gated for lymphocyte-sized cells on the basis of forward and side scatter, and then analyzed using CellQuest software (BD Biosciences). ProB cell pools were defined as CD79a⁺/μ⁺/H9260; preB cell pools were identified as CD79a⁺/μ⁻/H9262; and γδ T-lineage cells were defined as μ⁻/μ⁻/H11003. 293T cells followed by Western blot analysis of lysates (1 μg of Western blot protein per lane) were probed with a mouse anti-human CD79a/FITC goat Fab antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as a control and the following monoclonal antibodies were used: mouse anti-rabbit μ-chain/streptavidin-allophycocyanin, and mouse anti-rabbit μ-chain/streptavidin-PE. Three-color immunofluorescent staining was performed first with mouse anti-rabbit μ-chain/FITC goat Fab anti-mouse IgG. Cells were then fixed and permeablized and stained with biotinylated mouse anti-human CD79a.

**Detection of DJ, VDJ, and VJ gene rearrangements by PCR**

Pools of 50 cells were sorted into 96-well V-bottom plates containing 1 × 10⁴ bone marrow cells (600 ng of total genomic DNA) were amplified with these primer sets is DJ, VDJ, and VJ gene rearrangements. Pools of 50 cells were sorted into 96-well V-bottom plates containing 1 × 10⁴ bone marrow (100 μg/ml proteinase K in 1× PCR buffer (Promega)) using a FACS-Star Plus (BD Biosciences) cell sorter. Cells were lysed at 37°C for 30 min and incubated at 97°C for 10 min to inactivate the protease K. The PCR mixture (200 μM dNTP, 100 μM each primer; 1× PCR buffer) was added to the samples, with 2.5 U Jumpstart Taq polymerase (Sigma-Aldrich, St. Louis, MO): the PCR was performed for 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. Aliquots (2 μl) of the first-round PCR products were transferred to 48 μl of the second-round PCR mixture (containing nested primers), and 40 additional PCR cycles were performed under the same conditions. PCR products were analyzed by PAGE. Primers used in nested PCR amplification of DJ, VDJ, and VJκ gene rearrangements are illustrated in Fig. 1.

**Competitive PCR amplification for quantitation of BREC s**

VD recombinant signal sequence excision circles were quantified by competitive PCR using primers shown in Fig. 1. Briefly, BREC s from 2 × 10⁴ bone marrow cells (600 ng of total genomic DNA) were amplified with [³²P]dCTP with varying numbers of copies of a competitor plasmid, which results in a larger PCR product than the BREC target using the same primers. To quantitate BREC s, the log of the number of copies of competitor plasmid in each reaction was plotted against the log of the ratio of cpm incorporated into target and competitor PCR products. To enhance the sensitivity of BREC detection in some experiments, we used circular DNA isolated from bone marrow (as a template for PCR instead of total genomic DNA, and we added [³²P]dCTP (0.5 μCi) to the second round of PCR amplification. To isolate circular DNA, bone marrow cells were lysed in 1% alkaline-SDS (pH 12.45) and the chromosomal DNA was sheared by vortexing, denatured in the alkaline-SDS solution, and removed from the circular DNA by phenol extraction. The DNA circles were recovered by ethanol precipitation and subjected to PCR amplification. PCR products were separated by PAGE analysis and quantified by phosphomager analysis on the Typhoon 8600 imager (Pharmacia, New York, NY). The identity of BREC s was confirmed by nucleotide sequence analysis and is available from GenBank (accession number AY455933).

**Cloning, expression, and RT-PCR amplification of VpreB and λ5**

Rabbit VpreB and λ5 probes were generated by PCR amplification of bone marrow cDNA using primers to conserved regions of the mouse and human VpreB and λ5. The primers used for VpreB were 5′-CTACTGTTACCG CAGAGGCG-GC3′ (sense) and 5′-TCTTCTCCCTCATCTCCTC3′ (antisense); and the primers used for λ5 were 5′-CTGCTGTGGTCTGTG3′ (sense) and 5′-GTTGTGGTCTG3′ (antisense). PCR products were cloned into pGEMT EZ (Promega), and their identities were determined by nucleotide sequence analysis using the BigDye termination technique with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). These clones were used to screen a genomic library (~10⁶ PFU) (26); VpreB and λ5 phage clones were purified, and the nucleotide sequences of VpreB and λ5 were determined. Genomic Southern blot analysis was performed on HindIII- or XhoI-restricted genomic DNA according to standard protocols. The VpreB probe included probes were generated by PCR amplification of DJ, VDJ, and VJκ gene rearrangements illustrated in Fig. 1. To identify A5 and VpreB genes expressed in bone marrow, cDNA was PCR-amplified using two pairs of primers for each: for A5, A = (sense) 5′-TAAAGCTTTAAGGCACCCGGAGTGGCC-3′ (antisense) 5′-AATC TAGAAGGACACAGTGCAGGGCCCA-3′ from the first and last 20 bp of the gene; and B = (sense) 5′-TATCTCAAGTCTCCAGAGGA-3′ (antisense) 5′-GGGTGTGGTACTAGAGAGTT-3′ from the 5′ and 3′ untranslated regions. For VpreB, the primers were either pan-VpreB primers (sense) 5′-TTAGCTTATGCTGACTTGC3′ (antisense) 5′-GCACCGATCTTGTGGTCCTCAA-3′; or the pan-VpreB sense primer and the VpreB2-specific antisense primer, 5′-TCTCCCTCTCTTCTTCTCT-3′.

For in vitro expression of VpreB and λ5, the genes were PCR-amplified using primers 5′ and 3′ of the open reading frames; VpreB, (sense) 5′-TTAAGCTTTAAGGCACCCGGAGTGGCC-3′ (antisense) 5′-TATCTCAAGTCTCCAGAGGA-3′; and λ5, primer pair A (above). The nucleotide sequences were confirmed and the genes were inserted into the mammalian expression vector pCDNA3.1A-Myc-His (Invitrogen, Carlsbad, CA). Expression of each gene was confirmed by transfection into 293T cells followed by Western blot analysis of lysates (1 × 10⁶ cell equivalents) using 16% polyacrylamide gels. The blot was probed with anti-myc-epitope Ab (mAb 9E10) and HRP-conjugated goat anti-mouse Ig and developed with the ECL chemiluminescent reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

For RT-PCR amplification of VpreB and λ5, cDNA was prepared as described (27) from 249 cells of FACS-sorted proB and preB cells (MHC class II, surf-μ⁺). Two rounds of 40 cycles of PCR amplification were performed on the cDNA using the following sets of nested primers: VpreB primers were 5′-TATCTCAAGTCTCCAGAGGA-3′ (sense inside primer), 5′-ATGCTTATGCTGACTTGC3′ (sense inside primer), 5′-GCACCGATCTTGTGGTCCTCAA-3′ (antisense inside primer), and 5′-GTTGTGGTACTAGAGAGTT-3′ (antisense inside primer). PCR primers were 5′-TATCTCAAGTCTCCAGAGGA-3′ (sense outside primer), 5′-ATGCTTATGCTGACTTGC3′ (antisense outside primer), and 5′-GGGTGTGGTACTAGAGAGTT-3′ (antisense outside primer).

**FIGURE 1.** Illustration of PCR primers used to amplify DJ, VDJ, VJκ, and VD BREC gene rearrangements. The primer sequence used in each PCR amplification is shown on the right. So, sense outside primer; Si, sense inside primer; Ao, antisense outside primer; Ai, antisense inside primer. The expected product size is: DJ = 100 bp; VDJ = 500 bp; VJκ = 400 bp; VD BREC = 150 bp. The percentage of rearrangements expected to be PCR-amplified with these primer sets is DJ = −50%; VDJ = −100%; VJκ = −90% (41); VD BREC = −50%.
Immunoprecipitation of VpreB, A5, and μ-chain

A full-length rabbit μ-chain gene was generated by PCR amplification of cDNA from the rabbit B cell line 55D1 (28). The μ-chain gene was subcloned into pCDNA3.1-c-myc-His and confirmed by nucleotide sequence analysis. For immunoprecipitation, cell lysates from 293T cells transfected with μ, A5, and VpreB in pCDNA3.1, or as control, A5 and VpreB, were obtained and incubated with Sepharose-Gammabind-goat anti-μ antibody (H11011). PreB and B cells were isolated by magnetic bead enrichment followed by flow cytometry, purified by negative selection, and fixed with 4% paraformaldehyde. Data were analyzed with a FACSCalibur with CellQuest software. Western Blots were probed with anti-c-myc-epitope mAb as described above.

In vivo BrdU labeling

One-week-old rabbits were injected i.p. with 3 mg of BrdU in PBS at 12-h intervals for a 24-h period. Bone marrow cells were harvested 12 h following the last injection and stained for expression of CD79a, cyto-μ, and incorporation of BrdU. Briefly, 1 × 10^6 bone marrow cells were fixed with Cytofix/Cytoperm, and resuspended in freezing medium (10% DMSO and 90% FBS). Following a single freeze/thaw cycle, cells were subjected to a fixation and expression of a surrogate L chain

VpreB. To search for surrogate L chain genes, VpreB and A5, we screened a rabbit genomic phage library using probes described in Materials and Methods, and identified several phage clones that hybridized with these probes. From the VpreB phage we identified and sequenced two genes, VpreB1 and VpreB2 (Fig. 3). On the basis of the nucleotide and deduced amino acid sequences, VpreB1 and VpreB2 are 93% identical, differing by only 17 nucleotides and 10 amino acids. They are also similar in structure to mouse and human VpreB, showing conserved framework regions as well as a non-Ig domain. Within the Ig-like region, the deduced amino acid

Table I. Number of productive (P) and nonproductive (NP) Ig gene rearrangements in proB and preB cells populations

<table>
<thead>
<tr>
<th></th>
<th>VDJ</th>
<th>VJκ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProB</td>
<td>P</td>
<td>NP</td>
</tr>
<tr>
<td>PreB</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

To confirm the identity of proB and preB cells in rabbit bone marrow, we FACS-sorted progenitor B cells and assessed their VDJ and VJκ gene rearrangement status by PCR and nucleotide sequence analysis. Both DJ and VDJ gene rearrangements were PCR-amplified from proB cells and by nucleotide sequence analysis, we found as expected, almost all VDJ genes (10 of 11) were nonproductive (Table I). In one experiment, we PCR-amplified a single productive VJκ gene from proB cells. From preB cells, we recovered both VDJ and VJκ genes and found, as expected, that all of the VDJ genes were productive (14 of 14) and most VJκ genes were nonproductive (6 of 7) (Table I).

Taken together, the immunofluorescence data and Ig gene rearrangement analyses suggest that the progenitor B cell populations identified by flow cytometric analysis (Fig. 2) are proB cells (CD79a^+ , cyto-μ^+ ) which have DJ and nonproductive VDJ gene rearrangements and preB cells (CD79a^+ , cyto-μ^-, surf-μ^-) which have productive VDJ and nonproductive VJκ gene rearrangements.

Results

Identification of ProB and PreB lymphocytes in bone marrow

We searched for progenitor B cells within bone marrow from newborn rabbits by using flow cytometry. Because Abs to rabbit B220 and CD19 are not available, we used expression of CD79a, cyto-μ, and incorporation of BrdU as markers of B-lineage cells. We obtained evidence that CD79a is a B-lineage-specific marker in rabbits by performing two-color immunofluorescence on spleen cells from an adult rabbit, and finding that all μ^+ cells were CD79a^+ , and that all CD79a^+ cells were μ^+ (data not shown). To identify progenitor B cells in rabbit bone marrow, we performed two-color intracellular immunofluorescence staining with anti-μ and anti-CD79a Abs and identified two CD79a^+ cell populations, μ^- and μ^+. We designated these populations proB and (preB + B) cells, respectively (Fig. 2A). Nearly 70% of CD79a^+ cells were proB cells (μ^-), and only ~30% were preB^+ /B cells (μ^+). These data are in marked contrast to both mice and humans in which proB cells account for only 5–15% of B-lineage cells in bone marrow (29, 30). The proportions of preB and B cells within the CD79a^+ cyto-μ^- population were determined by analyzing surface μ expression (Fig. 2B). Most cyto-μ^- cells (~80%) were surf-μ^- preB cells, and only ~20% were surf-μ^+ B cells. We identified comparable B-lineage populations using three-color immunofluorescence staining for CD79a, cyto-μ, and surf-μ (Fig. 2C).

FIGURE 2. Representative flow cytometric profile of bone marrow cells to identify proB, preB, and B cell populations. Total bone marrow cells from a newborn (A and B) or 1.5-wk-old rabbit (C) were fixed, permeabilized, and stained for: A, cytoplasmic CD79a and μ-chain; B, cytoplasmic and surface μ-chain; and C, cytoplasmic CD79a, surface μ-chain, and cytoplasmic μ-chain. Inset, Cytoplasmic μ staining of CD79a^+ surf μ^- cells. Data were gated for lymphocyte-sized cells, and in A and B, the percentage that each population represents of total gated cells is noted in the lower right corner of the quadrants.
sequence of rabbit VpreB1 is 70% identical (82% similar) to both mouse and human VpreB1 (Fig. 4). The non-Ig region is much less conserved, having only 29 and 43% amino acid identity to that of mice and humans, respectively.

Southern blot analysis of genomic DNA using the VpreB probe resulted in three hybridizing bands (Fig. 5) suggesting that the rabbit genome contains at least three VpreB genes. To determine whether one or more of these genes is expressed during B cell development, we RT-PCR-amplified VpreB from newborn bone marrow cDNA using pan-VpreB primers and determined their nucleotide sequences. We found that each of 10 PCR products encoded VpreB1 (data not shown). To determine whether VpreB2 is expressed, we PCR-amplified and cloned bone marrow cDNA using VpreB2-specific primers and found that each of seven clones encoded VpreB2 (data not shown). Although these data demonstrate that both rabbit VpreB1 and VpreB2 are expressed in newborn bone marrow, VpreB1 is the predominantly expressed VpreB gene during B cell development. We found no evidence for a third VpreB molecule expressed in bone marrow even though we observed three VpreB-hybridizing bands by Southern blot analysis.

FIGURE 3. Restriction maps of VpreB1 and VpreB2 phage and comparison of deduced amino acid sequences. A, Restriction maps of phage 9 and phage 19; H, HindIII; S, SacI; B, BamHI; R, EcoRI; K, KpnI; X, XhoI; Z, XhoI. HindIII is not mapped in phage 19; XhoI is partially mapped in phage 19 and not mapped in phage 9 (B). The nucleotide sequence and amino acid translation of VpreB1 are compared with the deduced amino acid sequence of VpreB2. Positions of amino acid identity are denoted by a dot (●). To facilitate amino acid comparison, the leader was spliced to framework 1 on the basis of the splice site found in VpreB cDNA clones. The sequences of VpreB1 and VpreB2 are accessible at GenBank under accession numbers AY351269 and AY351268.

FIGURE 4. Comparison of deduced amino acid sequences of rabbit, human, and mouse VpreB1. The nucleotide sequence and amino acid translation of rabbit VpreB1 are shown on top. Positions of amino acid identity are denoted by a dot (●). Dashes (-) were introduced to maximize homology.
To determine whether two different newborn rabbits, we cannot rule out the possibility that another gest that only a single contained on phage 2 (data not shown). Although these data suggested the second hybridizing band found by genomic Southern blot residing in the non-Ig region, and (B) exon 1 of H. No HindIII or XhoI sites are present in VpreB or λ5 genes. Identical results were obtained from each of five rabbits. Sizes of λHindIII markers are shown at the left.

By Southern blot analysis, we found two hybridizing bands in each of five different rabbits, indicating that the genome contains two λ5 genes (Fig. 5B). We PCR-amplified, cloned, and sequenced λ5 genes from bone marrow cDNA of newborn rabbits using two different pairs of λ5-specific primers (see Material and Methods) to determine whether two different λ5 molecules were expressed. We found that the nucleotide sequences of four clones obtained from each set of primers were identical to that of the λ5 gene contained on phage 2 (data not shown). Although these data suggest that only a single λ5 gene is expressed in bone marrow of newborn rabbits, we cannot rule out the possibility that another λ5 gene is expressed but did not PCR amplify with either primer set. Alternatively, perhaps there is only a single λ5 gene in rabbits, and the second hybridizing band found by genomic Southern blot residing in the non-Ig region gene, as is found in humans (31).

PreBCR

To determine whether progenitor B cells express a preBCR, we performed RT-PCR for VpreB and λ5 on groups of FACS-sorted proB and preB cells. Because of the difficulty performing RT-PCR on fixed cells, we did not use the intracellular marker CD79a to isolate proB and preB cells, and instead searched for alternative surface molecules that would allow us to obtain progenitor B cells. We found that all CD79a+ bone marrow cells were also MHC class II+ and vice versa (Fig. 7A), suggesting that we could use MHC class II instead of CD79a to isolate B lineage cells. We stained total bone marrow cells with anti-MHC class II-PE and FACS-sorted progenitor B cells (MHC class II+, surf-μ-). By RT-PCR amplification, we found that VpreB and λ5 were expressed in these cells (25 cells/pool), but not in MHC class II- cells (100 cells/pool) (Fig. 7B). We conclude that VpreB and λ5 are expressed in proB and/or preB cells but not in other cell lineages.

To determine whether VpreB and λ5 can associate with the μ-chain, as would be expected if they are part of a preBCR complex, VpreB and λ5 proteins were myc-tagged and assayed for their ability to pair with the μ-chain in 293T cells transfected with VpreB, λ5, and μ-chain genes. Immunoprecipitation of the μ-chain from 293T cell lysates followed by Western blot showed that two proteins of ~18 and 22 kDa, as expected for VpreB and λ5, respectively, were coimmunoprecipitated with the μ-chain (Fig. 8). These two proteins were not observed in anti-μ immunoprecipitates of 293T cells transfected with VpreB and λ5 in the absence of μ. These results demonstrate that the μ-chain associates with VpreB and λ5 to form a preBCR-like protein complex.

FIGURE 5. Genomic Southern blot analysis of rabbit VpreB (A) and λ5 (B). Genomic DNA was digested with HindIII or XhoI, and the blots were probed with (A) a fragment of VpreB including framework region 2 through the non-Ig region, and (B) exon 1 of λ5. No HindIII or XhoI sites are present in VpreB or λ5 genes. Identical results were obtained from each of five rabbits. Sizes of λHindIII markers are shown at the left.

FIGURE 6. Restriction map of λ5 phage and comparison of deduced amino acid sequence encoded by rabbit, human, and mouse λ5 genes. A, The restriction map of phage 2-1 containing a λ5 gene; II, HindIII; S, SarI; B, BamHI; R, EcoRI; K, KpnI; X, XhoI. Z = XhoI. Exons of λ5 are designated as I, II and III; R, the nucleotide sequence and amino acid translation of rabbit, human, and mouse λ5. Positions of amino acid identity are denoted by a dot (•). Dashes (-) were introduced to maximize homology. The sequence of rabbit λ5 is accessible at GenBank under accession number AY351267.
In contrast to proB and preB cells, essentially no B cells were found in bone marrow at birth. Their numbers increased slowly over time, until B cells constituted all B-lineage cells in the bone marrow at 16 wk of age (Fig. 9B).

To confirm the decline in B lymphopoiesis observed by flow cytometric analyses, we quantitated the levels of V→DJ gene rearrangement leading to the formation of proB cells (Fig. 10A). We found that the number of BRECs at birth was ~300 per 3 × 10⁵ cells and reached maximum levels (~1100 per 3 × 10⁵ cells) at ~3 wk of age, when we see the highest percentage of proB cells in bone marrow (Fig. 9A). After 3 wk, BRECs declined steadily and were undetectable by 16 wk of age. Because in previous studies (6), it appeared that low levels of BRECs were detectable in adult rabbits, we increased the sensitivity of the analysis by using circular DNA (enriched for BRECs) and by adding [³²P]dCTP to the PCR amplification. In two adult rabbits (4 mo and 18 mo of age), we detected BRECs by phosphomager analysis but not by ethidium bromide staining. We compared the levels of BRECs in these adult rabbits with the levels found in bone marrow of newborn rabbits and found that BRECs were decreased ~200-fold (99.5%) at 4 mo (data not shown) and 500-1000 fold by 18 mo of age (Fig. 10B). Consistent with the greatly decreased number of BRECs in bone marrow of adult rabbits, we did not detect proB or preB cells by flow cytometric analysis, even though we analyzed 10 times more cells than in previous experiments (data not shown). Taken together, these data suggest that by 16 wk of age, B lymphopoiesis in rabbit is decreased over 99%, and by adulthood, is at a level ~<0.2% of that in newborn rabbits.

**BrdU labeling of ProB cells**

The above experiments demonstrate that at birth, most (~70%) B-lineage cells (CD79α⁺) are proB cells. ProB cell levels increase slowly and reach their highest levels only after 3 wk of age. This result is surprising because in mice, proB cells differentiate into preB cells within a 24-h period (36–39). Two possible explanations for the apparent delay in proB cell accumulation are that: 1) the majority of proB cells are quiescent and require a few weeks to differentiate into preB cells; or 2) ProB cells differentiate with kinetics similar to that of mice; however, the vast majority of proB cells die, thereby requiring a 3-wk time period for preB cells to accumulate and/or proliferate. To distinguish between these possibilities, we performed BrdU-labeling experiments. If the proB cell population is quiescent, we expected the majority of proB cells would not be labeled within 24 h following BrdU injection. We found that following two injections of BrdU over a 24-h period, the majority of proB cells (~70%) labeled with BrdU (Fig. 11), implying that proB cells in rabbits are not quiescent. We conclude that the slow accumulation of proB cells is likely due to massive cell death within the proB and/or preB cell populations during differentiation.

**Discussion**

Before this study, rabbit proB cells had been identified by several investigators (5, 22, 23); however, neither proB cells nor the status of proB cell accumulation was reported. Our studies show that in rabbits, the proB cell population is largely quiescent and relatively abundant only early in development. However, the percentage of proB cells in the bone marrow steadily declines until adulthood. These results suggest that proB cells are not quiescent and that they accumulate in bone marrow for a short period of time, to a level of ~300 per 3 × 10⁵ bone marrow cells. One possible explanation for this observation is that proB cells are labeled in vivo by BrdU, but are not detected by flow cytometric analysis because they are not proliferation competent. To test this hypothesis, we performed BrdU-labeling experiments and found that proB cells are labeled in vivo by BrdU. These results suggest that proB cells are not quiescent and that they accumulate in bone marrow for a short period of time, to a level of ~300 per 3 × 10⁵ bone marrow cells.
of Ig gene rearrangements in precursor cells was described. Although we and others assumed that B cell development proceeded through the ordered pathway, which predicts the existence of a preBCR, neither the VpreB nor λ5 gene of the surrogate L chain had been identified. Further, we knew that robust B cell development did not continue throughout life (6), but the timing at which B lymphopoiesis arrested had not been determined. In the present study, we cloned genes encoding VpreB, neither the preBCR, nor λ5 gene of the surrogate L chain had been identified. Further, we knew that robust B cell development did not continue throughout life (6), but the timing at which B lymphopoiesis arrested had not been determined. In the present study, we cloned genes encoding VpreB and λ5, found them expressed in progenitor B cells, and provided evidence that they can form a preBCR complex in vitro. On the basis of CD79a and μ-chain expression and Ig gene rearrangements, we identified both proB and preB cells within bone marrow and showed that the percentage of proB and preB cells peaked at ∼2–3 wk of age. Further, we found that the vast majority (99.5%) of B lymphopoiesis arrested by 16 wk of age.

Pathway of B cell development

The expression of VpreB and λ5 in early B-lineage cells and the ability of these molecules to pair with the μ-chain suggests that, as in mice and humans, early preB cells in rabbits likely express a preBCR. The finding that D gene segments rearrange in all three RF in proB cells but are found predominantly in RF1 in preB cells (presumably late stage preB cells) demonstrates that selection for D in the preferred RF, RF1, has occurred between the proB and preB cell stages. Presumably, this selection is mediated by the preBCR in early preB cells as it is in mice (32, 33), indicating that the preBCR in rabbits is important for B cell development.

The presence of a functional preBCR and the ordered rearrangement of H and L chain genes in proB and preB cells, respectively, suggests that rabbit B cell development proceeds primarily through the classical, or ordered, pathway. The finding that H and L chain genes do not rearrange simultaneously is further supported by the observation that essentially all μ H chains but only ∼40% of κ L chains contain N-nucleotide addition (40, 41). Although most B cells seem to develop through the classical pathway, we suggest that, as in mice, a small number of B cells develop by the alternative, or stochastic, pathway because we occasionally found a proB cell containing a VDJ gene rearrangement without a H chain gene rearrangement. However, if a significant proportion of developing B cells used this alternative pathway, we would expect to find cells expressing L chain in the absence of H chain. We and other researchers have not been able to identify such a cell type (Ref. 23 and P. J. J. and K. L. K., unpublished observations).

Arrest of B lymphopoiesis in bone marrow

After 3 wk of age, the levels of both proB and preB cells decrease in rabbit bone marrow, and by 16 wk of age, essentially all B-lineage cells in bone marrow are B cells. The increase in B cells is presumably due to homing of peripheral B cells back to the bone marrow rather than to continued B lymphopoiesis. The decline in progenitor B cells in bone marrow was corroborated by a decline in V→D BRECs. In a previous study (40), we found evidence that occasionally, IgH gene rearrangements seem to occur through a VD intermediate rather than through a DJ intermediate. Even though we think that this occurs infrequently and contributes little to lymphopoiesis, such rearrangements would be identified in the V→D BRECs. Taking together the immunofluorescence and BRECs, data, we conclude that B lymphopoiesis effectively terminates in bone marrow by 16 wk of age. This is in marked contrast to mice and humans in which B lymphopoiesis continues at a high level throughout life (39, 42).

Although B lymphopoiesis in rabbits is vastly reduced by 16 wk of age, we detected trace levels of BRECs in adult rabbit bone marrow (18 mo of age), estimated to be 500- to 1000-fold lower than that seen in newborn rabbits. By flow cytometric analysis, we were unable to detect proB and preB cells in bone marrow of these adult rabbits even after analysis of ∼1 × 10⁶ cells. We conclude that the vast majority of B cells in adult rabbits are generated from...
inlaid within the and histograms representing anti-BrdU staining of these populations are cytometric analysis. ProB and preB and B populations were gated as shown (PE), BrdU (FITC), and cytometric analysis.

FIGURE 11. BrdU labeling of proB, preB, and B cells in 1-wk-old rabbit bone marrow. Rabbits received two injections of BrdU at 12-h intervals and total bone marrow cells were triple-stained for expression of CD79a (PE), BrdU (FITC), and cyto-μ (allophycocyanin), and analyzed by flow cytometric analysis. ProB and preB and B populations were gated as shown and histograms representing anti-BrdU staining of these populations are inlaid within the figure.
the bursa and ideal Peyer's patch involute within weeks or months of age. Through the study of B cell development in different species, it is clear that a variety of mechanisms are used in B cell development and generation of the Ab repertoire. The rabbit, in particular, provides an opportunity to study a mechanism by which B lymphopoiesis occurs via the classical pathway in bone marrow, terminates during early development, and then the B cell repertoire is expanded and diversified in GALT giving rise to a competent humoral immune system for the life of the animal.

References