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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 BYPCR 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.

The pathway of B cell development in mice and humans is well-described. In mice, B lymphopoiesis occurs through development and occurs early in development (1–6). In these species, the Ab repertoire is expanded and diversified in gut-associated lymphoid tissue (GALT) thereby forming the primary or preimmune Ab repertoire (7–9). If GALT is removed early in ontogeny, development of the B cell repertoire is severely impaired. In chickens, embryonic bursectomy results in severe agammaglobulinemia (10–12), and in sheep and rabbits, neonatal removal of the ileal Peyer’s patch or GALT results in life-long B cell deficiency or a severely reduced number of B cells (13–15).

The pathway of B cell development in mice and humans is well-described. In mice, B-lineage precursors progress through several ordered developmental stages which are defined by the status of V(D)J gene rearrangement and expression of specific cell surface markers. The ordered rearrangement of Ig genes begins in proB cells where D→J gene rearrangements occur. These cells progress to preB cells upon productive V→DJ gene rearrangement and must express the preB cell receptor (preBCR) to continue development (16). The preBCR is a complex comprised of a μ-chain in association with a surrogate L chain, which is encoded by VpreB and λ5. Signaling through the preBCR is required for the developmental progression of early preB to late preB cells, which then become immature B cells upon productive L chain gene rearrangement (16). In addition, a small percentage of B cells in mice develop through an alternative preBCR-independent pathway in which the L chain rearranges independently of the H chain (17–21). In rabbits, the only progenitor B cells described are preB cells, which have been identified in fetal liver, fetal spleen, and neonatal bone marrow (5, 22, 23). The presence of a μ-chain and the absence of the L chain in virtually all preB cells suggests that B cell development in rabbit likely occurs through the classical, or ordered, pathway, mediated by a preBCR. However, neither a surrogate L chain nor a preBCR has been identified.

In contrast to mice, in which preB cells are found in bone marrow throughout life, in rabbits the number of preB cells is highest around birth (22) or at 2–3 wk of age (5) and then steadily decreases until they are undetectable in adults (5). To confirm these data, Crane et al. (6) examined levels of B cell recombination excision circles (BRECs) in bone marrow of young and adult rabbits. The authors found high levels of BRECs in bone marrow DNA from young rabbits and almost none in adult rabbits, suggesting that by adulthood, lymphopoiesis had largely arrested. Although these data indicate that B lymphopoiesis largely arrests early in ontogeny, the timing of the decrease in B lymphopoiesis in rabbits remains unknown.

In the present study, we identified progenitor B cells, determined the Ig gene rearrangements within these cells, and found evidence of a preBCR. We also quantitated the decline in B lymphopoiesis during the first several months of life by searching for B cell progenitors and BRECs.

Materials and Methods

Immunofluorescent staining of bone marrow cells

Antibodies. The Abs and indirect reagents used in this study were as follows: mAb anti-rabbit μ-chain (clone 367) (15); FITC-conjugated (24) goat anti-rabbit L chain Ab (15); mouse anti-human CD79a (Ig-α; mb-1),
which cross-reacts with rabbit CD79a (BD PharMingen clone H447; 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 × 10⁸) 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 anti-mouse IgG. Subsequently, cells were fixed and permeabilized 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 anti-mouse IgG. Cells were then fixed and permeabilized and stained with biotinylated mouse anti-rabbit μ-chain/streptavidin-allophycocyanin, and finally with PE 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 cells were defined as CD79a+cytomic μ- (cytomic μ-) surface μ- (surf-μ-); preB cells were defined as CD79a+cytomic μ+surf-μ-. B cells were defined as CD79a+cytomic μ+surf-μ. Detection of DJ, VDJ, and VJκ gene rearrangements by PCR

Pools of 50 cells were sorted into 96-well V-bottom plates containing 1× lysis buffer (100 µg/ml proteinase K in 1× PCR buffer (Promega)) using a FACSStar 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 proteinase K. The PCR mixture (200 pM 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**

VD recombination signal sequence excision circles were quantified by competitive PCR using primers shown in Fig. 1. Briefly, BREC's from 2 × 10⁸ 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 quantitated by phosphoimager 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′-CTACTGGTACTCACGAGAGGCGC-3′ (sense) and 5′-CTCTTTCCACCCATTCCCTC-3′ (antisense); the primers used for λ5 were 5′-CTGCTGCTGTTGTTGCT-3′ (sense) and 5′-GTGTTGTTGTTGGTTGGTTGGT-3′ (antisense). PCR products were cloned into pGEMT-EZ (Promega), and their identities were confirmed by plasmid sequence analysis.

For in vitro expression of VpreB and λ5, the genes were PCR-amplified using two pairs of primers for each: for A5, (sense) 5′-TTAAGCTTATGAGGCCCCGAGTGCCCT-3′, (antisense) 5′-AATCATTAGAGAAGCATGTCGGAGGAGGAG-3′ from the first and last 20 bp of the gene, and for B (sense) 5′-TATCTACAGGTCGTTAAGGAGGA-3′, (antisense) 5′-GGTTGTTGTTGAGAAGTTTTG-3′ from the 5′ and 3′ untranslated regions. For VpreB, the primers were either pan-VpreB primers (sense) 5′-TTAAGCTTATGGTTGTTGAGAAGTTTTG-3′ and (antisense) 5′- GCAGGCAGCTTGTTGCTTCTCA-3′ or the VpreB-sense primer and the VpreB2-specific antisense primer, 5′-CTCTCCCTCTTCTTCTCT-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′-TTAAGCTTATGGTTGTTGAGAAGTTTTG-3′ and (antisense) 5′-TTTCTACAGGCCCCGAGTGCCCT-3′; λ5, primer pair A (above). The nucleotide sequences were confirmed and the genes were cloned into the mammalian expression vector pCDNA3.1-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 previously (27) from groups 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 primers: VpreB primers were 5′-TGGTGTTGTTGAGAAGTTTTG-3′ (sense outside primer), 5′-ATGCATTACAGGTTGTGTTCCCTC-3′ (sense inside primer), 5′-GCACCGGAGCAGTTGCTGCTTCTCA-3′ (antisense outside primer), and 5′-GGTTGTTGTTGAGAAGTTTTG-3′ (antisense inside primer); λ5 primers were 5′-TATCTACAGGTTGAGGAGGAG-3′ (sense outside primer), 5′-ATGGGTTGAGGAGGAGGAG-3′ (sense inside primer), 5′-GGTTGTTGTTGAGAAGTTTTG-3′ (antisense outside primer), and 5′-GAGCTGTTGGATTGTTGCTTCTCA-3′ (antisense inside 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 \( \mu \)-chain

A full-length rabbit \( \mu \)-chain gene was generated by PCR amplification of cDNA from the rabbit B cell line 55D1 (28). The \( \mu \)-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 \( \mu \), A5, and VpreB in pCDNA3.1, or as control, A5 and VpreB, were obtained and incubated with Sepharose-Gammabind-goat anti-\( \mu \)-chain; and cytoplasmic and surface \( \mu \)-chain; and cytoplasmic \( \mu \)-chain.

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-\( \mu \)-, and incorporation of BrdU. Briefly, \( 1 \times 10^7 \) bone marrow cells were fixed with Cytofix/Cytoperm, and re suspended in freezing medium (10% DMSO and 90% FBS). Following a single freeze/thaw cycle, cells were subjected to a second round of fixation in Cytofix/Cytoperm. DNase (12.5 U) was added and cells were incubated for 1 h at 37°C and subsequently stained for CD79a, cyto-\( \mu \)-, and incorporation of BrdU, and analyzed as described above.

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 as a marker 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 \( \mu ^+ \) cells were CD79a\(^+ \), and that all CD79a\(^+ \) cells were \( \mu ^+ \) (data not shown). To identify progenitor B cells in rabbit bone marrow, we performed two-color intracellular immunofluorescence staining with anti-\( \mu \)- and anti-CD79a Abs and identified two CD79a\(^+ \) cell populations, \( \mu ^- \) and \( \mu ^+ \). We designated these populations as proB and (PreB + B cells), respectively (Fig. 2A). Nearly 70% of CD79a\(^+ \) cells were proB cells (\( \mu ^- \)), and only \( \sim 30\% \) were preB\(^+ \)/B cells (\( \mu ^+ \)). These data are in marked contrast to both mouse and humans in which proB cells account for only 5–15% of B-lineage cells in bone marrow (29, 30). The proportions of proB and B cells within the CD79a\(^+ \) cyto-\( \mu ^- \) population were determined by analyzing surface \( \mu \) expression (Fig. 2B). Most cyto-\( \mu ^- \) cells (\( \sim 80\% \)) were surf-\( \mu ^- \) preB cells, and only \( \sim 20\% \) were surf-\( \mu ^+ \) B cells. We identified comparable B-lineage populations using three-color immunofluorescence staining for CD79a, cyto-\( \mu \)-, and surf-\( \mu \) (Fig. 2C).

To confirm the identity of proB and preB cells in rabbit bone marrow, we FACS-sorted progenitor B cells and assessed their VDJ and V\( \kappa \) gene rearrangements 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 V\( \kappa \) gene from proB cells. From proB cells, we recovered both VDJ and V\( \kappa \) genes and found, as expected, that all of the VDJ genes were productive (14 of 14) and most V\( \kappa \) genes were nonproductive (6 of 7) (Table I).

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-\( \mu ^- \)) which have DJ and nonproductive VDJ gene rearrangements and preB cells (CD79a\(^+ \), cyto-\( \mu ^+ \), surf-\( \mu ^- \)) which have productive VDJ and nonproductive V\( \kappa \) gene rearrangements.

Identification 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>
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<tr>
<th>VDJ</th>
<th>P</th>
<th>NP</th>
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<tr>
<td>PreB</td>
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</tr>
<tr>
<td>ProB</td>
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<th>V( \kappa )</th>
<th>P</th>
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<td>1</td>
</tr>
<tr>
<td>ProB</td>
<td>14</td>
<td>6</td>
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</table>

a ProB and/or preB cells were FACs-sorted into pools of 50 cells and VDJ and V\( \kappa \) gene rearrangements were PCR-amplified and sequenced. VDJ, H chain gene rearrangements; V\( \kappa \), L chain gene rearrangements; P, productive; NP, nonproductive.

b Representative sequences of productive and nonproductive VDJ and V\( \kappa \) genes are available from GenBank (accession numbers AYY55932 and AY445934–38).

c One productive V\( \kappa \) gene was obtained from a total of six pools of 50 cells each.
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. 5A) 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.

The /H92615-hybridizing phage clones were restriction-mapped, and by Southern blot analysis, we found a phage (/H92782-1) that contained all three exons of a /H92615 gene (Fig. 6). From the deduced amino acid sequence, this gene appears functional and shares 57 and 66% amino acid identity with mouse and human /H92615, respectively, within the Ig-like region (Fig. 6). Similar to the results found with VpreB, the non-Ig region of /H92615 is significantly less-conserved across species, demonstrating 44 and 58% amino acid identity with mice and humans, respectively.

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, XbaI; Z, XhoI. HindIII is not mapped in phage 19; XbaI 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 suggest through the non-Ig region, and (data not shown). By Southern blot analysis, we found two hybridizing bands in ve rabbits. Sizes of VpreB are present in VpreB and V5 genes from bone marrow cDNA of newborn rabbits using two different pairs of V5-specific primers (see Material and Methods) to determine whether two different V5 molecules were expressed. We found that the nucleotide sequences of four clones obtained from each set of primers were identical to that of the V5 gene contained on phage 2 (data not shown). Although these data suggest that only a single V5 gene is expressed in bone marrow of newborn rabbits, we cannot rule out the possibility that another V5 gene is expressed but did not PCR amplify with either primer set. Alternatively, perhaps there is only a single V5 gene in rabbits, and the second hybridizing band found by genomic Southern blot re-

By Southern blot analysis, we found two hybridizing bands in each of five different rabbits, indicating that the genome contains two V5 genes (Fig. 5B). We PCR-amplified, cloned, and sequenced V5 genes from bone marrow cDNA of newborn rabbits using two different pairs of V5-specific primers (see Material and Methods) to determine whether two different V5 molecules were expressed. We found that the nucleotide sequences of four clones obtained from each set of primers were identical to that of the V5 gene contained on phage 2 (data not shown). Although these data suggest that only a single V5 gene is expressed in bone marrow of newborn rabbits, we cannot rule out the possibility that another V5 gene is expressed but did not PCR amplify with either primer set. Alternatively, perhaps there is only a single V5 gene in rabbits, and the second hybridizing band found by genomic Southern blot re-

FIGURE 5. Genomic Southern blot analysis of rabbit VpreB (A) and V5 (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 V5. No HindIII or XhoI sites are present in VpreB or V5 genes. Identical results were obtained from each of five rabbits. Sizes of HindIII markers are shown at the left.

PreBCR
To determine whether progenitor B cells express a preBCR, we performed RT-PCR for VpreB and V5 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 CD79α+ 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 anti-μ-FTGC Abs, and FACS-sorted progenitor B cells (MHC class II+, surf-μ+). By RT-PCR amplification, we found that VpreB and V5 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 V5 are expressed in proB and/or preB cells but not in other cell lineages.

To determine whether VpreB and V5 can associate with the μ-chain, as would be expected if they are part of a preBCR complex, VpreB and V5 proteins were myc-tagged and assayed for their ability to pair with the μ-chain in 293T cells transfected with VpreB, V5, 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 V5, 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 V5 in the absence of μ. These results demonstrate that the μ-chain associates with VpreB and V5 to form a preBCR-like protein complex.

FIGURE 6. Restriction map of V5 phage and comparison of deduced amino acid sequence encoded by rabbit, human, and mouse V5 genes. A, The restriction map of phage 2-1 containing a V5 gene; II, HindIII; S, SacI; B, BamHI; R, EcoRI; K, KpnI; X, XhoI; Z = XhoI. Exons of V5 are designated as I, II and III; R, the nucleotide sequence and amino acid translation of rabbit, human, and mouse V5. Positions of amino acid identity are denoted by a dot (●). Dashes (-) were introduced to maximize homology. The sequence of rabbit V5 is accessible at GenBank under accession number AY351267.
FACS-sorted MHC class II B flow cytometric analysis of newborn bone marrow cells stained for CD79a

FIGURE 8. Western blot of anti-mouse IgM mAb. 293T cells transfected with myc-tagged rabbit A5 and VpreB, with (+) or without (−) μ. The blot was probed with anti-myc mAb.

Table II. D RF of VDJ genes cloned from proB and preB cells

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<th>RF1</th>
<th>RF2</th>
<th>RF3</th>
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</tr>
<tr>
<td>PreB</td>
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<td>0</td>
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*D gene RF usage was determined from the deduced amino acid sequence; RF1 encodes primarily G and Y; RF2 encodes primarily M, V, and L; RF3 encodes primarily W, L, and C; and/or a stop codon.

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→D BRECs as a measure of V→D gene rearrangement leading to the formation of preB 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 preB 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 phosphoimager 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 PreB cells

The above experiments demonstrate that at birth, most (~70%) B-lineage cells (CD79a+) are proB cells. PreB 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 preB cell accumulation are that: 1) the majority of proB cells are quiescent and require a few weeks to differentiate into preB cells; or 2) PreB 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 preB cells is likely due to massive cell death within the proB and/or preB cell populations during differentiation.

Discussion

Before this study, rabbit preB cells had been identified by several investigators (5, 22, 23); however, neither proB cells nor the status
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 \( \lambda 5 \) 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 \( \mu \) chain and discovered that they can form a preBCR complex in vitro. On the basis of CD79a and \( \mu \)-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 \( \sim 2-3 \) 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 \( \lambda 5 \) in early B-lineage cells and the ability of these molecules to pair with the \( \mu \)-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 \( \mu \) H chains but only \( \sim 40\% \) of \( \kappa \) 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 V\( \kappa \) 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 the number of BREC cells. 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\( \rightarrow \)D BREC analysis. Taking together the immunofluorescence and BREC 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 BREC cells 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 \( \sim 1 \times 10^6 \) cells. We conclude that the vast majority of B cells in adult rabbits are generated from...
B lymphopoiesis occurring between birth and 16 wk of age. However, the low level of B lymphopoiesis in adult rabbits may, over time, contribute marginally to the B cell repertoire. These ideas are consistent with several seemingly contradictory observations: although our laboratory finds that essentially all VDJ genes in adult rabbits show evidence of somatic diversification, suggesting that these cells are not newly generated B cells, Mage and colleagues (43) have reported the existence of a small percentage of splenic B cells with undiversified VDJ genes. These undiversified VDJ genes could be derived from newly formed B cells or B cells that have self renewed since early in ontogeny. However, we think that lymphopoiesis in adults contributes little, if any, to the repertoire because of allotype suppression experiments in which neonatal or in utero treatment of rabbits heterozygous for Cκ or VH allotypes with Abs against one of the allelic allotypes resulted in life-long suppression of IgM of that allotype (44, 45). These data demonstrate that B cells expressing the suppressed IgM allotype do not reemerge over time despite trace levels of B lymphopoiesis in adult rabbits. Although we cannot rule out the possibility that B lymphopoiesis occurs at other anatomic sites, we have not found progenitor B cells or BRECs in tissues such as appendix, spleen, and liver of adult rabbits (P. J. J., M. K., and K. L. K., unpublished observations).

Although it might appear that the arrest of B lymphopoiesis would result in adult rabbits with low B cell numbers and a limited Ab repertoire, this is not the case. Rabbits likely compensate for the lack of new B cell production by B cell expansion and somatic diversification of Ig genes in GALT, thereby eliminating the need for continuous B cell development. It follows that if few B cells are produced in rabbits after 16 wk of age, then rabbit B cells must be long-lived and/or self-renewing.

Models of B cell development

Through the study of different species, two major strategies of B cell development have been identified—continuous B lymphopoiesis, as found in mice and humans, and short-term B lymphopoiesis followed by expansion in GALT, as found in chickens, rabbits, sheep, and cows (reviewed in Refs. 46 and 47). In species where continued B lymphopoiesis occurs, B cells develop primarily in the bone marrow and fetal liver, and Ig gene diversity is generated on-site through the process of combinatorial V(D)J joining. In the GALT species, in which B lymphopoiesis is brief, an initial pool of B cells is formed early in life in tissues such as the yolk sac and spleen, and Ig genes diversify in GALT. Although somatic diversification of Ig genes begins before birth in chickens, sheep, and cows, it is likely that exogenous Ag helps drive diversification of the Ab repertoire (48–51), as is the case for rabbits (52). Because B lymphopoiesis rapidly arrests, the B cell compartment must expand and diversify quickly to generate Abs with biologically relevant specificities. In species in which B lymphopoiesis continues throughout life, there may be less pressure to produce a B cell compartment quickly because new B cells with novel specificities are continuously being made. Consequently, there may be no need for GALT in generating the primary Ab repertoire.

Rabbits have features of B cell development similar to those in mice and humans as well as those of GALT species. We consider the rabbit primarily as a member of the GALT species, however, the timing of B lymphopoiesis is extended, lasting ~16 wk. In addition, the primary location of B lymphopoiesis in the rabbit appears to be fetal liver and bone marrow, similar to that of mice and humans. Another interesting characteristic of rabbits is that they use specific members of the intestinal microflora to assist in the formation and selection of the B cell repertoire as rabbits lacking conventional flora show almost no somatic Ig gene diversity (52). Furthermore, GALT does not involute quickly after expansion and diversification of the primary Ab repertoire, which is in contrast to other GALT species, such as chickens and sheep, where
the bursa and ileal 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