

# Evidence for Limited B-Lymphopoiesis in Adult Rabbits

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## Summary

Rabbits are born with a limited VDJ gene repertoire formed primarily by rearrangement of one  $V_H$  gene,  $V_H1$ . The VDJ genes are undiversified at birth but become diversified by  $\sim 2$  mo of age. To investigate more closely the time during which this diversity occurs, we determined the nucleotide sequences of VDJ genes from peripheral blood leukocytes taken from young rabbits at various time points, and we examined the extent of the diversification of the VDJ genes. At 4 wk of age there were, on average, 3 nucleotide changes per  $V_H$  region, with  $\sim 75\%$  of the genes showing some diversification. The number of nucleotide changes per  $V_H$  region increased to 12 by 6–8 wk of age, and all but 1 of the 35 sequences analyzed were diversified. Because only a limited number of genes can be examined by nucleotide sequence analysis, we used an RNase protection assay to examine a large number of genes and we determined the level of undiversified  $V_H1$  mRNA in lymphoid organs of both young and adult rabbits. In young rabbits, we found a high level of undiversified VDJ genes, but the level was greatly reduced by 2 mo of age. By adulthood, essentially all VDJ genes of cells from appendix, peripheral blood, and bone marrow were diversified. Because we had expected B lymphopoiesis to be ongoing in the bone marrow of adult rabbits, we were surprised not to find undiversified VDJ genes from the newly generated B cells. Therefore, we searched for evidence of ongoing B lymphopoiesis in bone marrow by isolating and examining circular DNA for the presence of VD and DJ recombination signal joints. We found highly reduced levels of recombination signal joints in bone marrow of adult rabbits relative to the levels found in bone marrow of newborn rabbits. These data indicate that limited VD and DJ gene rearrangements occur in bone marrow of adult rabbits, and we therefore suggest that B lymphopoiesis is limited in adults.

A crucial event in the development of a functional immune system is the generation of a primary antibody repertoire diverse enough to recognize a vast array of antigens. Many species use combinatorial joining of multiple V, (D), and J gene segments as a means of forming their primary antibody repertoire whereas rabbits use only one  $V_H$  gene in most of their VDJ gene rearrangements. (1, 2) The use of a small number of  $V_H$  genes in VDJ gene rearrangements limits the extent to which combinatorial joining contributes to the diversity of IgH chains in rabbit. As shown by Becker and Knight (3), much of the diversity of rabbit VDJ genes results from a somatic gene conversion-like mechanism in which  $V_H$  genes 5' of the  $V_H$  gene used in the VDJ gene rearrangement are used as donor genes. We identified presumed gene conversion events that diversify regions of VDJ genes spanning more than 125 bp (4).

Consequently, a single gene conversion event can markedly alter the nucleotide sequence of the VDJ gene and lead to changes in the antibody binding site.

Chickens also use somatic gene conversion as a mechanism to generate antibody diversity. In chickens, the 3'-most  $V_H$  and  $V_L$  gene segments are the only functional V genes in the germline, and they are used in all V(D)J gene rearrangements (5, 6). The other V gene segments serve as donor genes in a gene conversion process that diversifies the rearranged VDJ and VJ genes (7, 8). This diversification occurs in the bursa during fetal development so that by the time they hatch, chickens have a large, diverse primary antibody repertoire. In rabbits, the somatic diversification does not occur during fetal development, rather it occurs after birth. Friedman et al. (9) showed that, at birth, all VDJ genes of rabbit B cells are undiversified. In preliminary experiments Knight and Crane (10) found that VDJ genes cloned from peripheral blood leukocytes (PBL) of 7–8-wk-old rabbits were extensively diversified. In the present study we continued these experiments and examined the

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time course of somatic diversification of VDJ genes in normal rabbits during the first few weeks of life. During the course of these studies we found that essentially all VDJ genes of adult rabbits were diversified. These results led us to search for evidence of ongoing B lymphopoiesis in the adult rabbit.

## Materials and Methods

**Rabbits.** The rabbits used were from the colony maintained by K.L. Knight at Loyola University Chicago. The adult rabbits used in these studies were from 1 to 2 yr of age. The 4–8-wk-old rabbits used in the PCR amplification studies and in the RNase protection studies were progeny of  $V_{H2}$  allotype rabbits. Bone marrow cells used for detecting VD and DJ recombination circle DNA were obtained from progeny of  $V_{H3}$  allotype rabbits.

**Cloning and Nucleotide Sequence Analysis of VDJ Genes.** PBL RNA was obtained from ~25 ml of blood. The blood was centrifuged at 2,000 rpm for 20 min, and the cells from the buffy coat were collected. Red blood cells were lysed in hypotonic phosphate-buffered saline and the leukocytes were suspended in guanidine thiocyanate (GIT) (4 M guanidine thiocyanate, 0.025 M sodium acetate, pH 6.0). RNA was isolated by ultracentrifugation through CsCl (11). The cDNA was synthesized from RNA (3  $\mu$ g) by using oligo dT as primer and reverse transcriptase (12). The cDNA was PCR amplified by using as 5' primer,  $V_{H1}$ -B, (5'**CTG-CAGCTCTGGCACAGGAGCTC**3') located 75 bp upstream from the ATG of the leader region of  $V_{H1}$  and as 3' primer,  $C_{H1}$ - $\mu$ , (5'**TCAGGAATTCGGAGACGAGCGGTACAGAGT**3') located 19 bp downstream of the 5' splice site of  $C_{H1}$  exon 1. (Bold lettering indicates the restriction sites incorporated into each primer.) The PCR products were cloned into M13mp19, and their nucleotide sequences were determined (13, 14).

**$V_{H1}$ -using  $V_H$  Genes.** The nucleotide sequences of the VDJ genes from rabbit PBL were compared with the nucleotide sequences of genes known to be used in VDJ gene rearrangements,  $V_{H1}$ ,  $V_{H2}$ ,  $V_{H3}$ , and  $V_{H4}$  (1, 9) and the used gene was identified by its similarity to the framework regions of these four germline  $V_H$  genes. Only the  $V_{H1}$ -using genes were further analyzed in this study. To search for potential donor genes for gene conversion-like events, we compared the regions of the clones that differed from  $V_{H1-a2}$  ( $V_{H1}$  from an  $a2$  allotype rabbit) with the nucleotide sequences of  $V_{H2}$ – $V_{H9}$ , the eight  $V_H$  genes immediately upstream of  $V_{H1}$  from the  $a2$  chromosome (accession numbers M93172, M93175, M93178, M93182, and U51025–U51029).

**RNase Protection Assay.** Tissue RNA was obtained by homogenizing 4 g of tissue in GIT buffer and pelleting through CsCl in the ultracentrifuge (11). Germline  $V_{H1-a2}$ , including the leader exon and the leader intron, was cloned as a 550-bp PstI fragment into pGEM3 vector. The clone was subsequently used to generate the probe for the RNase protection assay. The  $^{32}$ P-labeled antisense RNA probe, which was transcribed from 1  $\mu$ g of linearized template plasmid by using T7 RNA polymerase, was hybridized overnight with up to 20  $\mu$ g of total cellular RNA (15, 16). The leader region of  $V_H$  genes is nonpolymorphic and generally remains undiversified. Consequently, the leader-encoding regions of total  $V_H$  mRNA protect the leader portion of the probe from digestion by RNase and, in this way, serve as a control for the total amount of Ig mRNA. After the  $^{32}$ P-labeled antisense RNA probe was hybridized with total cellular RNA, the mixture was treated with RNase and subsequently electropho-

resed on 5% polyacrylamide gels (15). RNase degrades the leader intron of the probe, leaving fragments of 120 bp if the leader region is protected as well as fragments of 250 bp if the  $V_H$  region is protected (Fig. 3 A). On the basis of the ability to protect the leader portion of the probe, the total Ig content was adjusted to a similar level in each lane of the gel.

In experiments with RNA from B cells, mesenteric lymph node cells were labeled with FITC-conjugated goat anti-rabbit Ig and B cells were purified by FACS<sup>®</sup>. Subsequently the cells were shown by flow cytometric analysis to be >95% Ig<sup>+</sup>.

**Detection of VD and DJ Recombination Signal Joints.** Closed circular DNA was isolated from bone marrow of newborn and adult rabbits by the method of Carroll et al. (17). Bone marrow was collected and  $10^7$  cells were lysed in 1% alkaline-SDS (pH 12.45). The chromosomal DNA was sheared by vortexing, denatured in the alkaline-SDS solution, and then removed from the circular DNA by phenol extractions. The DNA circles were recovered by ethanol precipitation. The VD signal joints were PCR amplified (30 cycles of amplification) from the circle DNA by using as 5' primer, 5'D2b (5'AGAGACTCACCACACTTCACCATGCTGCAG3') and as 3' primer, 3' $V_{H1a3A}$  (5'AATCTGGGCTCAGT-GCGTCTTGTGCG3') (Fig. 4 A, primers D and C, respectively). The DJ signal joints were PCR amplified (30 cycles of amplification) by using, as 5' primer, 5'J3 (5'CTCTCTCTG GAGTCCTCTGGTTC3') and, as 3' primer, 3'D2b (5'CACTAG-GATCCTGGGCCGCTGAGAATCTGT3') (Fig. 4 A, primers B and A, respectively). (Bold lettering indicates a restriction site incorporated into the primer.) To detect the PCR products that may be present at a low level, 0.05  $\mu$ Ci [ $^{32}$ P]dCTP was added to each of the PCR reactions. The products were then analyzed by electrophoresis on 5% polyacrylamide gels (PAGE) and by autoradiography. The specificity of the PCR was confirmed by Southern blotting and hybridization with three different probes, 500-bp SalI fragment from 3' of  $V_{H1}$ , pJ5 (18), and 500-bp probe from germline D2b (18). Further the specificity of the PCR product was established by cloning them into pGEMT vector and determining the nucleotide sequence.

To control for the efficiency of the recovery of circle DNA among different preparations, we added a constant amount of a control plasmid to each sample of  $10^7$  bone marrow cells before the alkaline lysis step in the purification of circle DNA. The control plasmid contained a VD joint that was interrupted by an unrelated sequence of 120 bp. Therefore, PCR amplification of the control plasmid results in a larger product (270 bp) than the product obtained from amplification of the VD joints from bone marrow (156 bp).

DJ recombination signal joints in bone marrow cells of mouse were determined in a manner similar to that described above. Closed circular DNA was isolated from  $10^7$  bone marrow cells from eight 1-wk-old and two 4-mo-old (adult) Balb/c mice. The DJ circles were PCR amplified (30 cycles of amplification) from the circle DNA by using as 5' primer, 5'MuJ4 (5'TAATGTCTG-AGTTGCCCAAGGGTGA-3') taken from the nucleotide sequence 50 bp upstream of germline J4 and as 3' primer, 3'DQ52 (5'-TTCTCTGCACTGTCTCCTGACCTCC-3'), taken from the nucleotide sequence 130 bp downstream of germline DQ52. The PCR was performed with [ $^{32}$ P]dCTP and the products were analyzed by PAGE and autoradiography. We confirmed that the PCR-amplified products were signal joints by Southern hybridization of the products separated by PAGE, using a probe of 200 bp that was PCR-amplified from genomic DNA of Balb/c mice using the 3'DQ52 primer and a 5'DQ52 primer (5'-ACCCAT-ACTCTGTGGCTAGTGTGAG-3') taken from 70 bp upstream

of germline DQ52. The nucleotide sequence of this probe was established after cloning into pGEMT vector.

## Results

**Time Course of VDJ Gene Diversification in B Cells of Young Rabbits.** We cloned VDJ genes from PBL of 4–8-wk-old rabbits and examined the extent of somatic diversity. We used PBL in this experiment so that we could examine the progression of diversification of VDJ genes in individual rabbits. The nucleotide sequence of each VDJ-C $\mu$  gene was compared with that of germline  $V_H1$  to identify the VDJ genes that used  $V_H1$ . We then analyzed those genes to determine whether they had been somatically diversified. Whereas previous experiments by Friedman et al. (9) showed that the VDJ genes of newborn to 1-week-old rabbits were essentially undiversified (Fig. 1 A), we found that at 4 wk of age, the VDJ genes in each of three rabbits examined were diversified, albeit to a limited extent (Fig. 1 B). The average number of mutations per  $V_H$  region was 3.3, with four  $V_H$  genes having no mutations. In rabbits 6–8 wk of age, we found a much higher level of diversity, an average of 11.5 mutations per  $V_H$  region. In the older rabbits, only one gene was completely undiversified. These data show that, until 4 wk of age, the VDJ genes remain relatively undiversified but that they then diversify rapidly, such that by 6–8 wk of age, nearly all of the sequences are diversified.

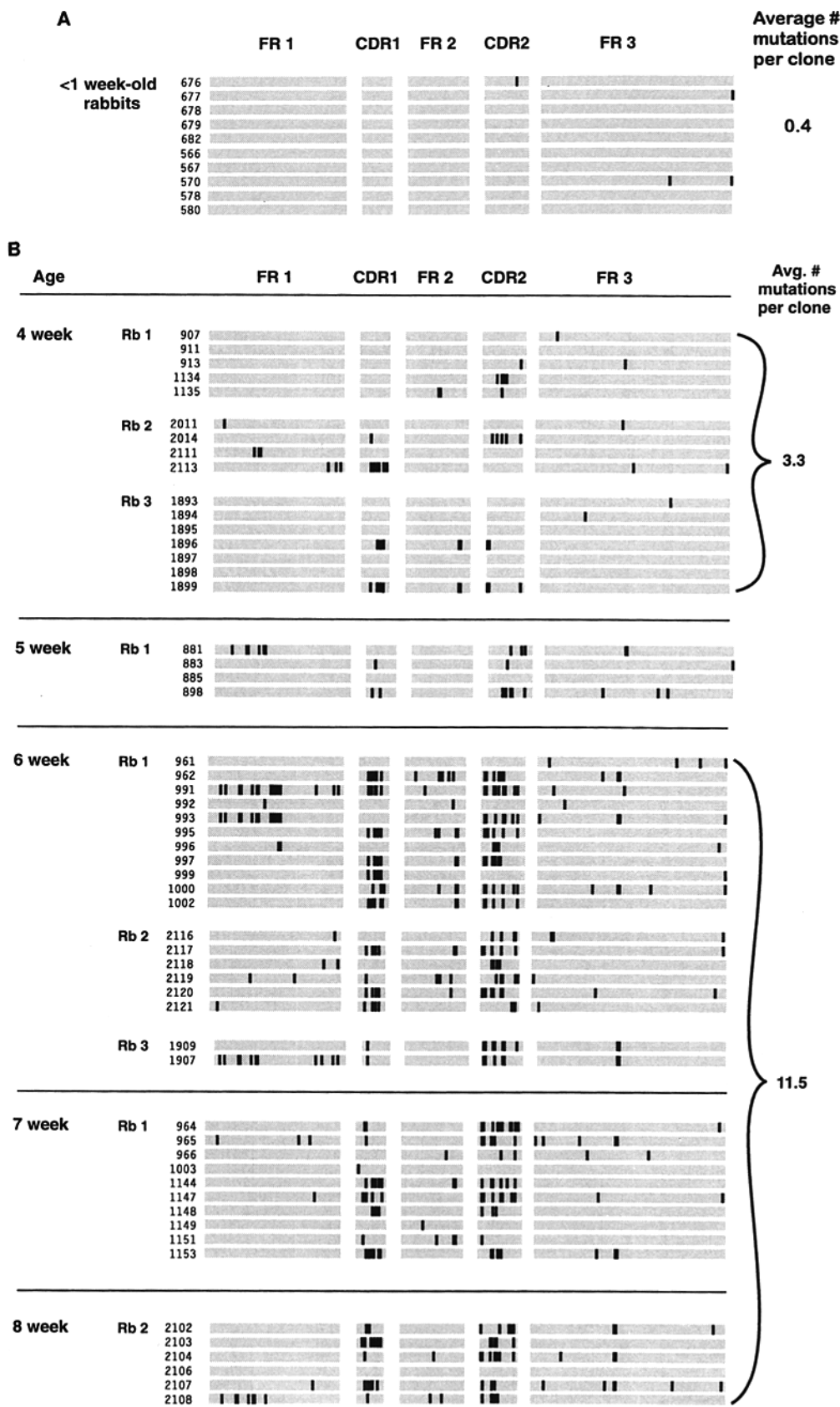
The somatic diversity in the  $V_H$  region of the VDJ genes was found predominantly in CDR1 and CDR2, although the framework regions were also somewhat diversified. To determine whether some of the diversification could be explained by somatic gene conversion, we selected regions of the VDJ genes that had clusters of nucleotide changes, and we searched our  $V_H$  genebank from 2 rabbits for  $V_H$  genes that had nucleotide sequences identical to the diversified regions. We found potential  $V_H$  donor genes for several clusters of nucleotide changes (Fig. 2). For example, we found eight clones in which the nucleotide changes were identical to the sequence of  $V_H3$ , a pseudogene located ~10 kb 5' of  $V_H1$  (19). We concluded that these VDJ genes were diversified by a gene conversion-like mechanism that used  $V_H3$  or a  $V_H3$ -like gene as donor. Similarly, we found one clone, 1909, that may have used  $V_H9$  as a donor in a gene conversion event that spanned at least 134 bp. We also found seven clones in which  $V_H7$  may have been used as a donor gene. Both  $V_H7$  and  $V_H9$  are pseudogenes located 30 kb and 40 kb 5' of  $V_H1$ , respectively (19 and unpublished data). Because these examples include VDJ genes from B cells of 4-wk-old rabbits, we conclude that the onset of gene conversion coincides with the onset of somatic diversification.

**Search for Undiversified VDJ mRNA by RNase Protection Assay.** The data from the time course experiment suggested to us that in rabbits 6 wk of age and older, most of the VDJ genes from PBL were diversified. Because we could examine only a limited number of VDJ genes by nucleotide sequence analysis, we extended these studies by using the RNase protection assay,

an assay that would allow us to screen VDJ genes from a large number of B cells. We synthesized an mRNA probe from germline  $V_H1$ , which contained both the nonpolymorphic leader region and the region spanning FR1 through FR3 (Fig. 3 A). The  $V_H$  portion of the probe was protected from RNase digestion only by mRNA derived from undiversified  $V_H1$ -using VDJ genes. We measured the level of undiversified  $V_H1$  mRNA from various lymphoid tissues in rabbits whose age ranged from 17 d to adult. For mRNA isolated from the appendix, we found that much of the  $V_H$  probe was protected by mRNA obtained from young rabbits (17 d to 5 wk old), but as the rabbits aged, the amount of protection decreased (Fig. 3 B). By 17 wk of age, very little of the  $V_H$  portion of the probe was protected. By adulthood, virtually none of it was protected, indicating that most of the VDJ genes in the B cells of the appendix of adult rabbits were diversified. Even though the amount of total  $V_H$  mRNA is somewhat less in samples from the older rabbits, as evidenced by the amounts of protected leader portion of the probe, the differences between the amounts of total mRNA cannot explain the lack of protected  $V_H$  probe in the older rabbits. Rather, the decrease in the level of  $V_H1$  RNA probe must be from increased diversity of the VDJ genes in B cells that use  $V_H1$  in their VDJ gene rearrangements. To confirm the data obtained from studying appendix cells, we tested whether the  $V_H1$ -using VDJ genes of PBL from adult rabbits are also diversified. We found that mRNA from PBL pooled from four adult rabbits also did not protect the  $V_H$  probe (Fig. 3 C), suggesting that most, if not all,  $V_H1$ -derived mRNA in the periphery is diversified in adult rabbits.

Ig-secreting plasma cells, most of which probably have diversified VDJ genes, reportedly have ~100 times more Ig mRNA than resting B cells (20). Therefore, we were concerned that the reason we detected little, if any, undiversified  $V_H1$  RNA in B cells was because the relative amount of mRNA derived from B cells was low in comparison to that obtained from plasma cells. To eliminate this possibility, we purified B cells and tested whether the RNA protected the  $V_H1$ -RNA probe from digestion by RNase. In each of two experiments we found that the B cell RNA did not protect the  $V_H1$  probe (Fig. 3 D), indicating that the VDJ genes of most, if not all, B cells from adult rabbits are diversified.

We expected to find undiversified VDJ genes in bone marrow cells of adult rabbits because we assumed that B-lymphopoiesis was ongoing and that newly generated B cells would have undiversified VDJ genes. In previous experiments we isolated mRNA from bone marrow of young and adult rabbits and tested whether the mRNA protected a  $V_H1$ -RNA probe from digestion by RNase (10). We found that, whereas the mRNA from bone marrow of a 5-wk-old rabbit protected a considerable amount of the  $V_H$  probe, mRNA from each of three adult rabbits protected only a trace amount of the  $V_H$  probe (Fig. 3 E). These data indicate that bone marrow contains few, if any, B-lineage cells with undiversified VDJ genes.



**Figure 1.** Nucleotide sequence analysis of V regions of PCR-amplified  $V_H1$ -using VDJ genes from spleen and appendix of newborn and from PBL of 4–8-wk-old rabbits. (A) Newborn to 7-d-old rabbits from Friedman et al. (9). (B) 4–8-wk old rabbits. Vertical bars represent nucleotides different from germline  $V_H1$ . The average number of mutations per  $V_H$  region (excluding D and J regions) is shown.

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      CDR1          FR2          CDR2          FR3
VH1 CCTCAGTAGCAATGCAATAAGCTGGTCCGCCAGGCTCCAGGGAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCT
995 .....T...G.G.G.....
999 .....T...G.G.G.....
2120 .....T...G.G.G.....
1899 .....T...G.G.G.....AC...TT....
997 .....T...G.G.G.....AC...TT....
2117 .....T...G.G.G.....AC...TT...A...G...A.....
1896 .....G.G.G.....AC...TT....
1000 .....G.G.G.....AC...TT...A...G...A.....
Ψ VH3 .....T...G.G.G.....AC...TT...A...G...A.....

      CDR1          FR2          CDR2          FR3
VH1 TCCCTCAGTAGCAATGCAATAAGCTGGTCCGCCAGGCTCCAGGGAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGGCAAAAGCCGATCCACCATCACCAGAAACCAACCTGAACACGGT
1909 .....T.....AT...AA...TA...A.....GA.....
Ψ VH9 .....T.....AT...AA...TA...A.....GA.....

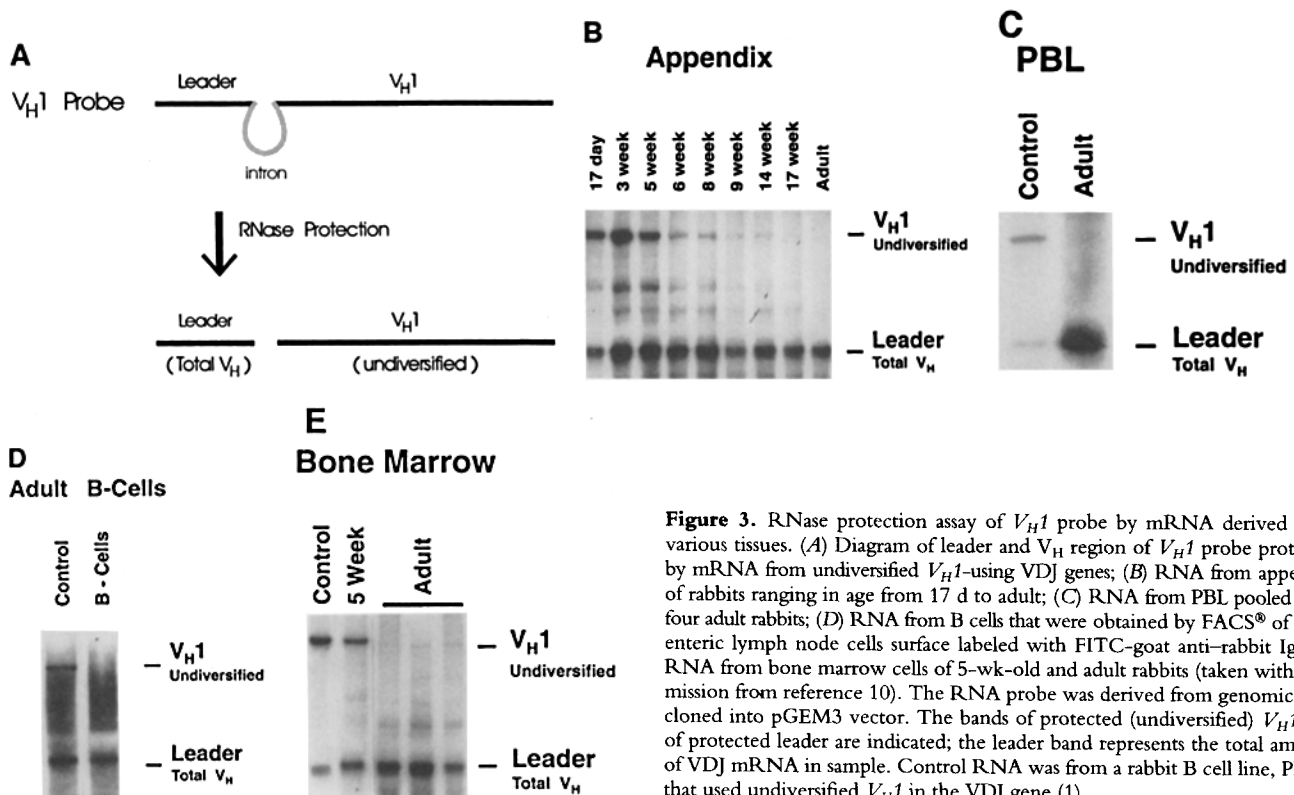
      FR1          CDR1          FR2          CDR2
VH1 AGTCGGTGAAGGAGTCCGAGGGAGGCTCTCTTCAAGCCAACGGATACCCCTGACACTCACCCTGCACAGTCTCTGGATTCTCCCTCAGTAGCAATGCAATAAGCTGGTCCGCCAGGCTCCAGGGAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTAC
881 .....G.....AG.....C.GA.....
2108 .....G.....AG.....C.GA.....T.....
991 .....C.G.....AG.....C.GA.....TGGA.GA.....C.....A.A.....T.C.AC...G.....
2113 .....C.....AG.....C.GA.....TGGA.GA.....C.....A.A.....T.C.AC...G.....
962 .....T.C.AC...G.....A.....G.A...G.....TA...A..TA.G.....
2119 .....T.C.AC...G.....A.....G.A...G.....TA...A..TA.G.....
1134 .....C.G.....AG.....C.GA.....TGGA.GA.....C.....A.A.....T.C.AC...G.....A.....G.....TA...A..TA.G.....
Ψ VH7 .....C.G.....AG.....C.GA.....TGGA.GA.....C.....A.A.....T.C.AC...G.....A.....G.....TA...A..TA.G.....

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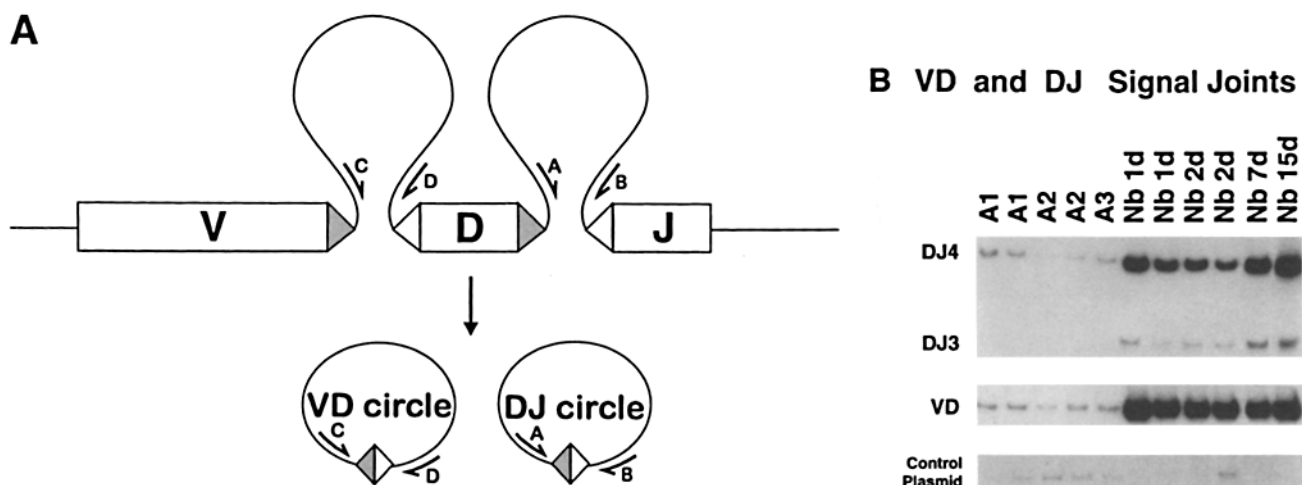
**Figure 2.** Nucleotide sequences of segments of VDJ genes (taken from Fig. 1) from young rabbits compared with germline  $V_H1$  and with germline  $\psi V_H3$ ,  $\psi V_H7$ , and  $\psi V_H9$  which may have been used to diversify the VDJ genes by somatic gene conversion.

*Search for Ongoing VDJ Gene Rearrangement in Adult Rabbits.* Because we found few, if any, B-lineage cells with undiversified VDJ genes in the bone marrow of adult rabbits, we postulated that new B cells were not being produced. To examine this possibility, we searched for evidence of VDJ gene rearrangements in bone marrow by testing for the pres-

ence of VD and DJ recombination signal joint circles produced during VDJ gene recombination. We isolated closed circular DNA from bone marrow of neonatal and adult rabbits and performed PCR, in the presence of [ $^{32}$ P]dCTP, with primers designed to amplify the recombination signal joints (Fig. 4 A). Although large amounts of VD and DJ



**Figure 3.** RNase protection assay of  $V_H1$  probe by mRNA derived from various tissues. (A) Diagram of leader and  $V_H$  region of  $V_H1$  probe protected by mRNA from undiversified  $V_H1$ -using VDJ genes; (B) RNA from appendix of rabbits ranging in age from 17 d to adult; (C) RNA from PBL pooled from four adult rabbits; (D) RNA from B cells that were obtained by FACS<sup>®</sup> of mesenteric lymph node cells surface labeled with FITC-goat anti-rabbit Ig. (E) RNA from bone marrow cells of 5-wk-old and adult rabbits (taken with permission from reference 10). The RNA probe was derived from genomic  $V_H1$  cloned into pGEM3 vector. The bands of protected (undiversified)  $V_H1$  and of protected leader are indicated; the leader band represents the total amount of VDJ mRNA in sample. Control RNA was from a rabbit B cell line, PBL1, that used undiversified  $V_H1$  in the VDJ gene (1).



**Figure 4.** Autoradiograph of VD and DJ circular DNA generated by V, D, and J gene rearrangement. (A) Diagram of VD and DJ circular DNA indicating the regions from which PCR primers A, B, C, and D were derived. (B) PAGE autoradiograph of PCR-amplified VD and DJ recombination signal joints from circle DNA of bone marrow from newborn and adult rabbits. Bone marrow-derived circle DNA was from three adults (A1, A2, and A3) and four newborns at 1, 2, 7, and 15 d of age (Nb 1d, Nb 2d, Nb 7d, and Nb 15d). PCR amplification of DNA from A1, A2, Nb 1d, and Nb 2d was performed in duplicate. DJ3 and DJ4 refer to bands representing PCR-amplified circle DNA generated from rearrangements of D2b to J3 and J4, respectively, which were the only products of DJ circles found at a significant level in this study. VD refers to a band representing PCR-amplified product from circle DNA generated from rearrangements of  $V_H$  to D2b, a frequently used D gene segment. The control plasmid controls for efficiency of the isolation of circle DNA (see Materials and Methods). All circle DNA samples for this experiment were isolated simultaneously, with each one receiving the same amount of control plasmid prior to isolation of circle DNA. Similar results were obtained from duplicate circle DNA preparations made for each tissue sample and from replicate PCR amplification of all circle DNA preparations. Several dilutions of each circular DNA preparation were used for PCR amplification and in each case the amount of amplified product was proportional to the dilution.

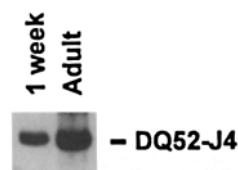
signal joints were amplified from DNA isolated from each of four bone marrow samples from neonatal rabbits, the amount of VD and DJ circles amplified from DNA isolated from each of three bone marrow samples from adult rabbits was much less (Fig. 4 B). We controlled for the loss of circle DNA during the isolation procedure by adding a constant amount of control VD plasmid to each sample before preparation of the circle DNA. The amount of PCR-amplified control VD plasmid was generally greater in the samples from adults than in the samples from newborns, indicating that the purification of circle DNA from the adult samples was at least as efficient as that from the newborn samples (Fig. 4 B). The decreased level of VD and DJ signal joints in bone marrow samples from adult rabbits indicates that there were many fewer ongoing VDJ gene rearrangements in bone marrow of adult rabbits than in bone marrow of newborn rabbits. The paucity of ongoing VDJ gene rearrangements in cells of bone marrow from adults indicates that B lymphopoiesis in adults is limited.

In control experiments, we tested for DJ recombination circles in bone marrow of adult mice because B lymphopoiesis in mice continues into adulthood. We isolated circle DNA from bone marrow of young and adult mice and PCR-amplified DJ signal joints by using primers derived from 3' of germline DQ52 and 5' of JH4. We found ample amounts of PCR-amplified DJ recombination signal joints in samples from both adult and young mice (Fig. 5) suggesting that in contrast to rabbit bone marrow in which we found evidence for only limited ongoing VDJ gene rearrangements in adults, IgH gene rearrangements are abundant in bone marrow of adult mice.

## Discussion

We performed a time course experiment to determine the extent of diversification of  $V_H$  genes in rabbits of various ages by using nucleotide sequence analyses and RNase protection assays. Nucleotide sequence analyses showed that at 4 wk of age, 75% of the cloned  $V_H$  regions were diversified, with an average of three mutations per  $V_H$  region, and that by 6–8 wk of age, 97% of the sequences were diversified, with an average of 12 mutations per  $V_H$  region. Using the RNase protection assay, we found that the amount of mRNA from undiversified  $V_H1$ -using VDJ genes decreased with age in both B-lineage cells of the periphery, such as those in blood and the appendix, and in B-lineage cells of the bone marrow. By adulthood, which in rabbits is reached at  $\sim 5$  mo of age, we found little or no undiversified  $V_H1$  mRNA indicating that essentially all of the VDJ genes in B cells that use  $V_H1$  in their VDJ gene rearrangements were diversified.

If B cells were continuously generated in the bone marrow of adult rabbits, one would expect to find undiversi-



**Figure 5.** Detection of murine DJ circular DNA generated by rearrangement of DQ52 and JH4 gene segments. Autoradiograph of PCR-amplified DJ recombination signal joints from circle DNA of bone marrow from young (1–2 wk old) and adult (4 mo old) mice. The PCR-amplification was performed with [ $^{32}$ P]dCTP and the products were analyzed by PAGE. The observed bands on the autoradiograph were confirmed to be DJ recombination signal joints by nucleotide sequence analysis of the cloned product and by Southern hybridization of the PCR-amplified products using the 200-bp DQ52 probe (see Materials and Methods).

fied VDJ genes in bone marrow throughout the life of the rabbit. However, we found almost no undiversified genes in rabbits older than 8 wk of age, which suggests that generation of new B cells in adult rabbits is very limited. We therefore searched for evidence of B lymphopoiesis by testing for ongoing VD and DJ gene rearrangements in bone marrow. By PCR, we found extensive amounts of VD and DJ signal joints in newborns but little or none in adults. This is in contrast to the situation in mice in which large amounts of DQ52-J4 signal joints are found in adult mice. It is important to note that detection of large amounts of these signal joints occurs even though the DQ52 gene segment is not frequently used in adult mice (21). This indicates to us that this assay is sufficiently sensitive to detect signal joints from infrequently rearranged IgH gene rearrangements. Therefore, even if  $V_{H1}$ , D2b and J4 were rearranged at low levels in adult rabbits, we think that we would detect them in our assay. In our experiments, D2b is the most important of these gene segments because the detection of both VD and DJ gene rearrangements relies on rearrangement of D2b. Although we cannot rule out the possibility that VD and DJ gene rearrangements occur in adult rabbits and that D2b is not used in these rearrangements, nucleotide sequence analysis of VDJ genes from adult rabbits does not support this possibility. Not only do we not find new D segments used, but we find that D2b is used in 25–30% of VDJ genes in both young and adult rabbits (4, 9).

Because of the limited VD and DJ gene recombination in bone marrow of adult rabbits, we wonder whether such recombination occurs in other tissues. In preliminary experiments, we have not found evidence for VD and DJ signal joints in peripheral lymphoid tissues including appendix, spleen, Peyer's patches, sacculus rotundus, mesenteric lymph node, and PBL. Therefore, we think that little, if any, B lymphopoiesis occurs in adult rabbits. If new B cells are not generated throughout the lifetime of the rabbit, then the B cells that were produced early in ontogeny may be long-lived and/or self-renewing. To evaluate this possibility, we need to determine directly the production rate of the various precursor B cell populations in the bone marrow and the life span of peripheral B cells.

Most PBL in humans and mice are IgM<sup>+</sup>IgD<sup>+</sup> naive B cells, and human and mouse VDJ and VJ genes are undiversified, even in adults (22–24). In contrast, nearly all of the VDJ genes in PBL of adult rabbits are highly diversified and yet the adult rabbit is very capable of mounting primary antibody responses to previously unseen antigens. This suggests that rabbits do not seem to have naive B cells in the same way as do mice and humans. These observations have led us to propose a model for the development of the antibody repertoire in rabbit that is different from the way that other species generate their antibody repertoires. In our model, rabbit B cells with undiversified VDJ genes are generated in the bone marrow during the early part of life (19) and then within the first few weeks of life, they migrate to the gut-associated lymphoid tissue (GALT)<sup>1</sup> where

they diversify their VDJ genes (see below). We suggest that these B cells are the rabbit's naive B cells because when they migrate to the periphery, even though their VDJ genes are already diversified, they are capable of mounting primary antibody responses. Because it appears there is a limited amount of B-lymphopoiesis in adult rabbits, we also postulate that rabbit B cells are long-lived and/or self-renewing. In this case the B cells that emigrate from GALT with diversified VDJ genes are the ones that maintain the functional antibody repertoire in the adult rabbit.

Data supporting this model for development of the antibody repertoire was reported by Weinstein et al. (25) who showed that at least some of the diversification of the VDJ genes occurs in the appendix. Although we do not know what factors trigger the diversification process, because of results from germ-free rabbits in which investigators found that both the anatomy and the function of the immune system were altered by the lack of microbial stimulation (26–28), we suggest that the normal microbial flora of the gut plays a role in stimulating diversification (10). In the germ-free rabbits, the secondary lymphoid organs were dramatically underdeveloped, with no germinal centers present until at least three and a half months of age. Also, the total numbers of lymphoblasts and lymphocytes were less than those of normal rabbits. These germ-free rabbits produced only low levels of antibodies, either spontaneously or after immunization with antigen. Together these observations indicate that a normal microbial flora plays an essential role in the development of the humoral immune response. We suggest that germ-free rabbits do not develop a normal B cell repertoire because they are missing the stimulus from the microbial flora that normally drives the B cells to proliferate and diversify. Pospisil et al. (29) have recently shown that in the appendix of rabbit, B cells that express  $V_H$  genes encoding characteristic framework regions are positively selected. We suggest that some component of the microbial flora binds to a  $V_H$  framework region and serves as the stimulus for B cells not only to proliferate but also to diversify their VDJ genes.

GALT has now been shown to be important for diversifying Ig genes for chicken, sheep, and rabbit. Chicken Ig genes diversify before hatching by somatic gene conversion in the bursa (30), and the Ig genes of sheep undergo somatic diversification by somatic mutation in ileal Peyer's patches (31). The VDJ genes of rabbit, however, diversify by a somatic gene conversion-like mechanism, some of which occurs in the appendix (3, 25). This diversification does not occur until a few weeks after birth. While we postulate that the diversification of Ig genes in rabbit probably requires exogenous stimuli, such as microbes, the VDJ genes of chickens and sheep diversify independent of exogenous stimuli (32). Regardless of the importance of exogenous stimuli, we suggest that GALT is an important site for generating the antibody repertoire in many, if not all, species.

The rabbit provides an interesting system for studying B cell development and the generation of antibody diversity. Several important issues need to be addressed. These include (a) the turnover rate of progenitor B cells in bone mar-

<sup>1</sup>Abbreviation used in this paper: GALT, gut-associated lymphoid tissue.

row of adults; (b) the trafficking patterns of newly generated B cells; (c) the role of GALT (and/or other tissues) in the development of the primary antibody repertoire; (d) what factors, if any, trigger the VDJ genes of B cells to di-

versify; and (e) whether rabbit B cells are long-lived and/or self-renewing. The resolution of these issues will expand our knowledge of antibody repertoire development in rabbits as well as in other species.

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