BIASED EXPRESSION OF JH-PROXIMAL VH GENES OCCURS IN THE NEWLY GENERATED REPERTOIRE OF NEONATAL AND ADULT MICE

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The large diversity of antibody molecules produced by B lymphocytes is accomplished in part by the somatic assembly of multiple germline gene segments that encode the variable region of the molecule. Variable region genes are assembled during the antigen-independent phase of B cell differentiation, which in mammals occurs in primary B cell differentiation organs (fetal and neonatal liver and bone marrow) (reviewed in reference 1). Precursor B (pre-B) lymphocytes assemble and express heavy and light chain genes to become surface Ig+ B cells. The set of variable genes expressed by the population of newly formed B lymphocytes is the "newly generated" antibody repertoire, which is unselected by external antigens (reviewed in reference 2). B lymphocytes migrate to peripheral lymphoid organs, such as the spleen and lymph nodes, where they may mature into Ig-secreting cells (plasma cells) if appropriately stimulated (1).

The heavy chain variable region is assembled from three germline elements: variable (VH), diversity (D), and joining (JH) segments (reviewed in reference 3). The estimated 100-1,000 (4, 5) murine VH gene segments have been grouped into 11 families of related genes based on nucleotide sequence homology (4, 6-8). Murine VH gene families are generally clustered on the chromosome, but there is extensive interspersion of some families (9-13). The relative chromosomal positions of VH gene families have been determined to be as follows: (VHJ558-VHJ3609)-VHJ606-(VHJAM3.8-VHJ36-60-VHJ24-VHJ3609)-DJH (11-13); families with significant interspersion of members are indicated in parentheses (9-13). Analysis of Abelson murine leukemia virus-transformed pre-B cell lines (10, 14, 15) and fetal liver hybridomas (14, 16) suggested that selection of VH gene segments for VH to DJH rearrangements in pre-B cells was biased; VH segments from the most JH-proximal VH gene families were preferentially used. More recently, we demonstrated that the VH repertoire expressed in neonatal liver is also biased with dis-
proportionately high expression of JH proximal gene segments (17). Together, the data from the cell lines and neonatal liver suggest that developing pre-B cells generated a biased VH repertoire as a result of mechanistic constraints on the Ig gene assembly process that leads to preferential rearrangement and expression of JH-proximal VH segments.

The pattern of VH gene family expression in the adult spleen differs dramatically from that observed in the neonatal liver (17). Several different analyses have shown that the VH repertoire in adult spleen is "randomized" such that expression of VH gene families correlates approximately with the size (i.e., number of individual members) of the family encoded in the genome, suggesting that individual VH gene segments are represented at equal frequencies (17-20). Although strain-dependent variations that may not be due to the size of the VH gene family have been observed (17, 20-22), for simplicity we will refer to the shift away from the position-related VH repertoire of the neonatal liver to the distribution observed in adult spleen as the "randomization" of the repertoire. Randomization could be effected by several, not mutually exclusive, mechanisms. One possibility is that programmed changes in recombinational mechanisms may alter the frequency with which different VH segments are used during development; according to such a model the rearrangement mechanism operating in fetal pre-B cells would preferentially use proximal VH gene segments, while that operating later in ontogeny randomly use all VH gene segments (14, 16, 17, 23). Alternatively (and not mutually exclusively), rearrangement frequencies of VH gene segments in pre-B cells could be the same throughout ontogeny but cellular selective interactions acting subsequent to V gene rearrangement would normalize the representation of the various VH gene families in mature B cell populations. To elucidate mechanisms that operate to shift the VH repertoire from the biased pattern observed in the neonatal liver to the more random pattern observed in the adult spleen, we assayed the patterns of VH expression in primary and peripheral lymphoid organs at various times in ontogeny. We find that the biased VH repertoire is apparent early in fetal ontogeny and is constant throughout fetal and neonatal development until 5-7 d after birth. Furthermore, we find that the primary repertoire generated in adult bone marrow is also biased with respect to expression of JH-proximal VH gene segments. Together, these findings suggest that selection, rather than changes in rearrangement frequency, is the major contributor to randomization of the VH repertoire in adult splenic B cells.

Materials and Methods

Mice. BALB/cByJ and C57BL/6J mice of specified age were purchased from The Jackson Laboratory (Bar Harbor, ME). Fetal and newborn samples were derived from timed-pregnant matings purchased from The Jackson Laboratory. C.B-17 and C.B-17scid mice (hereafter designated scid mice) were maintained in specific pathogen-free animal facilities at the Institute for Cancer Research (Philadelphia, PA) and were provided by Dr. M. Bosma and G. Bosma (Fox Chase Cancer Center, Philadelphia, PA). The scid mutation occurred in the C.B-17 inbred strain, a congenic partner of BALB/cAnICR that has the Igh^h haplotype from C57BL/Ka. Unless otherwise indicated, spleen and bone marrow samples were pooled from two to three mice and fetal and neonatal liver samples were pooled from three to five litters. Adult samples were removed at 5-6 wk of age; otherwise samples were removed at the age indicated in the text.

Preparation of Tissue Samples. Spleens and livers were dissected and RNA isolated without
Bone marrow cell suspensions were prepared from femurs and tibias by perfusion with media containing FCS and washed once in PBS.  

**VH and Cp Probes.** The VH-specific probes representative of each of the VH gene families and the Cp probe (μS) have been described in detail previously (17). The VH81X and VH.B4 (17) probes were used to identify the VH7183 and VH.J558 families, respectively. The μS probe is the 1.4-kb Pst I fragment isolated from the genomic DNA clone μ5 (24) which encodes the second exon and 3' untranslated portion of the membrane form of μ mRNA.

**Preparation of RNA and Northern Blotting.** Total and poly(A)* RNA were prepared as previously described (25). Northern blotting, probe preparation, and hybridization procedures have previously been described (25).

**VH Utilization Assay.** The assay to measure utilization of VH gene families in tissue samples has been described in detail previously (17). Briefly, total RNA from spleen or bone marrow or poly(A)* RNA from liver samples were titered for the level of Cp expression and amounts of RNA that gave approximately equal signals were assayed for hybridization to VH-specific probes. The amounts of RNA differed for the various tissue samples approximately as follows: 100-200 μg poly(A)* RNA from fetal and neonatal livers; 150-200 μg total RNA from spleens day 1 to day 7; 5 μg from spleens at 2-3 wk; 1-2 μg total RNA from adult spleen; 5-10 μg total RNA from adult bone marrow. Replicate Northern blots were hybridized to each of the VH or μ probes and signal intensities were quantitated. The VH-to-Cp ratio was calculated for each sample. Preference values are a comparison of the relative expression of a particular VH gene family in a primary lymphoid organ (that is, fetal liver, neonatal liver, or adult bone marrow) to that expressed in normal, adult spleen. Thus a preference value of 1 indicates that a VH family comprises an equivalent proportion of the μ mRNA in the experimental sample as in the adult spleen; a value >1 indicates the proportion to which that family is over-represented compared with adult spleen; and a value <1 indicates the proportion to which that family is under-represented compared with adult spleen. Within each experiment, all samples were compared with the same adult samples so that values between experimental samples can be directly compared. The percent expression of VH gene families was calculated by comparison of the VH to Cp ratio in the tissue samples to the ratio obtained in reference cell lines, as previously described (17).

**Densitometry.** Densitometry was performed on multiple exposures of the autoradiograms using the Joyce Loebl Chromoscan 3.

**Results**

**Expression of VH Gene Families Is Constant Throughout Fetal and Neonatal Development.** To determine if the relative expression of VH gene families changes during fetal development, we analyzed VH expression in fetal livers at different times during gestation. The predominant Cp-hybridizing mRNA species in fetal liver between days 15 and 17 fail to hybridize to VH gene probes (26) and most likely correspond to germline Cp transcripts (data not shown, 26-29). Because of the difficulty in resolving some germline transcripts from those mRNAs derived from fully assembled VH(D)JH-Cp Ig heavy chain genes (27-29), it was not possible to accurately standardize the expression level of VH-containing Cp mRNA transcripts for fetal liver samples of different ages. Therefore, replicate Northern blots containing equivalent amounts of poly(A)*-enriched RNA from fetal samples at different times in gestation were hybridized to VH probes specific for the VH7183 and VH.J558 families. The signal intensities were determined by densitometry and the ratio of the signals obtained with the VH7183 and VH.J558 probes for each of the fetal liver samples was compared with that of newborn liver, which was arbitrarily set at 1. Although the absolute signal intensities with the VH probes increased between day 15 and day 18 fetal liver (probably reflecting the increased frequency of pre-B and virgin B cells)
The relative expression of \( V_{H}7183 \) and \( V_{H}J558 \) was similar throughout fetal development until birth (Fig. 1).

To determine the expression levels of other \( V_{H} \) gene families in fetal development, fetal liver samples from day 18 of gestation were examined in more detail. In both BALB/c and C57BL/6 mice, the pattern of expression in day 18 fetal liver was similar to that observed in newborn liver for six \( V_{H} \) gene families examined (Fig. 2, compare lanes 1 and 2, 8 and 9). To facilitate comparison of \( V_{H} \) usage patterns in the different tissue samples, the relative expression of each \( V_{H} \) family for a standardized level of \( \mu \) mRNA was compared with that observed in adult spleen (Fig. 2, lanes 7 and 14) and preference values were calculated (Table I). A marked preference for expression of \( J_{H} \)-proximal \( V_{H} \) gene families was observed in fetal liver relative to adult spleen (Table I). The only change in expression between day 18 fetal liver and newborn liver is a two- to fourfold increase in expression of \( V_{H}J558 \) (Fig. 2 and Table I). Biased usage of proximal \( V_{H} \) gene families is still apparent in liver samples taken 3 and 7 d postnatally in both BALB/c (Fig. 2, lanes 3 and 4) and C57BL/6 (Fig. 2, lanes 10 and 11) mice. Thus, the \( V_{H} \) repertoire expressed in differentiating B cells in the liver is relatively constant throughout fetal development and the first days of postnatal life, suggesting that \( V_{H} \) segments continue to be rearranged and expressed at a rate that reflects chromosomal position.

**Normalization of \( V_{H}7183 \) Expression Occurs between 1 and 2 wk of Age.** To elucidate further the shift between fetal and adult \( V_{H} \) repertoires, we assayed the relative expression of \( V_{H}7183 \) in liver samples from day 15 to 19 of fetal development and from day 1 to 7 of postnatal life. The pattern of expression of \( V_{H}7183 \) in fetal liver was similar to that observed in the newborn liver samples (Fig. 1), and the relative expression of \( V_{H}7183 \) was similar to that observed in the newborn liver samples (Fig. 2). The signal intensities of \( V_{H}7183 \) hybridization were determined by densitometry, and were used to determine the \( V_{H}J558/V_{H}81X \) ratio for each of the samples; these ratios are expressed relative to the ratio in the NB liver sample which was arbitrarily set at 1. The signal intensities of \( V_{H} \) hybridization to day 15 fetal liver (lanes 1) were too weak for accurate densitometric scanning; however, the \( V_{H}J558/V_{H}81X \) ratio as assessed by visual examination of the original autoradiograms was similar to the liver samples taken later in ontogeny.

**Figure 1.** Expression of the \( V_{H}7183 \) and \( V_{H}J558 \) gene families during fetal development of BALB/c mice. 5-10 \( \mu \)g of poly(A)+ RNA prepared from pooled BALB/c livers removed at the indicated day of fetal development or the first day after birth (newborn, NB) were fractionated on agarose-formaldehyde gels and transferred to nitrocellulose filters. Duplicate filters were hybridized to either the \( V_{H}81X \) probe or the \( V_{H}B4 \) probe. Signal intensities were determined by densitometry, and were used to determine the \( V_{H}J558/V_{H}81X \) ratio for each of the samples; these ratios are expressed relative to the ratio in the NB liver sample which was arbitrarily set at 1. The signal intensities of \( V_{H} \) hybridization to day 15 fetal liver (lanes 1) were too weak for accurate densitometric scanning; however, the \( V_{H}J558/V_{H}81X \) ratio as assessed by visual examination of the original autoradiograms was similar to the liver samples taken later in ontogeny.
Figure 2. Expression of V_H gene families during neonatal development of BALB/c and C57BL/6 mice. Pooled samples of 18-day fetal livers (lanes 1 and 8), neonatal livers (lanes 2-4 and 9-11), and neonatal spleens (lanes 5-7 and 12-14) were isolated from BALB/c (lanes 1-7) and C57BL/6 (lanes 8-14) mice. Total RNA from the spleen samples and poly(A)^+ RNA from the liver samples was standardized for mRNA levels and amounts that gave approximately equal signals were fractionated. Replicate Northern blots were assayed for hybridization to V_H-specific probes as indicated. Densitometric analyses of multiple autoradiographic exposures of these Northern blots are summarized in Table 1.

representation of each V_H gene family in the \( \mu \) mRNA expressed in neonatal splenic B cells. The initial repertoire expressed in the neonatal spleen is biased for expression of J_H-proximal V_H families; relative expression of the six V_H families in the day 3 spleen is almost identical to that in newborn liver in both BALB/c (Fig. 2, compare lanes 2 and 5) and C57BL/6 (Fig. 2, compare lanes 9 and 12) strains. Changes in expression are observed in day 7 spleen: although still biased toward proximal families, relative expression of V_H7183 is decreased in BALB/c spleen (Fig. 2, lane 4) and expression of V_H606 and V_H558 are slightly increased in both BALB/c (Fig. 2, lane 4) and C57BL/6 (Fig. 2, lane 13) day 7 spleens.

To analyze when in ontogeny the V_H repertoire is normalized to adult levels, the absolute utilization of the V_H7183 and V_H558 gene families was determined in BALB/c splenic B cells at various times from neonate to adult. The level of V_H7183 expression remained constant until 5 d after birth; subsequently, the level decreased steadily and reached approximately adult levels by 2 wk after birth (Fig. 3, A and B). Although there is some variation in the absolute expression of the V_H558 family in the first week after birth, adult levels were reached by 2 wk of age.
TABLE I

VH Usage During Development in BALB/c and C57BL/6 Mice

| Mice          | Fetal   | Day 1 | Day 3 | Day 7 | Day 3  | Day 7  | Adult |
|---------------|---------|-------|-------|-------|--------|--------|-------|
| Liver         |         |       |       |       |        |        |       |
| BALB/c        |         |       |       |       |        |        |       |
| Vh7183        | 4-5     | 4-8   | 4-5   | 3-4   | 5-6    | 2-3    | 1.0   |
| VhQ52         | 0.5-2   | 0.8-0.8 | 0.7-0.8 | 0.7-1 | 0.9-1   | 1.0-1   | 1.0   |
| VhS107        | 0.1-0.2 | 0.4-0.5 | 0.3-0.4 | 0.3-0.3 | 0.2-0.3 | 0.4-0.5 | 1.0   |
| Vh36-60       | ND   | 0.6   | ND   | ND   | 0.6   | 1      | 1.0   |
| VhJ606        | 0.1-0.2 | 0.2-0.3 | 0.1-0.2 | 0.2-0.3 | 0.3-0.4 | 0.4-0.5 | 1.0   |
| VhJ558        | 0.2    | 0.5   | 0.5   | 0.5   | 0.5    | 0.6    | 1.0   |
| Spleen        |         |       |       |       |        |        |       |
| BALB/c        |         |       |       |       |        |        |       |
| Vh7183        | 15-20   | 7-10  | 10-20 | 9-20  | 10-25  | 10-20  | 1.0   |
| VhQ52         | 0.6-1.0 | 0.8-1 | 0.8-1 | 1-2   | 0.9-2  | 2-3    | 1.0   |
| VhS107        | 0.5-1   | 0.8-2 | 0.6-1 | 0.7-2 | 0.5-2  | 0.7-2  | 1.0   |
| Vh36-60       | 2-3     | 1-2   | 2-3   | 2-3   | 2-3    | 3-5    | 1.0   |
| VhJ606        | 0.1-0.1 | 0.2-0.3 | 0.2-0.4 | 0.3-0.4 | 0.2-0.6 | 0.6-1  | 1.0   |
| VhJ558        | 0.1-0.2 | 0.5-0.6 | 0.3-0.6 | 0.7-0.9 | 0.4-0.5 | 0.9-1  | 1.0   |
| C57BL/6       |         |       |       |       |        |        |       |
| Vh7183        | 15-20   | 7-10  | 10-20 | 9-20  | 10-25  | 10-20  | 1.0   |
| VhQ52         | 0.6-1.0 | 0.8-1 | 0.8-1 | 1-2   | 0.9-2  | 2-3    | 1.0   |
| VhS107        | 0.5-1   | 0.8-2 | 0.6-1 | 0.7-2 | 0.5-2  | 0.7-2  | 1.0   |
| Vh36-60       | 2-3     | 1-2   | 2-3   | 2-3   | 2-3    | 3-5    | 1.0   |
| VhJ606        | 0.1-0.1 | 0.2-0.3 | 0.2-0.4 | 0.3-0.4 | 0.2-0.6 | 0.6-1  | 1.0   |
| VhJ558        | 0.1-0.2 | 0.5-0.6 | 0.3-0.6 | 0.7-0.9 | 0.4-0.5 | 0.9-1  | 1.0   |

Northern blots as described in Fig. 2 were analyzed by densitometry. Signal intensities were determined and used to derive the preference values listed in the table. Preference values were calculated according to the following equation: Preference (VH) = [I>*VH/Cμ] / [I>*CA/Cμ]; where I*>VH and I>*CA denote the intensities of hybridization of a given VH probe and the Cμ probe to the indicated tissue samples. The range of values given for each comparison encompasses all the values obtained after densitometric analysis of multiple (2-4) autoradiographic exposures of each Northern blotting experiment (except for hybridization of the VH36-60 and VHJ558 probes to the BALB/c samples for which only one exposure suitable for densitometric scanning was obtained); numbers are rounded off to a single significant digit. Preference values are further described in Materials and Methods.

Alternative splicing of primary VH-DJH-Cμ transcripts results in two species of μ mRNA that contain different 3' exons: processed μ mRNAs of 2.7 and 2.4 kb encode the membrane-bound (μm) and the secreted (μs) forms of μ heavy chain proteins, respectively (24, 31, 32). B-lineage cells at different developmental stages produce different steady-state amounts of the two species of μ mRNA: pre-B cells and mature B cells produce equal or higher levels of μm mRNA than μs mRNA, whereas plasma cells produce predominantly μs mRNA (28, 29). Thus, the relative expression of μm versus μs mRNA in a given tissue is in part related to the maturation state of the cells expressing the predominant level of μ mRNA sequences in the tissue. To determine whether the shift in VH7183/VhJ558 expression was correlated with a shift in the pattern of μ mRNA produced, we quantitated more precisely the apparent shift in μ mRNA expression suggested from the VH hybridizations observed in day 1 and day 3 spleens (Fig. 3 A). For this analysis, the relative intensity to which BALB/c splenic RNA samples hybridized to a probe specific for the μm exons was compared with that obtained with a probe (μs2) that detects all μ mRNAs. When the amount of RNA electrophoresed for each sample is adjusted to give a relatively constant signal of total μ mRNA, the μm-specific hybridization steadily decreases after 3 d of age (Fig. 3 A). An estimate of the relative expression of μm mRNA was quantitated by comparing the ratio of the signal intensities for the two probes (μm/Cμ) obtained for each spleen sample to that obtained for an Abelson murine leukemia virus (A-MuLV)-transformed pre-B cell line (Fig. 3 B). These data clearly demonstrated that the decrease in VH7183 expression correlates with a gradual shift
in the expression of $\mu$ mRNA from predominantly $\mu_m$ to predominantly $\mu_\gamma$ in the adult spleen (Fig. 3B).

$V_H$ Gene Utilization in Differentiating Pre-B Cells in the Adult Is Also Biased to $J_H$-proximal Families. To determine if a repertoire biased toward utilization of $J_H$-proximal $V_H$ segments continues to be generated in differentiating B cells in the adult, we analyzed three independent pools of total bone marrow cells for $V_H$ gene expression.
Migration of B cells from the bone marrow to peripheral lymphoid organs is not unidirectional; B cells stimulated by antigen in the periphery may recirculate into the bone marrow and mature into \( \mu \)- and \( \gamma \)-secreting plasma cells (33-35). Plasma cells produce as much as 100-fold or more \( \mu \) mRNA, predominantly \( \mu_s \), than B-lineage cells of earlier stages (36). Thus, even a small number of contaminating plasma cells would prevent detection of mRNA contributed by the developing B cells. The number of plasma cells in the bone marrow accumulates with age (33). To minimize contamination of bone marrow with recirculating cells from the periphery, bone marrow samples were derived from young adult (4-5 wk old) BALB/c mice and only those samples that had a high proportion of \( \mu_m \) mRNA (indicative of pre-B and B cells) were tested. The relative representation of each \( \text{VH} \) gene family in the \( \mu \) mRNA of bone marrow cells was compared with that in the \( \mu \) mRNA of adult spleen. For comparison, newborn liver samples were assayed in the same experiments. The pattern of \( \text{VH} \) expression in adult bone marrow differs from that of spleen and is similar to but not identical to that of newborn liver (Fig. 4 and Table II). In particular, the \( \text{VH}7183 \) family is preferentially expressed in adult bone marrow relative to adult spleen; however, the relative \( \text{VH}7183 \) utilization in adult bone marrow is clearly less than that of newborn liver. The biased expression of proximal \( \text{VH} \) gene segments in adult bone marrow is consistent with the notion that these \( \text{VH} \) gene segments are also preferentially rearranged in differentiating pre-B cells in the adult.

A curious feature of \( \text{VH} \) expression in adult bone marrow was the high expression of the \( \text{VH}5107 \) family, which was higher than that expressed in both adult spleen and newborn liver (Fig. 4 and Table II). However, the bulk of \( \text{VH}5107 \)-containing \( \mu \) mRNA was the size of the \( \mu_s \) species of mRNA (2.4 kb), suggesting bone marrow contains a disproportionate number of plasma cells or secreting B cells producing antibodies with heavy chains encoded by \( \text{VH} \) segments from this family. This result also is consistent with the observation that adult mice naturally produce a high titer of antibodies bearing the T15 idiotype (37), which is encoded by a member of \( \text{VH}5107 \) family (38).

**Biased \( \text{VH} \) Gene Expression in Adult Splenics of scid(\( \mu^+ \)) Mice.** As another approach to studying the \( \text{VH} \) repertoire generated in adult mice, we analyzed expression in the mutant mouse strain that is homozygous for the scid mutation (scid mice; 39, 40). In general, scid mice lack T cells and B cells due to a mutation that does not allow developing B and T lineage cells to functionally rearrange their respective antigen receptor loci (41). However, a certain percentage of adult scid mice, termed “leaky” scid, produce significant amounts of serum Ig (42). To analyze the potential of this immunodeficient mouse strain to express \( \text{VH} \) genes, pooled or individual scid spleens were screened for \( \mu \) mRNA expression and \( \text{VH} \) usage analyzed in those samples that had detectable \( \mu \) expression. The majority of scid spleens had extremely low levels of \( \mu \) mRNA (>1,000-fold lower levels than those of normal C.B-17 of comparable age) (data not shown); however, 1 of 3 pooled scid spleen samples and 2 of 12 individual scid spleens had detectable levels of \( \mu \) mRNA (10-100-fold lower than that of normal C.B-17) (Fig. 5). These spleen samples also expressed complete \( \kappa \) mRNA transcripts in proportions similar to the level of \( \mu \) mRNA (data not shown), suggesting that expression is due to mature B cells and not to pre-B cells that may be present in spleens of some of these defective mice (43).
FIGURE 4. **V**<sub>H</sub> gene expression in bone marrow from adult BALB/c mice. Expression of **V**<sub>H</sub> gene families in total RNA prepared from pooled adult BALB/c bone marrow (BM) samples (lanes 2, 3, and 7) was compared with that observed in RNA from adult spleen (spl; lanes 4, 5, and 8) and poly(A)<sup>+</sup>-RNA from day 1 newborn liver (NBL, lanes 1 and 6) as described in Fig. 2. The lower **V**<sub>H</sub>-hybridizing bands observed in some samples (e.g., lanes 2 and 5) correspond to γ<sub>0</sub> mRNA and were not included in the densitometric analyses summarized in Table II. Lanes 1–5 represent Exp. 1 and lanes 6–8 represent Exp. 2. In Exp. 2, the NBL and adult spleen samples are the same as those depicted in Fig. 2.

The **V**<sub>H</sub> expression in the selected scid(μ<sup>+</sup>) spleens differed significantly from that of normal C.B-17 spleens (Fig. 5 and Table III). To facilitate comparison between normal and scid spleens, a ratio of the relative utilization of each **V**<sub>H</sub> gene family for a standardized amount of μ mRNA was derived by dividing the relative (**V**<sub>H</sub>/C<sub>μ</sub>) expression in the scid spleens by the relative expression in normal spleens (Table III). The **V**<sub>H</sub>7183 gene family was highly preferentially expressed in two of three scid spleens (Fig. 5 and Table III). The third scid spleen sample had substantially...
lower levels of μ mRNA, making quantitation of VH family expression difficult. However, the relative expression of the two VH families that were detectable (VHQS52 and VHJS558) was biased towards the JH-proximal family (VHQS52). Thus, the general pattern of VH gene expression in adult scid(μ+) spleens is biased in a manner reminiscent of that observed in pre-B cell lines derived from normal C.B-17 bone marrow (44) and in neonatal livers and neonatal spleens from normal BALB/c and C57BL/6 mice.

**Table II**

|          | NBL | BM1 | BM2 | BM3 |
|----------|-----|-----|-----|-----|
| VH81X    | 10-20 | 4-5 | 4-6 | 4-8 |
| VHQS52   | ND  | ND  | ND  | 0.8-0.8 |
| VHJS107  | 0.2-0.4 | 2-4 | 1-2 | 0.4-0.5 |
| VH36-60  | 0.8-1 | 0.3-0.4 | 0.4-0.5 | 0.6 |
| VHJ606   | ND  | ND  | ND  | 0.2-0.3 |
| VHJS558  | 0.3-0.7 | 0.5-1.0 | 0.7-1.5 | 0.5 |

The Northern blots described in Fig. 4 were analyzed by densitometry and preference values were calculated as described in Table I and Materials and Methods. The intensities of hybridization of the given VH probe and the C probe to the newborn liver (NBL) or adult bone marrow (BM) sample were compared with each adult spleen. The range of values for each comparison represents all the values obtained from densitometric analysis of multiple autoradiographic exposures; numbers are rounded off to a single significant digit.

**Figure 5.** VH utilization in spleens from adult scid(μ+) mice. 20 μg of total RNA prepared from adult scid spleens were screened for detectable levels of μ mRNA. One of 3 pools of scid spleen samples (lane 2) and 2 of 12 individual spleen samples (mouse no. 8203, lane 5; and mouse no. 8186, lane 6) were subsequently analyzed for VH gene expression as indicated and compared with expression observed in normal C.B-17 adult spleens (2 μg total RNA) (pooled sample, lane 1; individual samples, lanes 3 and 4). The signal intensities were determined and the results are summarized in Table III. Expression of VHJS558 in the scid spleen shown in lane 5 was re-examined in other Northern blots containing levels of μ mRNA more comparable to those in the normal samples so that a better comparison could be made (data not shown); the quantitation of this data is given in Table III.
TABLE III

VH Usage in Adult Spleens from Scid Mice

| Exp. 1 | Exp. 2 |
|--------|--------|
| Poolled spleen | Mouse 8203 | Mouse 8186 |
| VH81X | 10-20 | 7-10 | Not detected |
| VHQ52 | 0.2-0.3 | 0.3-0.5 | 0.2-0.3 |
| VHJ606 | Not done | <0.1 | Not detected |
| VHJ358 | 0.3-0.4 | 1-2 | <0.1 |

The Northern blots described in Fig. 5 were analyzed by densitometry. Values represent a comparison of the expression of VH gene families in scid(μ*) spleens relative to that observed in normal C.B-17 spleen for a given amount of μ mRNA. Values were calculated as follows: [Isc(VH)/Isc(Cp)] was divided by [IN(VH)/IN(Cp)]; where ISc and IN denote the intensities of hybridization to a given probe in scid and normal spleens, respectively.

Discussion

Expression of the VH Gene Repertoire in Fetal and Neonatal Development. We have demonstrated that the position-dependent bias of VH gene expression previously observed in neonatal liver (17) is also evident in earlier stages of fetal development. The relative expression of the VH7183 and VHJ558 gene families is constant from day 15 of fetal life through birth. Six VH gene families that are representative of regions throughout the VH locus (11-13) were expressed at the same relative level through fetal and neonatal liver development. These findings suggest that the rate at which particular VH segments are rearranged and expressed remains constant during early ontogeny and that this rate is influenced by chromosomal position.

An ontogenic analysis of VH gene expression in spleen revealed that the neonatal splenic repertoire was similar to that of neonatal liver. The shift away from the position-dependent VH repertoire was first apparent at ~1 wk of age. Although there is a transient appearance of pre-B cells in the spleen early in ontogeny, the total number of pre-B (cytoplasmic μ*, surface Ig*) cells plateaus at birth and is only a fraction of the number of B (surface Ig*) cells at 3 d postnatally (30, 45). Because B cells produce equal or greater amounts of steady-state μ mRNA than pre-B cells (28), B cells most likely contribute the predominant μ mRNA detected in the postnatal spleen. Thus the primary B cell repertoire expressed in the neonatal spleen reflects the biased repertoire generated by the position-dependent rearrangement of VH gene segments that occurred in the differentiating pre-B cells, suggesting that the mechanisms that “normalize” the VH repertoire to the random representation of VH genes in the adult act subsequent to expression of surface Ig. These data agree with other studies that found an over-representation of JH-proximal VH gene families in hybridomas derived from unstimulated neonatal spleens (46, 47), antigen-selected hybridomas (48), and in polyclonally activated B cells assayed by in situ hybridization (49). In the latter study, the representation of VH gene families reached approximately adult levels by 7 d after birth (49).

B cell responsiveness to some antigens is acquired in a temporally ordered fashion during ontogeny (50-59). Our finding that the utilization of VH gene families by differentiating pre-B cells remains constant during fetal and adult B cell differentiation implies that it is unlikely that the ordered appearance of antibody specificities...
can be explained solely on the basis of the absence of rearrangements of particular VH gene segments. However, it is possible that the relative bias in the representation of certain VH gene segments in the newly generated versus normalized repertoires might account for the usage of different antibody specificities in response to particular antigens in neonatal versus adult mice. For example, in neonatal antibody responses a more frequently rearranged V gene of lower affinity may predominate at the expense of a less commonly encountered V gene of higher affinity. Recently, it has been demonstrated that the representation of κ light chain families differs in B cell colonies derived from neonatal versus adult spleen (60). Thus, it is possible that biased rearrangements at light chain loci or combinatorial associations (e.g., 61) of particular heavy and light chains might contribute to the ordered acquisition of particular antibody specificities.

The gradual shift in VH gene expression in the spleen during the first 2 wk of life suggests that during this time in development other factors act on the initially biased repertoire of newly formed B lymphocytes to diversify the expressed VH repertoire. These mechanisms may include positive or negative regulatory forces, such as tolerance induction, immunoregulatory networks, and antigen selection. For example, hybridomas derived from neonatal B cells have been found to have reactivity to self antigens (46, 47, 62), including reactivity to variable regions of Ig molecules (i.e., idiotypes) (46, 62). The potential modulatory effects of these antibodies during neonatal development may play a role in the generation of a randomized adult repertoire (46, 62). Differential VH gene expression by distinct B cell subsets may also contribute to the developmental pattern of VH gene expression. For example, the Ly-1 B cell subset makes up a larger proportion of splenocytes in the neonate than in the adult (63); thus the shift away from expression of JH-proximal VH families may correlate with the decrease in proportion of Ly-1 B cells in the spleen. However, available evidence (64, 65) suggests that the VH repertoire expressed by the Ly-1 B cell subset does not significantly differ from the conventional B cell population (64, 65; and our unpublished data).

**Generation of the B Cell Repertoire in Adult Mice.** Unlike pre-B cell differentiation early in ontogeny, pre-B cell differentiation in adult mice occurs exclusively in the bone marrow. It has been suggested that there is a difference in the repertoire generated by pre-B cells derived from adult bone marrow versus fetal or newborn liver that leads to the developmental shift in VH gene expression. We found increased representation of VH 7183-related gene segments in the adult bone marrow relative to adult spleen but less than fetal or newborn liver. The bone marrow is a heterogeneous population of B-lineage cells that includes about equal numbers of pre-B and newly formed B cells (30, 35, 45), as well as memory B and plasma cells that have recirculated from peripheral lymphoid organs (33–35). Although μ-secreting plasma cells that have recirculated from the periphery may contribute to the μ mRNA signal and thus skew the VH repertoire measured, the fact that preferential expression of the VH 7183 family is observed suggests that the VH repertoire generated in differentiating B cells in the adult has a position-dependent bias similar to that early in ontogeny. This conclusion is consistent with the nonrandom usage of VH genes in rearrangements of pre-B cell lines derived from adult bone marrow (10, 14) or hybridomas
from long-term bone marrow cultures (66), as well as in nonproductive rearrangements of adult B lineage cells (15).

In contrast to these findings, another study found that polyclonally activated adult bone marrow cells analyzed by in situ hybridization displayed a pattern of VH usage similar to adult spleen (49). The different results in that study (49) versus the analysis presented here may be explained by differences in the cell populations assayed. The former study (49) examined functional B cells stimulated with LPS, whereas in our assay VH expression is contributed by pre-B and newly formed, unstimulated B cells. It is possible that regulatory mechanisms that influence the antibody repertoire in mature cells have already acted on the LPS-responsive cells in the adult bone marrow. Examination of purified populations of pre-B cells and unstimulated B cells from the bone marrow may clarify these apparent discrepancies.

**Biased VH Expression in Spleens of Leaky scid(μ+) Mice.** The scid mutation is an autosomal, recessive mutation that arose spontaneously in an inbred strain of mice (39). Mice homozygous for this mutation are deficient in B and T lymphocytes; other hematopoietic cell types (erythrocytes, monocytes, granulocytes, and megakaryocytes) are normal (39, 40). The scid mutation appears to affect the ability of T and B lymphocytes to functionally rearrange their respective antigen receptor loci (41). The majority of scid mice lack detectable serum Ig due to the deficiency of functional lymphocytes. However, serological screening of scid mice have revealed circulating Ig in ~15% of the mice (42); mice with detectable serum Ig were denoted scid(Ig+). Analyses of circulating Ig in scid(Ig+) mice revealed a restricted κ light chain heterogeneity in most mice; however, some mice showed more complex serum Ig patterns, suggesting variable degree of leakiness among individual mice (42). In this study, we screened scid spleens for the presence of μ mRNA; a small percentage of mice had low, but detectable, levels of μ mRNA and are denoted scid(μ+). The μ mRNA detected was predominantly of the secreted type and κ mRNA was detected in proportion to the level of μ mRNA (data not shown). These observations suggest that the μ mRNA found in scid(μ+) spleens was produced by plasma cells and was not due to the presence of nonactivated B cells or pre-B cells, which can sometimes be recovered from scid spleens by transformation with A-MuLV (43). This conclusion is consistent with the lack of detectable B220+ cells in the spleens of scid(Ig+) mice (42).

The analysis of VH gene families in scid(μ+) spleens revealed that multiple VH gene families were expressed in individual spleen samples, suggesting that multiple clones of B cells were present. However, the pattern of VH expression in these scid(μ+) spleens was strikingly different from that observed in their normal counterparts. The higher expression of JH-proximal VH gene families in scid(μ+) adult spleens relative to normal adult spleens is similar to that observed in livers and spleen of normal neonatal mice. We suggest that the biased VH gene expression in the adult spleens of leaky scid mice reflects the position-dependent rearrangements occurring in pre-B cells of bone marrow. Potentially, a lack of immunoregulation due to the deficiency of normal numbers of B and T lymphocytes in scid mice may result in an unselected repertoire that becomes activated in the spleen.

Together our findings strongly indicate that the position-dependent VH repertoire
generated in differentiating B cells reflects constraints imposed by the Ig gene assembly process and that this process is unchanged during development. Regulatory and selection mechanisms acting subsequent to the rearrangement events appear to be the major pathway leading to randomization of the VH repertoire to adult levels.

Summary

We have previously demonstrated a dramatic preference for utilization of the most JH-proximal VH gene segments in the newborn liver versus adult spleen. We now examine in detail the relative expression of different VH gene families throughout ontogeny and in immunodeficient mice to gain insight into factors that cause the shift in VH usage. We find that the relative expression of VH gene families remains constant and biased throughout fetal and neonatal liver development. In addition, the primary VH repertoire expressed in neonatal spleen displays a similarly biased, position-dependent VH repertoire. The pattern of VH gene expression begins to change at 5–7 d postnatally and reaches the adult randomized pattern at ~2 wk of age. We also find biased expression of JH-proximal VH gene families in adult bone marrow and in spleens of adult leaky scid mice, suggesting that the spontaneously generated repertoire of adult mice is similar to that observed in neonates. Together, these data suggest that a position-dependent repertoire is generated in differentiating pre-B cells at all stages of ontogeny, at least in part, as a result of preferential rearrangement of proximal VH gene segments. Therefore, mechanisms subsequent to V gene rearrangement, such as regulatory interactions and antigen selection, must play a major role in normalizing the repertoire.

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