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ORIGINAL PAPER

D_H and J_H usage in murine fetal liver mirrors that of human fetal liver

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Abstract In mouse and human, the regulated development of antibody repertoire diversity during ontogeny proceeds in parallel with the development of the ability to generate antibodies to an array of specific antigens. Compared to adult, the human fetal antibody repertoire limits N addition and uses specifically positioned VDJ gene segments more frequently, including V6-1 the most D_H-proximal V_H, DQ52, the most J_H-proximal D_H, and J_H2, which is D_H-proximal. The murine fetal antibody repertoire also limits the incorporation of N nucleotides and uses its most D_H proximal V_H, V_H81X, more frequently. To test whether D_H and J_H also follow the pattern observed in human, we used the scheme of Hardy to sort B lineage cells from BALB/c fetal and neonatal liver, RT-PCR cloned and sequenced V_H7183-containing VDJCμ transcripts, and then assessed V_H7183-D_H-J_H and complementary determining region 3 of the immunoglobulin heavy chain (CDR-H3) content in comparison to the previously studied adult BALB/c mouse repertoire. Due to the deficiency in N nucleotide addition, perinatal CDR-H3s manifested a distinct pattern of amino acid usage and predicted loop structures. As in the case of adult bone marrow, we observed a focusing of CDR-H3 length and CDR-H3 loop hydrophobicity, especially in the transition from the early to late pre-B cell stage, a developmental checkpoint associated with expression of the pre-B cell receptor. However, fetal liver usage of J_H-proximal D_HQ52 and D_H-proximal J_H2 was markedly greater than that of adult bone marrow. Thus, the early pattern of D_H and J_H usage in mouse feta liver mirrors that of human.

Keywords Fetal mouse repertoire · CDR-H3 · Adult mouse repertoire

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Abbreviations

BM	bone marrow
CDR-H3	complementary determining region 3 of the immunoglobulin heavy chain
NL	neonatal liver
RT-PCR	polymerase chain reaction amplification of transcripts cloned into cDNA by reverse transcription of mRNA
TdT	terminal deoxynucleotidyl transferase
WT	wild type

Introduction

The ability of B-lymphocytes to create a polyclonal immunoglobulin repertoire with a diverse set of antigen-binding sites permits specific responses to a broad range of foreign and self-antigens. Each nascent immunoglobulin is the product of a complex series of V (D) J gene rearrangement and terminal deoxynucleotidyl transferase (TdT) catalyzed N addition that created an exponential repertoire of diverse heavy (H) and light (L) chains (Hozumi and Tonegawa 1976; Tonegawa 1983). Immunoglobulin rearrangement is a hierarchical process, with $D_H \rightarrow J_H$ rearrangement preceding $V_H \rightarrow DJ_H$ rearrangement, and followed by $V_L \rightarrow J_L$ rearrangement. Each stage of rearrangement marks a separate developmental checkpoint with early checkpoints focusing on generation of a functional immunoglobulin and later ones testing the specificity of the antigen-binding site (Huetz et al. 1993). Of the six hypervariable loops that are juxtaposed to form the antigen-binding site, the third complementary determining region of the heavy chain (CDR-H3) is the most diverse (Alt and Baltimore 1982; Rajewsky 1996; Tonegawa 1983; Yancopoulos et al. 1984). CDR-H3, which includes the carboxyl terminus of the V_H, the amino terminus of the J_H, and all of the D_H in its entirety, is the direct product of V(D)J joining and N addition. Its location at the center of the antigen-binding site permits CDR-H3 to often play a critical role in antibody specificity (Kabat et al. 1991; Padlan 1994; Xu and Davis 2000).

In humans, the ability to mount an effective humoral response to many antigens, including specific microorganisms and vaccines, is delayed until well after infancy (Stein 1992). This sequential acquisition of the ability to respond to particular antigens is a characteristic feature of the developing immune system in jawed vertebrates, humans and mice included (Silverstein 1977). These observations present a paradox in that they suggest an element of order to the development of the binding characteristics of the antibody repertoire even though the mechanisms that underlie the generation of the antibody were initially viewed as highly stochastic (Yancopoulos and Alt 1986). This paradox was resolved, at least in part, by the observation that the fetus does not make full use of all the mechanisms that are available for the generation of V domain diversity in general, and CDR-H3 diversity in particular (Feeney 1990; Gu et al. 1990; Schroeder et al. 1987; Schroeder et al. 1995; Yancopoulos and Alt 1986; Yancopoulos et al. 1984). More recent studies have shown that even in the adult the composition of CDR-H3 continues to demonstrate constraints on amino acid composition, length, and charge distribution (Ippolito et al. 2006; Ivanov et al. 2005b; Schelonka et al. 2005; Schelonka et al. 2008). Manipulation of these constraints can lead to altered patterns of B cell development, antigen-specific antibody production, and immune responses to pathogens.

Given the importance of CDR-H3 to antigen recognition, we sought to re-evaluate the process of CDR-H3 content control during perinatal B cell development in the expressed and functional mouse repertoire.

This new information is essential for a better understanding of the mechanisms that regulate the development of the early repertoire, the role of fetal repertoire restriction on perinatal B cell development, the role of fetal repertoire in perinatal responses to antigen, and the contribution of the fetal repertoire to the population of critical B cell niches in the adult, such as the peritoneal cavity. This research also facilitates evolutionary studies of repertoire development (Ivanov et al. 2005a).

We used surface expression of CD43, BP-1, CD19, IgM, and IgD to sort B lineage cells from BALB/c 18-days post conception and neonatal liver. After polymerase chain reaction amplification of transcripts cloned into cDNA by reverse transcription of mRNA (RT-PCR) amplification, we sequenced V_H7183-containing VDJCμ transcripts from these sorted cells and then compared the results to our previous studies of repertoire development in adult BALB/c mice (Ippolito et al. 2006; Ivanov et al. 2005b; Schelonka et al. 2005). As in the case of the adult BALB/c mice, we found that perinatal CDR-H3 content was biased from its inception, and then further focused with development. In contrast to a previous study using unsorted cells from the perinatal liver (Bangs et al. 1991), we also found that the changes in D_H and J_H usage between fetus and adults exhibited striking parallels to the pattern of D_H and J_H usage observed in human ontogeny. These results reinforce the value of the mouse as a model for the ontogeny of the development of immune competence in human.

Materials and methods

Mice

We obtained 18-day post-conception fetal liver and neonatal liver from two separate mice each on two separate occasions, for a total of four samples. The mice analyzed represent the progeny of BALB/c mice bred in the University of Alabama at Birmingham (UAB) vivarium. The mice were maintained in a specific pathogen-free barrier facility. All experiments with live mice were approved by and performed in compliance with the requirements of the UAB Institutional Animal Care and Use Committee.

Flow cytometry and cell sorting

Flow cytometric analysis and cell sorting from mononuclear cells from the liver was performed as previously described on mononuclear cells from the bone marrow of 8-week-old BALB/c mice (Ippolito et al. 2006; Ivanov et al. 2005b; Schelonka et al. 2005). A MoFlo instrument (Cytomation, Ft. Collins, CO, USA) was used for cell sorting. Developing B lineage cells in the liver were identified on the basis of the surface expression of CD19, CD43, IgM, BP-1, and/or IgD (Fig. 1).

Sorting, RNA preparation, RT-PCR, and sequence analysis

Total RNA isolation, V_H7183 specific VDJCμ RT-PCR amplification, cloning, sequencing, and sequence analysis was performed in the same way as previously described (Ippolito et al. 2006; Ivanov et al. 2005b; Schelonka et al. 2005), thus permitting a direct comparison between perinatal and adult *BALB/c* mice sequences. The sequences reported in this paper have been placed in GenBank database (accession number GU975849-GU976285). A listing of the 472 unique, in-frame perinatal sequences used for analysis in this work is provided in Supplemental Table (S1).

Statistical analysis

Differences between populations were assessed, where appropriate, by two-tailed Student's t test, two-tailed Fisher's exact test, χ^2, or Levene test for the homogeneity of variance. Analysis was performed with JMP version 7.0 (SAS Institute). The standard error of the mean accompanies means.

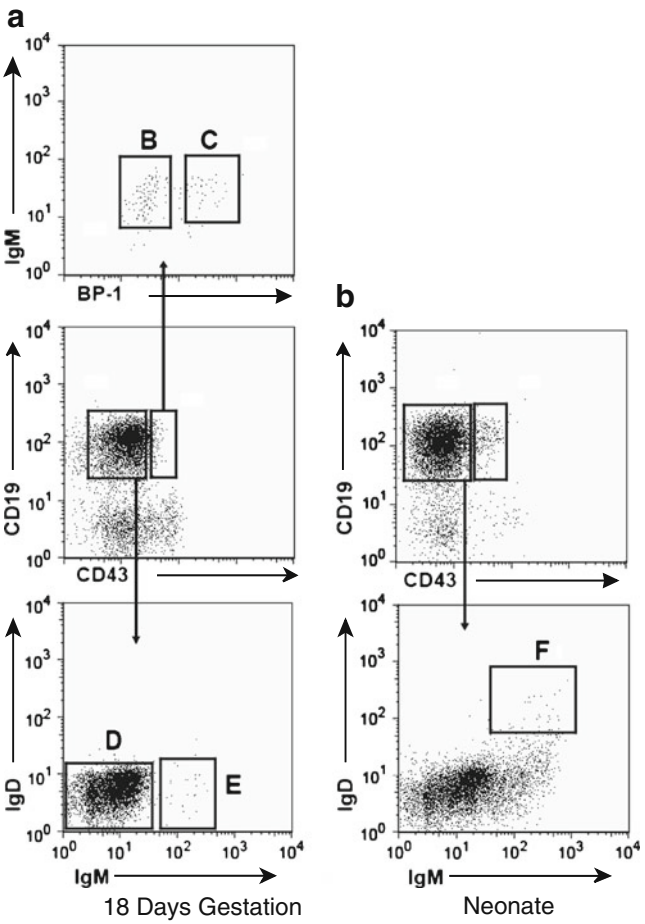


Fig. 1 Flow cytometric gates for the collection of fetal B lineage cells from 18-days post-gestation and neonatal BALB/cJ mice. Cells within the lymphocyte gate were first distinguished on the basis of the expression of CD19 and CD43. Early B-cell progenitors (CD19+ CD43+) were divided into fractions B and C on the basis of the expression of BP-1. Late pre-B, immature B, and mature B cells (CD19+ CD43−) were divided into fractions D, E, and F on the basis of the surface expression of IgM and IgD

Results

Isolation of B lineage cells and RT-PCR cloning of Ig transcripts from fetal and perinatal liver

We identified and isolated B lineage cells from Hardy fractions B through E (Hardy and Hayakawa 2001) from 18 days gestation fetal liver, but were unable to detect cells belonging to fraction F (Fig. 1, left). Subsequently, fraction F cells for our analysis were obtained from the livers of neonatal mice (Fig. 1, right). Following total RNA extraction, RT-PCR amplification was performed using 3′ Cμ and 5′ V_H7183-specific primers. The resulting cDNAs were cloned into pUC19 plasmids and then sequenced. We obtained 472 unique, in-frame, and open reading frame V_H7183-D-J-Cμ sequences. Of these, 53 fraction B (pro-B cells), 55 fraction C (early pre-B cells), 90 fraction D (late pre-B), and 142 Fraction E (immature B cells) sequences were obtained from 18-day post-conception liver, and 132 fraction F (mature B cells) sequences were obtained from neonatal liver. We compared these to 194, 373, 279, 255, and 254 previously published Fractions B through F unique, in-frame, open reading sequences from the bone marrows of 11 different 8-week-old adult BALB/c mice (Zemlin et al. 2007).

Usage of V_H81X (V_H7183.1) in fetal B lineage cells varies by developmental stage

Consistent with previous studies (Martin et al. 1997; Yancopoulos et al. 1984), we observed preferential use of D_H-proximal V_H81X (V_H7183.1) in fetal liver B lineage cells (Fig. 2). When compared to adult bone marrow B

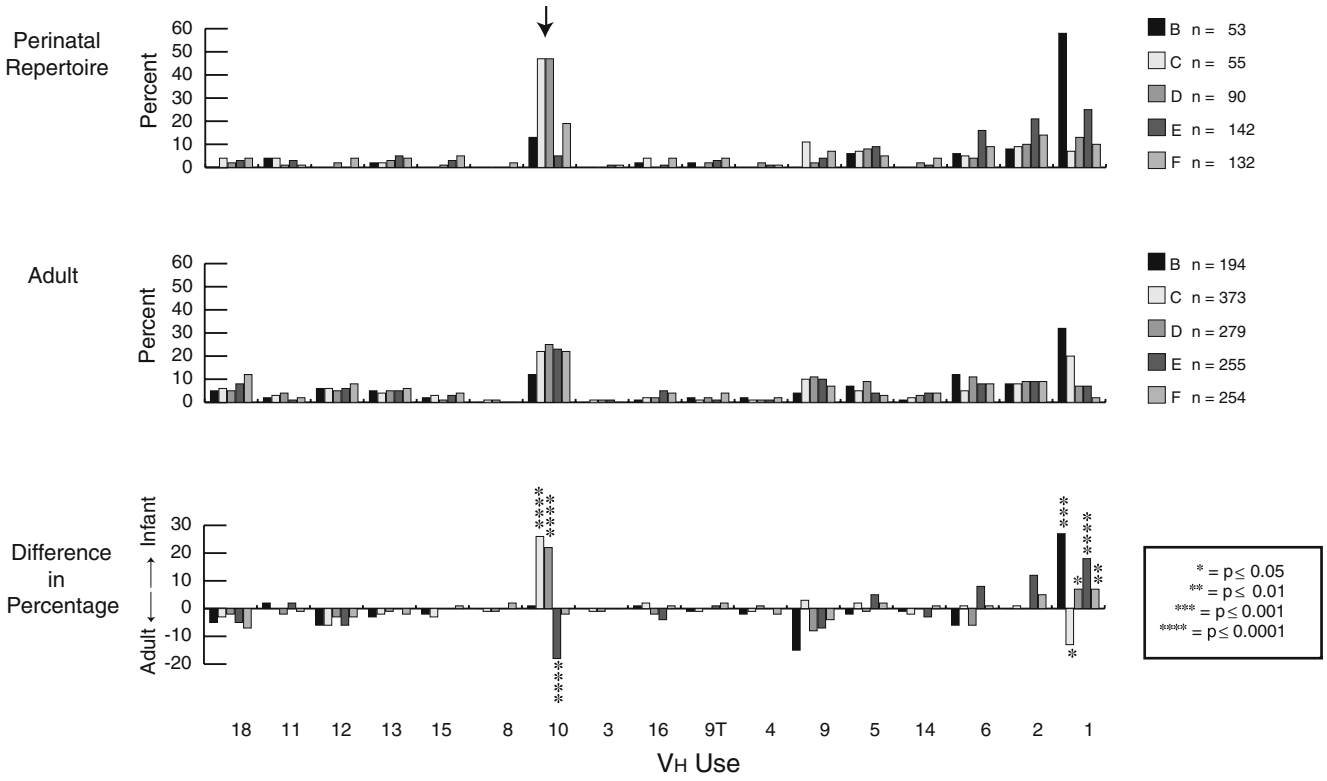


Fig. 2 V_H 7183 gene segment usage during B cell development in the perinatal liver versus the bone marrow of adults. V_H gene segments are arranged according to their position relative to the J_H locus in the genome, with V_H7183.1 (V_H81X), the most J_H proximal, at the right. *Top* Percent of unique, in-frame sequences using the V_H gene segment specified in the perinatal liver from Hardy fractions B ($CD19^+$ $CD43^+$ IgM^- BP-1), C ($CD19^+$ $CD43^+$ IgM^- $BP\text{-}1^+$), D ($CD19^+$ $CD43^-$ IgM^- IgD−), E ($CD19^+$ $CD43^-$ IgM^+ IgD^-), and F ($CD19^+$ $CD43^-$ IgM^{low} IgD^{high}) from BALB/c mice is displayed. *Middle* Percent of unique, in-frame sequences using the V_H gene segment specified in the bone marrow of 8-week-old BALB/c mice. *Bottom* Divergence in the percentage of V_H gene segment use between the perinatal liver and the young adult bone marrow is displayed

lineage cells, the use of V_H81X was greater in perinatal fractions B, D, E and F; but smaller in fraction C ($p<0.05$). In adult fraction C through fraction F, VH7183.10 proved the most heavily used V_H. In the perinatal period, we observed a dramatic increase in the relative use of V_H7183.10 in fractions C and D ($p\leq0.0001$), followed by a drop in fraction E ($p\leq0.0001$). By fraction F, however, the use of V_H7183.10 matched that of adult.

The effect of distance to D_H was also apparent in V_H gene segments other than V_H81X. We grouped the V_H gene segments distal to V_H7183.10 into one block (block 8–18); and the V_H gene segments proximal to V_H7183.10 but distal to V_H81X and V_H7183.2 into a second block (block 3–6). Although usage of the distal block in the perinatal period increased with development, it consistently lagged behind the usage observed in adult (Fig. 3, left); whereas in fractions C, E, and F usage of the proximal block was similar between perinatal liver and adult bone marrow (Fig. 3, right). Although within each fraction the numbers of sequences were insufficient to achieve statistical significance, when compared as a population the differences in the proportion of usage between the two blocks of sequences between the perinatal period versus the adult achieved statistical significance at $p=0.005$.

Preferential use of D_H DQ52 in fetal liver

The relative use of the D_H gene families in the perinatal liver reflected proximity to the J_H locus. When compared to adult bone marrow, we observed decreased use of J_H-distal DFL gene segments and enhanced use of J_H-proximal DQ52 (Fig. 4, left panel). While this pattern of preference based on physical location was observed throughout development, by Fraction F there was evidence of convergence with the overall pattern of D_H family usage nearly matching that of adult bone marrow fraction F.

Preferential use of J_H2 in perinatal liver

J_H utilization also followed proximity to DQ52. While use of J_H1 did not differ between the perinatal and adult periods, when compared to adult use of J_H2 was favored over J_H3 and J_H4 ($p\leq0.002$) at all stages of development except for fraction E. In human, there is a similar shift with use of J_H 2

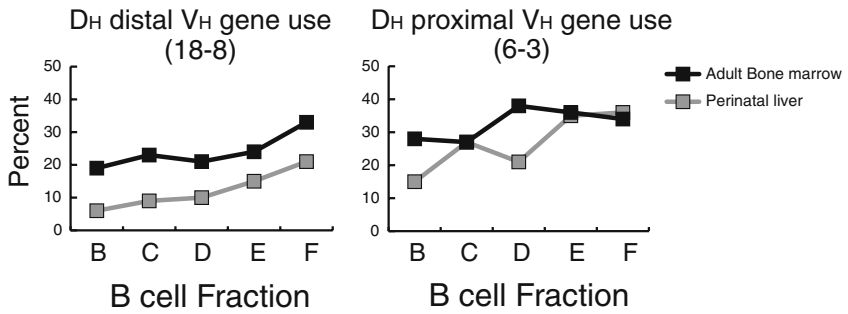


Fig. 3 D_H distal and proximal V gene usage during B cell development in the perinatal liver versus the adult bone marrow. We grouped the V_H gene segments upstream of $V_H7183.10$ into one block (block 8–18); and the VH gene segments downstream of $V_H7183.10$, but upstream of V_H81X and $V_H7183.2$, into a second block (block 3–6) of gene segments. V_H block usage is reported as the percent of the sequenced population of unique, in-frame, open transcripts from BALB/c mice perinatal liver and adult bone marrow Hardy fractions B through F

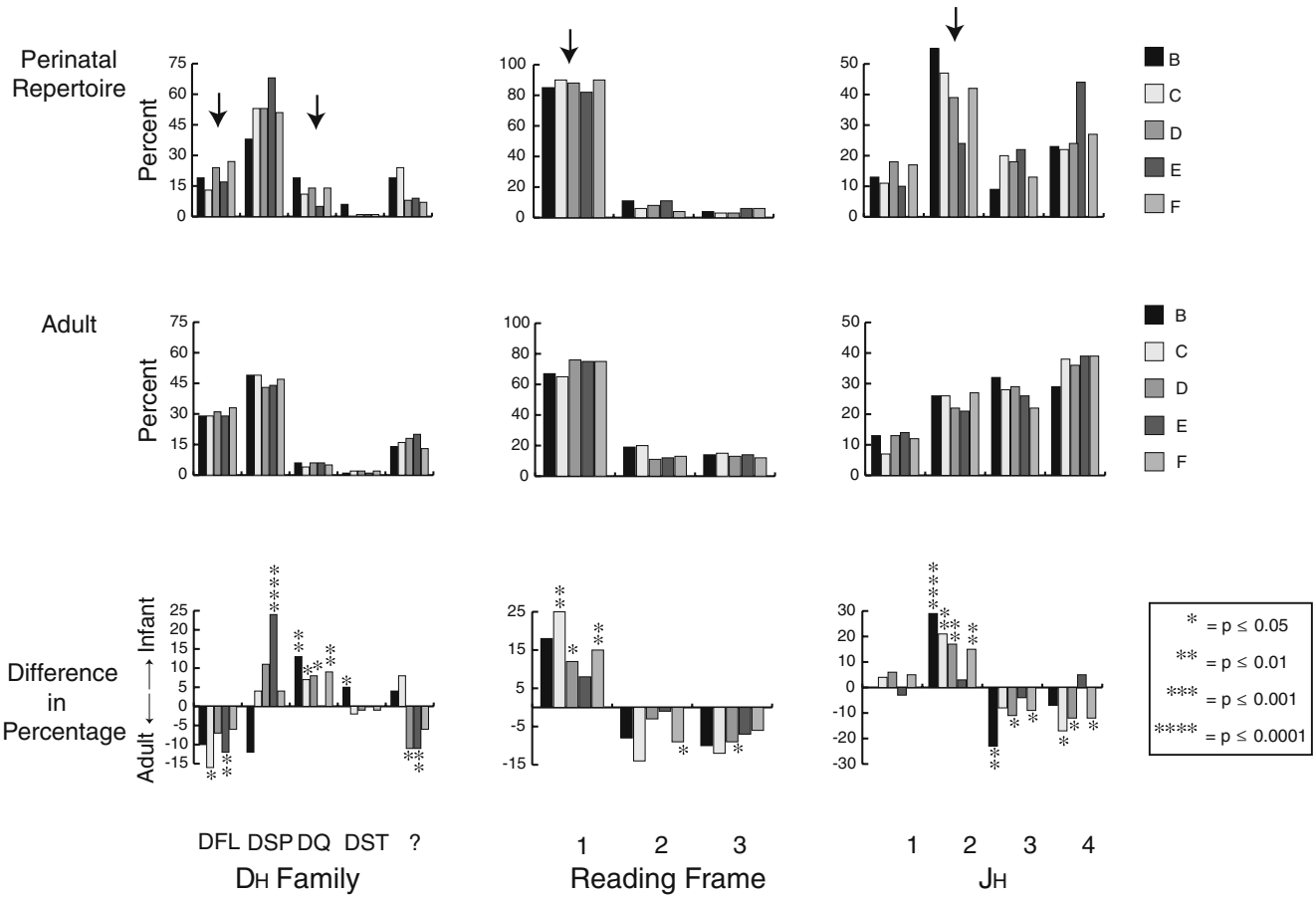


Fig. 4 D_H, J_H, and D_H reading frame usage during B cell development in the perinatal liver versus the bone marrow of adults. *Top left to right* The percent of sequences using members of the specified D_H family; the percent of sequences using DSP or DFL D_H gene segment family members in reading frames 1, 2, or 3; and percent of sequences using J_H1, 2, 3, or 4 among in-frame sequences cloned from the perinatal liver from Hardy fractions B through F is displayed. *Middle left to right* The percent of sequences using members of the specified D_H family; percent of sequences using DSP or DFL D_H gene segment family members in reading frames 1, 2, or 3; and percent of sequences using J_H1, 2, 3, or 4 among in-frame sequences cloned from 8 week old BABL/cJ bone marrow from Hardy fractions B through F is displayed. *Bottom left to right* Divergence in the percentage of D_H gene family, DSP and DFL, reading frame, and J_H between the perinatal liver and the young adult bone marrow is displayed. *Arrows* point to features of particular interest

and 3 in first trimester fetal liver preferred over J_H5 and 6, and use of J_H5 and 6 in adult bone marrow preferred over use of J_H2 and 3 (Baskin et al. 1998 Apr; Xue et al. 1997; Yamada et al. 1991).

Increased prevalence of D/J overlaps and use of reading frame 1 in perinatal liver

As previously reported (Kepler et al. 1996), CDR-H3 sequences with V/D and D/J overlaps were exceedingly common in the perinatal samples (Fig. 5), which essentially lack N nucleotide addition. The increased prevalence of sequences with V/D and D/J overlaps were associated with an increase in the use of reading frame 1 among CDR-H3s that used DSP or DFL gene segments (Fig. 4, middle panels).

Changes in the distribution of CDR-H3 length with development

The average length of perinatal CDR-H3 was significantly shorter than adult at all stages examined (Fig. 6a). However, an increase in the average length of CDR-H3 from fraction B (9.2±0.4 codons) to fraction F (10.2±0.2, $p<0.002$) was observed. This change was progressive, but was most marked in the transition from fraction C (9.2±0.4) to D (10.1±0.2, $p<0.02$).

A closer examination of the distribution of CDR-H3 lengths as a function of ontogeny and development (Fig. 7) revealed that the perinatal samples were completely devoid of CDR-H3s of greater than 15 codons. At the other end of the scale, we observed an increase in the prevalence of sequences of eight codons or less. In part, the reason for the major shift in length between fractions C and D reflected the reduced prevalence of CDR-H3 sequences with less than seven codons, including sequences without a recognizable D_H element (Fig. 4).

Altered patterns of amino acid usage in perinatal CDR-H3 loops

In the absence of N addition and with preferential use of reading frame 1, which is enriched for use of tyrosine, there was a striking divergence in the pattern of amino acid utilization in the CDR-H3 loops of the sequences obtained from fraction B, which is prior to the expression of the H chain in protein form (Fig. 8). When compared to adult, this early repertoire was enriched for use of tyrosine, histidine, and asparagine. Conversely, the perinatal repertoire was virtually devoid of arginine, lysine, glutamine, glutamic acid, proline, and phenylalanine. Use of leucine, valine, and isoleucine was also reduced. Of the five amino acids where little or no change was observed between fetus and adult, two, methionine and cysteine, were remarkable for their absence

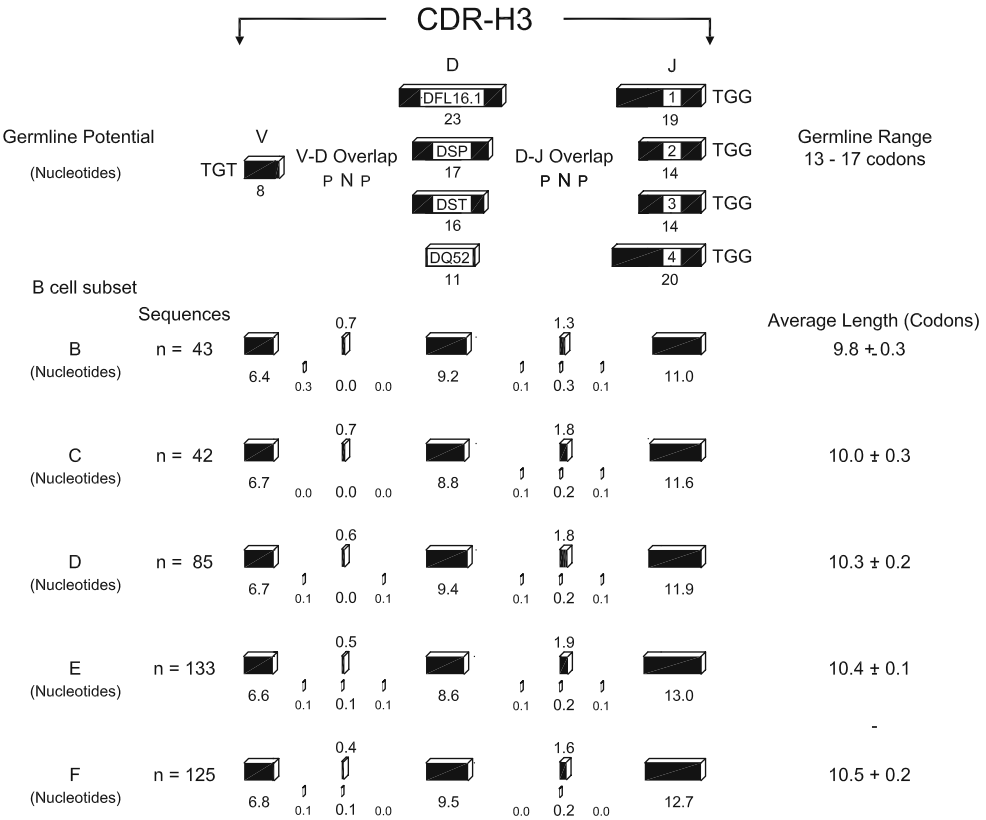


Fig. 5 Deconstruction of the contributing components to CDR-H3 length in sequences containing identifiable D_H gene segments as a function of B cell development in the perinatal liver. The potential distribution of the germline sequence of the V_H gene segment, P junctions, the D_H gene segment, V_H-D_H and D_H-J_H overlap, and the J_H gene segment to the CDR-H3 length is illustrated

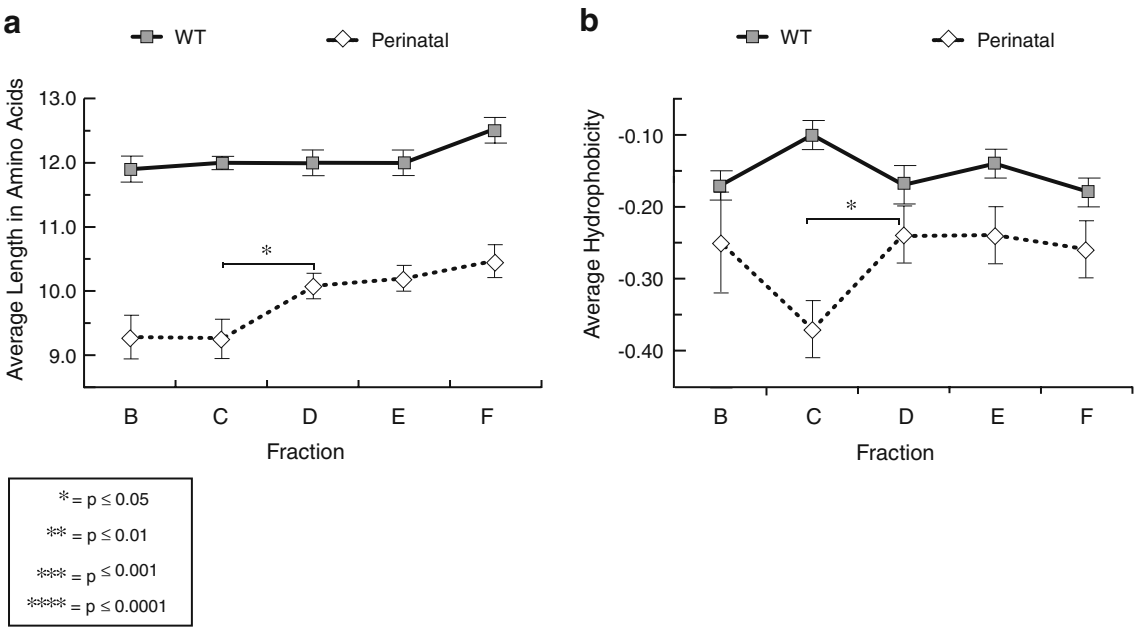


Fig. 6 Average CDR-H3 length and CDR-H3 loop charge as a function of B cell development in the perinatal liver as compared to that in the bone marrow of adult BALB/cJ mice. **a** Average CDR-H3 length and **b** average CDR-H3 loop hydrophobicity of the V_H7183DJCμ transcripts from perinatal liver and adult bone marrow. The standard error of the mean is shown

at both stages of life. The overall outline of amino acid usage remained relatively unchanged from fraction B to fraction F. However, closer inspection revealed subtle adjustments as the B cells passed through sequential development checkpoints. For example, use of aspartic acid was enhanced in fractions B and C, but declined to adult levels in fractions D, E, and F.

Variation of CDR-H3 hydrophobicity during fetal B cell development

We used a normalized Kyte–Doolittle scale to assess the relatively distribution of hydrophobicity in the CDR-H3 loop of perinatal versus adult CDR-H3 loops (Eisenberg 1984; Kyte and Doolittle 1982). In the absence of N addition and with increased use of reading frame 1, there was a shift in average hydrophobicity towards the charged end of the spectrum (Fig. 6b). Closer inspection revealed that this shift primarily reflected a paucity of sequences in the more hydrophobic range of the normal repertoire (Fig. 9). Indeed, the vast majority of the CDR-H3 loop sequences remained within the range that is preferred for recirculating fraction F B cells in adult; although unlike the adult fraction F repertoire, a few sequences continued to carry excess charge or hydrophobicity.

Discussion

In the bone marrow of adult BALB/c mice, the composition of the expressed immunoglobulin CDR-H3 repertoire is marked by constraints on length, amino acid utilization, and charge (Ivanov et al. 2005b). These constraints are first established in early B cell progenitors, and then focused as the B lineage cells progress through sequential developmental checkpoints. Many of these constraints are dependent on the specific sequence of the contributing gene segments and vary during ontogeny (Asma et al. 1986; Ippolito et al. 2006; Logtenberg et al. 1992; Schelonka et al. 2005; Terrell et al. 1977; Zemlin et al. 2008). In this work, we sought to re-examine the development of the expressed CDR-H3 repertoire in the perinatal liver in order to gain insight into the forces that shape the repertoire during its passage through developmental checkpoints at both a different stage of ontogeny and in a different tissue organ.

Fraction B is represents the developmental stage at which V➔DJ rearrangement begins. In both fetus and adult, the transition from fraction B to C is characterized by increased use of V_H gene segments other than V_H81X. For example, the use of V_H 7183.10 increases markedly, suggesting that by fraction C access to V_H gene segments other than V_H81X may be enhanced (Fig. 2). The greatest differences between the adult and perinatal repertoires occurred in fraction B (Fig. 10), which is the least likely to undergo selection on the basis of the H chain to bind to either endogenous or exogenous compounds. This would suggest that the differences that were observed largely reflected the effects of genetically controlled differences between the fetal and adult B cell progenitors in terms of the patterns of rearrangement and gene segment access.

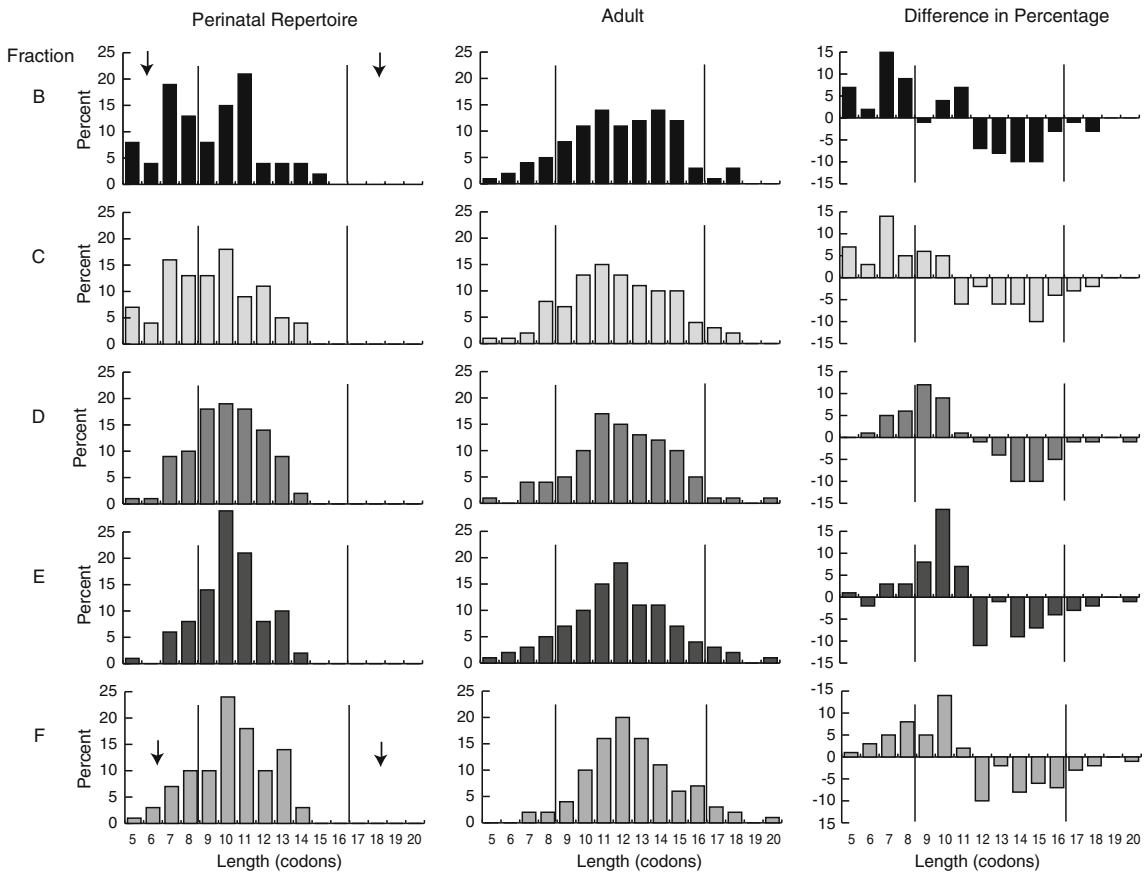


Fig. 7 Distribution of CDR-H3 lengths as a function of B cell development in the perinatal liver as compared to that in the bone marrow of adult BALB/cJ mice. *Left* Distribution of CDR-H3 lengths in V_H7183DJCμ transcripts from perinatal liver as a function of B cell development (Centers for Disease and Prevention). Distribution of CDR-H3 lengths in V_H7183DJCμ transcripts from adult bone marrow as a function of B cell development. *Right* Divergence in the distribution of CDR-H3 length between the perinatal liver and the young adult bone marrow is displayed. To facilitate visualization of the change in variance of the distribution, the *vertical lines* mark the preferred range of lengths in the bone marrow fraction F. *Arrows* point to features of particular interest

The transition from fraction C to D requires a successful interaction between the nascent H chain and (l5/VpreB) the pre-B cell receptor, which produces an appropriate survival and cell replication signal. The changes in average length and charge are most marked at this transition (Fig. 6), which reflect the loss of the shortest and the most highly charged CDR-H3s. The change in charge in adult bone marrow is also most marked at this transition; although in this case, there appears to be a greater reduction in the more hydrophobic rather than the most charged. These results would suggest that either the binding of H chains with short, highly charged, or highly hydrophobic CDR-H3s to VpreB/λ5 the pre-B cell receptor is less efficient or the signal that is transduced by H chains with these types of CDR-H3s is qualitatively or quantitatively different than that of CDR-H3 sequences of the preferred length, charge, or hydrophobicity.

The transition from fraction D to E reflects successful expression of a complete surface IgM B cell receptor. CDR-H3 length distribution, hydrophobicity, and charge are unchanged, supporting the view that the pre-B cell receptor has acted to select for acceptable physico-chemical CDR-H3 characteristics. However at this stage, we see a marked change in the use of V_H7183.10, which does not occur in the adult. It is possible that the antigens to which immature B cells that use V_H7183.10 are responding differ between fetus and adult or that the binding specificity of V_H7183.10 N-less CDR-H3s is not conducive to further B cell development. Examination of the pattern of V_H usage in sorted single cells would help distinguish between these possibilities, as would evaluation of repertoire diversification in the absence of TdT.

The transition from fraction E to F at this early stage of life comes in a setting where exposure to the external environment has been restricted to the womb. Subtle differences were observed in terms of V_H, D_H, and J_H usage, suggesting further antigen receptor-mediated selection in response to endogenous antigens.

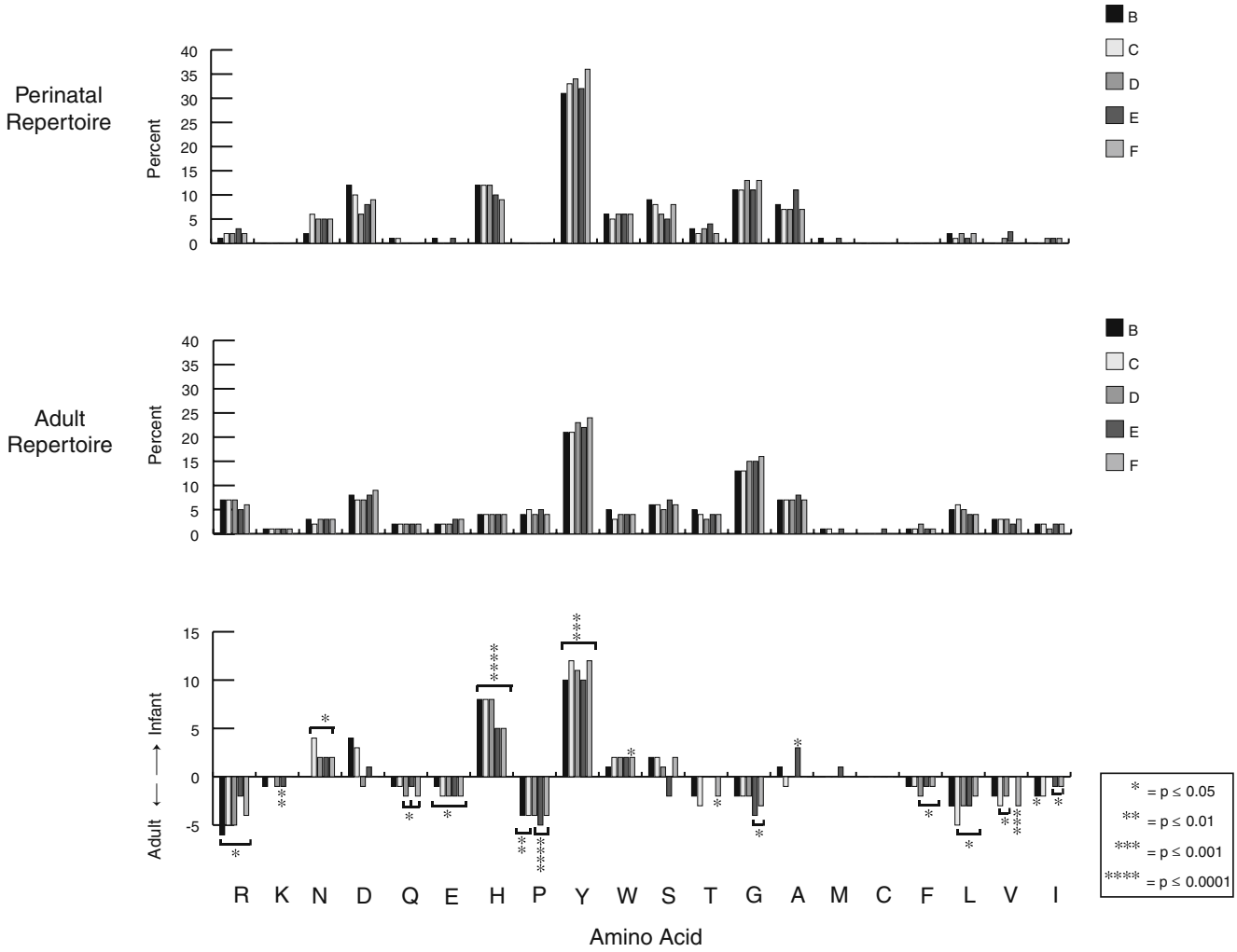


Fig. 8 Amino acid usage as a function of B cell development in the perinatal liver as compared to that in the bone marrow of adult BALB/c mice. The distribution of individual amino acids in the CDR-H3 loop of sequences as a function of B cell development in the perinatal liver. The Hardy bone marrow B lineage designation of fractions B through F (Hardy and Hayakawa 2001) was used throughout

By the time the B cells achieve fraction F status, the perinatal liver and adult bone marrow repertoires have drawn closer to each other in terms of V_H usage, D_H usage, J_H usage, and CDR-H3 length hydrophobicity and charge. The fetal repertoire is well known for its tendency to produce poly-reactive antibodies with self-specificities (Avrameas 1991; Avrameas and Ternynck 1995). Selection of the repertoire through idiotype/anti-idiotype interactions has been shown to differ between fetus and adult (Benedict et al. 2000; Benedict and Kearney 1999; Kearney et al. 1997). However, given the fact that the antibody repertoire in the perinatal period has developed in an environment protected from exposure to exogenous antigens, it may be that both the fetal and adult repertoires are being shaped, at least in part, by interactions with the same self-antigens.

In agreement with previous studies, we observed preferential use of the most D_H proximal V_H, V_H81X, in the earliest B cell progenitor examined. And we confirmed a progressive fall in the usage of this gene segment with development (Huetz et al. 1993; Yancopoulos et al. 1984). We also observed diminished use of the group, or block, of 7183 V_H gene segments most distal to D_H. These findings are in accordance with studies that report differential accessibility of portions of the V_H locus to rearrangement early in ontogeny (Perlmutter et al. 1985; Sen and Oltz 2006; Williams et al. 2001; Yancopoulos et al. 1984).

The importance of position to gene segment expression in the perinatal liver was underscored by the increased use of J_H-proximal DQ52 and D_H-proximal J_H2 and 3. This follows the positional pattern of usage that is observed in human (Sanz 1991; Schroeder et al. 1987) as well as in rhesus monkeys (Link et al. 2005). It has been shown that deletion of both DQ52 and the region immediately adjacent and its upstream promoter enriches use of D_H-proximal J_H gene segments in adult bone marrow (Nitschke et al. 2001;

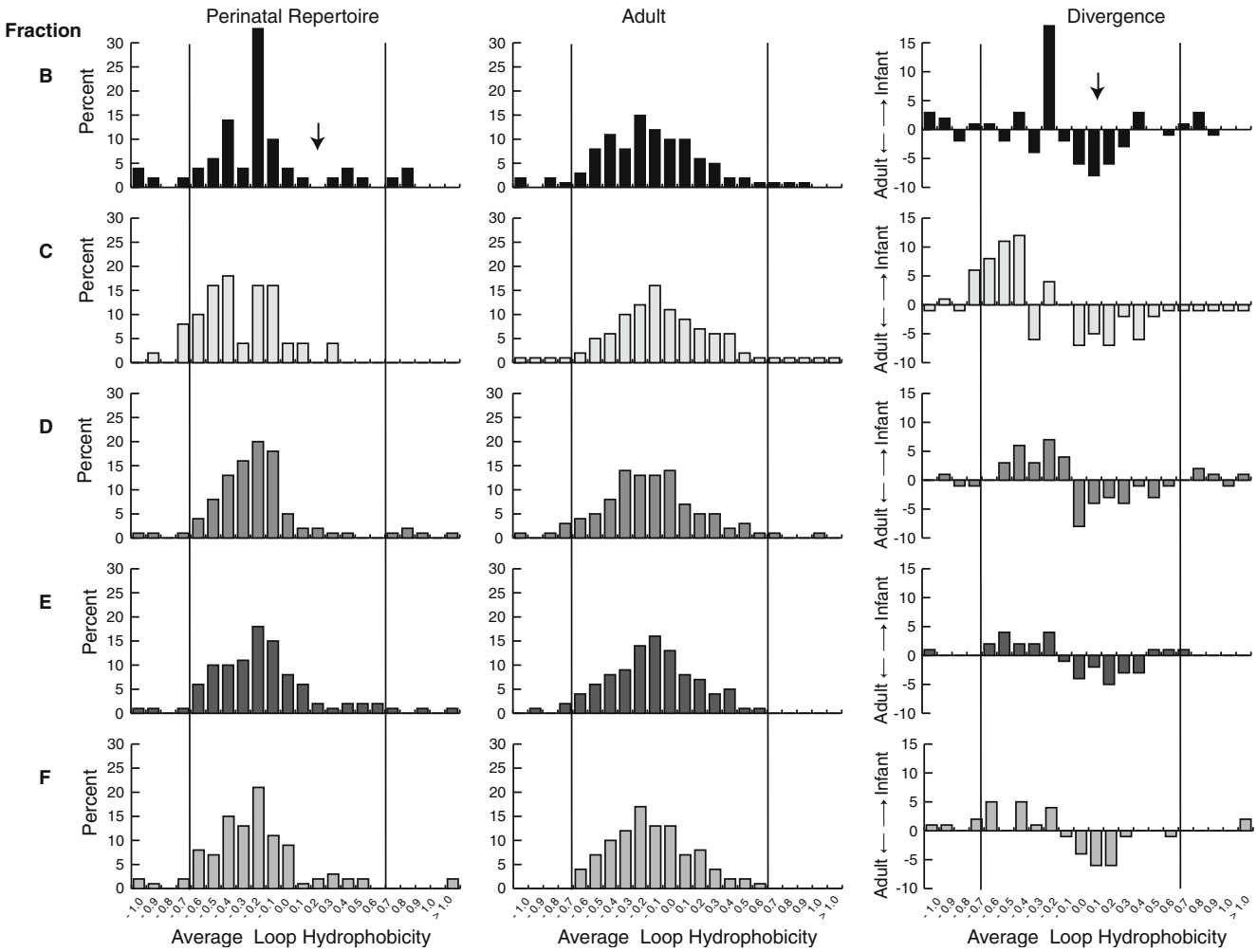
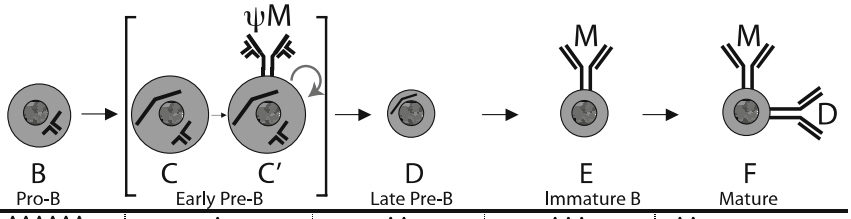


Fig. 9 Distribution of CDR-H3 loop charge as a function of B cell development in the perinatal liver as compared to that in the bone marrow of adult BALB/cJ mice. *Left* Distribution of average CDR-H3 hydrophobicities in VH7183DJCμ transcripts from perinatal liver as a function of B cell development (Centers for Disease and Prevention). Distribution of average CDR-H3 hydrophobicities in VH7183DJCμ transcripts from adult bone marrow as a function of B cell development. *Right* Divergence in the distribution of CDR-H3 loop hydrophobicity between the perinatal liver and the young adult bone marrow is displayed. The normalized Kyte–Doolittle hydrophobicity scale (Eisenberg 1984) has been used to calculate average hydrophobicity. Although this scale ranges from −1.3 to +1.7, only the range from −1.0 (charged) to +1.0 (hydrophobic) is shown. Prevalence is reported as the percent of the sequenced population of unique, in-frame, open transcripts from each B lineage fraction. To facilitate visualization of the change in variance of the distribution, the vertical lines mark the preferred range average hydrophobicity previously observed in wild-type fraction F (Ivanov et al. 2005b). *Arrows* point to features of particular interest

Schelonka et al. 2005), whereas deletion of a portion of the promoter region upstream of DQ52 only had no effect (Afshar et al. 2006). Together, this would support the view that the DQ52 region as a whole, including DQ52, plays a critical, evolutionarily preserved role in the regulation of initial D➔J immunoglobulin rearrangements (Kottmann et al. 1992).

In addition to restrictions in V_H usage, the perinatal H chain repertoire in mice is also marked by an absence of N nucleotides (Asma et al. 1986; Delassus et al. 1998; Li et al. 1993; Rusquet et al. 1987; Schroeder 2005; Shiokawa et al. 1999). In the absence of these non-germline-encoded terminal modifications, rearrangement occurs preferentially at sites of germline microhomology between the rearranging sequences (Chukwuocha and Feeney 1993; Feeney 1990; Feeney 1992; Huetz et al. 1997; Lieber et al. 1988). This has the effect of directing rearrangement into RF1 and further reducing CDR-H3 length by eliminating shared D_H and J_H sequence. Preferential use of RF1 diminishes access to the hydrophobic and charged amino acids encoded by reading frames other than RF1 (Ivanov et al. 2002). The absence of N addition limits incorporation of lysine, glutamic acid, glutamine, and proline while enriching for tyrosine, histidine, and asparagine. Together, these factors create a fetal pre-B cell H chain CDR-H3 repertoire that differs significantly

Parameter							
VH7183 usage: 81x	PNL[1]	↑↑↑↑↑↑	↑	↑↑	↑↑↑	↑↑	10%[2] **
	ABM	↑↑↑↑	↑↑↑	↑	↑		2%
10	PNL	↓	↑↑↑	↑↑↑	↓↓	-	19%
	ABM	↓	-	-	-		22%
Block 8-18 (D_H distal genes)[3]	PNL	↓↓↓↓↓	↓↓↓↓	↓↓↓	↓↓	↓	21%*
	ABM	-	-	-	-		33%
Block 3-6 (D_H proximal genes)[3]	PNL	↓↓	↓	↓	-	-	36%
	ABM	↓	↓	-	-		34%
D family usage: DFL	PNL	↓	↓↓	-	↓	-	27%
	ABM	-	-	-	-		33%
DSP	PNL	↓	-	-	↑	-	51%
	ABM	-	-	-	-		47%
DQ	PNL	↑↑	↑	↑	-	↑	14%*
	ABM	-	-	-	-		5%
D reading frame 1 use	PNL	↑	↑	↑	-	↑	90%*
	ABM	↓	↓	-	-		75%
JH element use: JH2	PNL	↑↑↑↑	↑↑↑	↑↑	-	↑↑	42%**
	ABM	-	-	-	-		27%
JH3	PNL	↓↓	↓	↓	-	↓	13%*
	ABM	↑	-	-	-		22%
JH4	PNL	↓	↓	↓	-	↓	27%*
	ABM	↓	-	-	-		39%
Amino acid use: Tyr, His	PNL	↑	↑	↑	↑	↑	36;9%****
	ABM	-	-	-	-		24; 4%
Arg	PNL	↓↓	↓	↓	↓	↓	2%****
	ABM	↓	↓				6%
Pro, Phe, Val[4]	PNL	nd	nd	nd	↓	nd	
	ABM	-	-	-	-		4;1;3%
Average length (codons)	PNL	↓↓↓	↓↓↓	↓↓	↓↓	↓↓	10.2****
	ABM	↓	↓	↓	↓		12.5
Prevalence of short CDR-H3 (<9 codons)	PNL	↑↑↑↑	↑↑↑↑	↑↑	↑↑	↑↑	21%
	ABM	↑	↑	↑	↑		45%
Average hydrophobicity (Kyte-Doolittle index)[5]	PNL	↓(-0.24)	↓↓↓ (-0.37)	↓ (-0.24)	(-0.21)	↓	-0.27**
	ABM	(-0.17)	↑↑(-0.1)	(-0.17)	(-0.14)		-0.18
Prevalence of charged loop (< -0.7)	PNL	↑↑↑	↑↑↑↑	↑	↑	↑↑	4%**
	ABM	↑↑	↑↑	↑↑	↑		0%
Prevalence of hydrophobic loop (>0.6)	PNL	↑↑	-	↑↑	↑	↑	2%**
	ABM	↑	↑↑	↑	↑		0%
Kinked CDR-H3 base	PNL	↓	-	-	-	-	93%
	ABM	-	-	-	-		96%
Intact hydrogen bond ladder	PNL	↑	↑↑	↑↑↑	↑↑↑	↑↑↑	100% ****
	ABM	-	-	-	-		47%

[1] PNL, perinatal liver; ABM, adult bone marrow; ↑Increased compared to ABM Fraction F (ABM F); ↓Decreased compared to ABM F; - (dash) undistinguished compared to ABM F;

[2] Numbers in the right column represent the actual data from either neonatal fraction F (NL F) or ABM F (reference value); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001;

[3] According to the organization in the V_H locus, we grouped the V_H gene segments upstream to V_H7183.10 into block 8-18 (D_H distal genes); and the V_H gene segments downstream to V_H7183.10 but upstream to V_H81X and V_H7183.2 into block 3-6 (D_H proximal genes);

[4] nd, not detected. The amino acid valine was detected but <1% prevalent in Fractions B, C and F and only 1 and 2% prevalent in Fr D and Fr E in fetal liver, respectively;

[5] Arrows represent the change in the average hydrophobicity index value of the CDR-H3 loops compared to ABM F.

Fig. 10 Summary of results. The adult and perinatal repertoires differ most markedly in fraction B; however, in fraction F the repertoires have drawn closer to each other in terms of VH usage, DH usage, JH usage, length, and hydrophobicity and charge

from its adult counterpart in its distribution of CDR-H3 lengths, amino acid content, and charge. The changes that we have observed in early repertoire development could thus reflect either differences in the cells and tissues in which these developmental processes are occurring or they could reflect the effects of the absence of N addition. A similar analysis of repertoire development in a TdT deficient state would help clarify the extent, if any, to which N addition influences the changes that we have observed in repertoire development between fetus and adult.

Studies of the anatomy of hot spots in protein interfaces (Bogan and Thorn 1998) have shown that tryptophan, tyrosine, and arginine contribute disproportionately to binding energies. All three of these amino acids share the ability to contribute multiple types of favorable interactions. Tyrosine, the most commonly used amino acid in both adult and fetal CDR-H3 loops, offers a large hydrophobic surface, aromatic p-interactions, and the hydrogen bonding ability of its 4-hydroxyl group. Tryptophan and arginine also offer these binding opportunities. Tryptophan offers a large hydrophobic surface, aromatic p-interactions, and a hydrogen-binding donor. Arginine offers the ability to form a hydrogen bond network with up to five H-bonds, a guanidinium motif that offers a pseudo-aromatic p system, and three methylene carbon groups, which are all hydrophobic in character. In addition, arginine offers the ability to create salt bridges by means of its positively charged guanidinium motif.

Given the importance of these three amino acids, it is remarkable that the pattern of their usage between the fetus and the adult is so different. Globally, the fetus is enriched for tyrosine and depleted of arginine. DQ52 is an unusual D_H gene segment in that it lacks tyrosine in all six reading frames, encoding tryptophan in its place (Ivanov et al. 2002). The inclusion of DQ52 uniquely leads to enrichment for tryptophan-containing antigen-binding sites. Indeed, in our studies, the increase in the inclusion of tryptophan achieved statistical significance in fraction F ($p=0.03$). The biologic consequences of the increased prevalence of tryptophan-enriched CDR-H3s amongst mature neonatal B cells remains unclear when compared to adult, but this pattern holds true for both human and mouse (Ohno et al. 1985). It seems likely that all of these differences could contribute to the differential control of immune responses that characterizes early development (Silverstein 1977). The extensive similarities that we have observed between the composition of the fetal versus adult repertoires in mice and humans would suggest that the mouse may be a better model for the evaluation of both the mechanistic and functional consequences of human fetal CDR-H3 repertoire restriction than previously appreciated.

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