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The Peritoneal Cavity B-2 Antibody Repertoire Appears To Reflect Many of the Same Selective Pressures That Shape the B-1a and B-1b Repertoires

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To assess the extent and nature of somatic categorical selection of CDR-3 of the Ig H chain (CDR-H3) content in peritoneal cavity (PerC) B cells, we analyzed the composition of V_{H1}7183DJC\_\mu transcripts derived from sorted PerC B-1a, B-1b, and B-2 cells. We divided these sequences into those that contained N nucleotides (N\(^+\)) and those that did not (N\(^-\)) and then compared them with sequences cloned from sorted IgM\_\mu IgD\(_{\mu}\) B cells from neonatal liver and both wild-type and TdT-deficient adult bone marrow. We found that the PerC B-1a N\(^-\) repertoire is enriched for the signatures of CDR-H3 sequences present in neonatal liver and shares many features with the B-1b N\(^-\) repertoire, whereas the PerC B-1a N\(^+\), B-1b N\(^-\), and B-2 N\(^+\) repertoires are enriched for adult bone marrow sequence signatures. However, we also found several sequence signatures that were not shared with other mature perinatal or adult B cell subsets but were either unique or variably shared between the two or even among all three of the PerC subsets that we examined. These signatures included more sequences lacking N nucleotides in the B-2 population and an increased use of D_{H1} reading frame 2, which created CDR-H3s of greater average hydrophobicity. These findings provide support for both ontogenetic origin and shared Ag receptor-influenced selection as the mechanisms that shape the unique composition of the B-1a, B-1b, and B-2 repertoires. The PerC may thus serve as a general reservoir for B cells with Ag binding specificities that are uncommon in other mature compartments. The Journal of Immunology, 2010, 185: 6085–6095.

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Diversity provided by arrays of V, D, and J gene segments, the D\(\rightarrow\)J and V\(\rightarrow\)D junctions are further somatically diversified by the variable loss or palindromic gain of terminal gene segment nucleotides (3) and, in postnatal tissues, the random addition of N nucleotides (4, 5).

Although at first glance VDJ rearrangement and N addition would appear to permit unrestricted CDR-H3 diversity, the CDR-H3 repertoires expressed by specific B cell subsets often exhibit characteristic categorical constraints. These may include biases in VDJ gene segment usage, in D_{H1} reading frame preference, and in the number (i.e., length) and physicochemical properties (e.g., hydrophobicity or charge) of the encoded amino acids (6–8).

Some of these categorical constraints are genetically predetermined (9–13). Others are progressively imposed by selective mechanisms as developing B cells pass through critical developmental checkpoints in both primary and secondary lymphoid tissues (6, 14–16). In either case, these biases create what appear to be preferred ranges of potential structures and Ag binding complementarity surfaces in each B cell subset. For example, in previous studies, we identified somatically imposed sequence signatures that distinguished the range of CDR-H3 repertoires expressed in the spleen by marginal zone (MZ) B cells from those expressed by follicular (FO) B cells (14).

In the current work, we have sought to test whether B cells residing in the peritoneal cavity (PerC) also display evidence of categorical repertoire selection. We specifically sought to compare the repertoires expressed by PerC B-1a and B-1b cells, both major sources of natural Abs, with that of PerC B-2 cells, which have long been assumed to correspond with the conventional, recirculating mature B cell pool (17), as exemplified by the IgM\_\mu IgD\(_{\mu}\) B cells of the BM. Some previous studies have identified alternative lineages of differing ontogenetic origin as the basis for B-1a, B-1b, and B-2 cell repertoire diversity (18, 19), whereas others have
found evidence of a somatic selection for the BCR based on Ag engagement and signal strength (20, 21). Having recently performed an analysis of the pattern of categorical selection exhibited by the CDR-H3 repertoires expressed in BALB/c perinatal liver and in TdT-deficient adult BM (15, 16), we postulated that a comparative analysis of PerC B cell CDR-H3 repertoires might yield new insights into the derivation and selective pressures that give rise to the signature range of Ig repertoire diversity within these key PerC subsets.

Our analysis of the IgH sequences reported in this work provides support for both the ontogenetic and the Ag-driven somatic selection models of PerC B cell repertoire development. In support of the ontogenetic model, we found that each PerC B cell subset includes aspects of CDR-H3 signatures that are characteristic of their presumed ontogenetic origin (i.e., perinatal or adult). However, we also found support for the somatic selective model in that the CDR-H3 repertoires of B-1a, B-1b, and B-2 cells appear to share sequence characteristics that distinguish them from the repertoires expressed by mature B cells of either the perinatal liver or the postnatal BM. The latter results raise the possibility that B cells expressing BCRs enriched for specific categories of CDR-H3 amino acid sequence content are favored for entry or survival both in the PerC and that this selective advantage occurs regardless of ontogenetic origin or the expression of CD5.

Materials and Methods

Mice
The mice analyzed represent the progeny of a mixed 129/C57BL6 founder that had been back-crossed for 10 generations onto BALB/cJ (stock no. 000651; The Jackson Laboratories, Bar Harbor, ME) (6). Our initial analysis of BM CDR-H3 repertoire development had been performed on four separate mice derived from the same breeding pool (6). Additional samples were independently obtained from five individual 8- to 10-wk-old BALB/cJ mice bred in our own mouse colony (6, 11, 12, 14). All studies were performed in accordance with University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations.

Flow cytometry and cell sorting
Peritoneal B cells were isolated from two different mice. Single-cell suspensions were prepared by washing the PerC with 10 ml ice-cold FACS buffer (1× PBS with 2% heat-inactivated FCS). Cells were washed and resuspended in an appropriate volume of FACS buffer for counting and staining. Total peritoneal cells from individual mice were incubated in 1 ml fluorescently labeled Abs in FACS buffer. Sorting was performed on a MoFlo instrument (DakoCytomation, Ft. Collins, CO). The following mAbs were used to isolate peritoneal B cells into the B-1a, B-1b, and B-2 subpopulations (Supplemental Fig. 1) anti-IgM (Cy5) (Jackson ImmunoResearch, West Grove, PA), anti-CD19 (Spectral Red) (Southern Bio-technology, Birmingham, AL), anti-Mac-1 (FITC) (BD Pharmingen, San Diego, CA), and anti-CD5 (PE) (BD Pharmingen).

Sorting, RNA preparation, RT-PCR, and sequencing
For each peritoneal B cell population, 2 × 10^6 cells were sorted directly into RLT lysis buffer (RNeasy mini-kit; Qiagen, Valencia, CA). Because the lysis precluded reanalysis of the cells, we routinely used companion unfixed sorted cells to confirm the purity of the sorted populations. Based on “tight gating” for surface expression of IgM, CD19, Mac-1, and CD5, this purity of the populations was typically 99% for all peritoneal B cell subsets (data not shown).

RNA isolation, RT-PCR amplification, and sequencing were performed as previously described (6). A listing of the 383 unique, in-frame V H 7183DJC 4 sequences used for analysis in this work is provided in Supplemental Table I. All unique sequences have been placed in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) under the accession numbers HM132462–HM132836. The sequences from adult BM fraction F (BM F; BM mature IgM + IgD + B cells) used in this work were previously reported (6, 11, 12, 14). The sequences from NL fraction F (NL F; neonatal liver CD19 + IgM + IgD + B cells) used for comparison were previously reported (15), as were the sequences from TdT-deficient adult BM (16).

**Sequence analysis**
CDR-H3 was identified as the region between (but not including) the 3' terminal V H - TGT codon for the conserved Cys at Kabat (1) position 92 (international ImMunoGeneTics information system [IMGT] 104) and the 5' terminal J H TGG codon for the conserved Trp at Kabat position 103 (IMGT 118). CDR-H3 was separated into two components, the base (Kabat aa 93 and 94 [IMGT 105 and 106; typically alanine and arginine]) and Kabat aa 100–102 [IMGT 115–117; typically phenylalanine, aspartic acid, and tyrosine]) and the loop (the intervening amino acids).

**Structural analysis**
We used the “H3 rules,” as published by Shirai et al. (22, 23), to predict structural features of the CDR-H3 base and loop, as previously described (14). Briefly, the structure of the CDR-H3 base (termed kinked, extra-kinked, or extended) can be predicted in sequences that contain a minimum of five amino acid residues, including IMGT positions 105–118 (Kabat positions 93–103). In ~25–30% of the sequences with a kinked or extra-kinked CDR-H3 base, the H3 rules can predict whether an intact hydrogen bond ladder may be formed within the loop of the CDR-H3 region or whether the hydrogen bond ladder is likely to be broken. For example, proline residues tend to inhibit formation of a stable hydrogen bond ladder, whereas the presence of a V γ -encoded arginine at the N terminus of CDR-H3 in conjunction with a J H -encoded aspartic acid at the C terminus permits formation of a salt bridge that stabilizes the base. Glycine residues permit greater flexibility (23).

**Statistical analysis**
Differences between populations were assessed by two-tailed Student t test, two-tailed Fisher’s exact test, χ^2, or Levene’s tests for the homogeneity of variance, as appropriate. Analysis was performed with JMP version 7.0 (SAS Institute, Cary, NC). Means are reported with the SEM.

**Results**
Creation of a PerC B-1a-derived, B-1b-derived, and B-2-derived V H 7183DJC 4 sequence database
Initial studies of PerC CDR-H3 sequences identified the extent of N nucleotide addition as a characteristic feature that could be used to distinguish between Ig sequences derived from PerC B-1a (IgM +CD19 +Mac-1 −CD5 +) B cells versus those obtained from the alternative B-2 (IgM +CD19 +Mac-1 −CD5 −) population (24–26). B-1a cells characteristically demonstrate a paucity of N addition, whereas B-2 cells demonstrate an extensive array of N nucleotides in CDR-H3. These classic observations contributed greatly to the perception that many PerC B-1a cells were the progeny of fetal progenitors, which lack TdT activity; whereas B-2 cells were the progeny of postnatal BM-derived progenitors. However, in the absence of a detailed comparative analysis of the CDR-H3 repertoires expressed by conventional BM B cells; these initial studies left open the question whether the PerC B-2 repertoire was unrestrictedly drawn from the conventional B cell repertoire, as represented by the mature, recirculating BM IgM + IgD + B cells (Hardy fraction F) (27) or whether the B-2 subset might also be subject to somatic PerC-specific categorical selection.

To distinguish between these possibilities, we used RT-PCR to randomly clone V H 7183DJC 4 transcripts from sorted PerC B-1a, B-1b (IgM +CD19 +Mac-1 −CD5 +), and B-2 cells. We chose to focus our studies on the V H 7183 family because it comprises 10% of the repertoire, it is a V H family that is expressed in both fetal and adult B cell progenitors, and it does not contain any of the iconic PerC sequences that are overrepresented in transcripts using members of the V H11, V H12, or V H107 families (28–30). Indeed, we have generated an extensive database of V H11783 expressed by multiple other tissues and B cell subsets that can be used as a standard for comparison. These comparison populations include mature CD19 + IgM + IgD − B cells from NL F and BM F.

For the current study, we obtained a total of 379 in-frame, open, and unique sequences (see Supplemental Table I). Of these, 147 were derived from sorted B-1a cells, 118 from B-1b cells (CD19 +)}
Mac-1+CD5−), and 114 from B-2 cells (Supplemental Fig. 1). We compared these sequences as a population to 254 unique BM F sequences (6, 11, 12, 14) and 132 unique sequences from NL CD19+IgM+IgD+ fraction F (NL F) (15). Also available for comparison were 167 sequences from fraction B (pro-B), 313 from C (early pre-B), 229 sequences from D (late pre-B), and 203 from E (immature B) from wild-type (WT) TdT-deficient BM; 73 unique sequences from TdT-deficient BM F (BM F TdT−/−); BM mature IgM+IgD+ B cells from TdT-deficient mice); and 102 unique CDR-H3 sequences from splenic transitional T1, 97 unique CDR-H3 sequences from splenic transitional T2, 97 unique CDR-H3 sequences from splenic FO, and 151 unique CDR-H3 sequences from splenic MZ B cells (6, 11, 12, 14, 16).

Given that the absence of N addition in many B-1a CDR-H3 sequences is typically attributed to their fetal origin (31), we used N addition as an additional variable by grouping sequences into those that lacked N nucleotides (N−) and those that contained them (N+). These additional comparative groups included PerC B-1a, B-1b, and B-2 N− and N+ sequences, NL F cell N− and N+ sequences, and BM F N− sequences. Only 5 of the 254 (2%) adult BM F sequences from the TdT-deficient WT mice lacked N nucleotides, a number too small to permit a meaningful comparison. Thus, to control for the effect of an absence of N nucleotides on the adult BM repertoire, we further included 62 N− sequences obtained from TdT-deficient BM (16), with a focus on mature fraction F (BM F TdT−/− N−).

N− sequences are more common in the PerC B-2 subset than in BM F

In accordance with previous observations (26), when compared with the B-1a CDR-H3 repertoire, the B-2 CDR-H3 repertoire contained more sequences with N nucleotides (p < 0.0001) (Fig. 1). However, when compared with the CDR-H3 repertoires of BM immature B cells (fraction E), mature BM F B cells, or splenic FO B cells, the PerC B-2 repertoire proved to also be 5- to 7-fold enriched for CDR-H3s that lacked N regions (p < 0.001; χ2). The increased prevalence (15%) of N− CDR-H3s in the B-2 repertoire lay intermediate between that of the PerC B-1b (23%) and the splenic MZ B cell (10%) repertoires (p = 0.15 and p = 0.24, respectively; Fig. 1). The difference in N addition between B-1b cells and splenic MZ B cells achieved statistical significance at p = 0.007, as did the difference between B-1a cells (39%) and B-1b cells (23%; p = 0.005).

**Increased use of Vh7183.18 in all three of the peritoneal B cell subsets examined**

Differences in the Ig repertoires expressed by the PerC B cell subsets extended beyond CDR-H3 to the V domain as a whole, as represented by VH usage. The fetal liver H chain repertoire is heavily enriched for use of members of the DH-proximal VH7183 family (32–34). Within that family, which numbers 17 active members in the BALB/c IgM+ haplotype, VH81X (Vh7183.1) is the most highly expressed. For example, in our database of perinatal liver VH7183-containing sequences, VH81X-containing transcripts composed 10% of the NL F B cell VH7183-containing clones (Fig. 2, top) (15). When compared as a whole irrespective of N addition, only 2% (p < 0.001) of the B-1a, B-1b, or B-2 cloned cDNAs used VH81X. This matched the prevalence of this “fetal” sequence in the CDR-H3 repertoire of mature adult B cells (BM F; Fig. 2, top).

In young adults, BM fraction E (BM E) and BM F B cells preferentially express Vh7183.10 (6) and this VH continues to be overexpressed in both the FO and MZ B cell populations of the spleen (6, 14). In the PerC B cell subsets, Vh7183.10 usage was equivalent to that observed in BM E, BM F, splenic FO, and splenic MZ B cells (Fig. 2 and data not shown). Thus, when taken as a whole, all three PerC populations were marked by the two major VH usage characteristics most representative of the repertoires expressed by a large array of adult B cell subsets.

However, although the pattern of usage of VH81X and Vh7183.10 was similar to BM F and other adult subsets, several other VH gene segment signatures were found that were shared by all three PerC subsets but not by the BM or spleen B cells. For example, the most prominent member of the Vh7183 family expressed by the PerC B cells proved to be VH7183.18. It contributed to more than 20% of PerC B cell sequences (p < 0.001 and p < 0.05 compared with NL F and BM F, respectively).

The usage of individual Vh7183 gene segments in fetal versus adult B cell progenitors has been found to be heavily influenced by their physical location relative to the DH locus (35, 36). Fetal cells preferentially use DH proximal VH7183 gene segments rather than DH distal ones. Vh7183.18 is the most DH distal member of the Vh7183 family, again suggesting an “adult” influence on the repertoire of all three PerC B cell subsets. Intriguingly, the transitional T1 B cells of the spleen also exhibited a high prevalence of the use of this gene segment (17%; data not shown), suggesting that either there might be shared selection pressures acting on T1 and PerC B cells or that the transitional 1 stage is a branch point for selection (14).

**Vh7183 gene usage in N− B-1a transcripts followed the perinatal pattern**

When the three PerC B cell repertoires were analyzed by the presence or absence of N nucleotides, the extent of correlation between “fetal” and “adult” usage was found to differ by subset. Among the B-1a-derived clones, the pattern of Vh7183 usage in the
N- repertoire proved highly similar to NL F (Fig. 2, middle), demonstrating an increased use of VH81X and decreased use of VH7183.10. Indeed, only two of the other 15 VH7183 gene segments diverged significantly in prevalence from that observed in NL F B cells. Use of VH7183.13, which is Dl proximal, was uniquely significantly increased, whereas use of VH7183.2, which is Dh proximal, was significantly decreased but matched that of the N- B-1b and B-2 repertoires. Among the N+ compartment, use of VH81X and VH7183.10 followed the pattern expressed by adult BM F B cells, and enhanced use of Dh distal VH7183.18 was evident.

Unlike B-1a, the B-1b-derived N- repertoire followed an “adult” rather than a “fetal” pattern, with VH usage closely resembling that of adult BM F TdT-/- N- sequences. None of these N- sequences used VH81X, and use of VH7183.10 and VH7183.18 matched that of TdT-/- BM F N+. Two outliers in the N- subset were: VH7183.6 in B-1b and VH7183.14 in B-2, both of which are Dh proximal. The N+ PerC B-1b-derived VH repertoire also resembled BM F more than NL F (Fig. 2, middle). Among the sequence signatures that diverged from BM F, an increased use of Dh distal VH7183.18 was prominent. Dh distal VH7183.13 was also increased, whereas use of Dh proximal VH7183.16 and VH7183.14 was decreased.

Among the B-2 N- derived sequences, the pattern of VH usage, including VH81X and VH7183.10, again largely matched that of BM F TdT-/- N-. Exceptions included an increased use of Dh distal VH7183.18 and VH7183.11. Not unexpectedly, VH4 usage among the B-2 N- sequences largely matched WT BM F. However, the N+ repertoire was again enriched for the use of Dh distal VH7183.18; and there was also a decrease in the use of Dh proximal VH7183.16 (Fig. 2).

Altered patterns of Dh and Jh usage in B-2 cells

Signature differences in individual Dh and Jh gene segments are also apparent in the fetal and adult CDR-H3 repertoires (15).

Perinatal B cells use the DQ52 gene segment at a greater frequency than that of adult B cells; whereas adult B cells use DFL family gene segments at a greater frequency than that of perinatal B cells.

We examined Dh usage in the presence or absence of N nucleotides and found that both the B-1a and B-1b N- repertoires contained DQ52 more frequently than the B-1a N+ and B-1b N+ cells, respectively; whereas B-2 cells matched the pattern of adult BM DQ52 usage irrespective of the presence or absence of N nucleotides (Fig. 3, middle). With regards to DFL gene segments, B-1a and B-1b B cells demonstrated an adult usage pattern, and B-2 cells used DFL gene segments more frequently than adult BM again irrespective of the presence or absence of N nucleotides.

The increased use of DFL family members in B-2 cells achieved statistical significance compared with B-1a (p < 0.01), B-1b (p < 0.01), NL F (p < 0.01), and BM F (p < 0.05; Fig. 3).

Within the Jh locus, perinatal B cells demonstrate a relative preference for use of Jh2, whereas adult cells use Jh3 and Jh4 more frequently. Although B-1a N- sequences demonstrated an increased use of Jh2 and a diminished use of Jh3 and Jh4 relative to adult, the differences in Jh2 between B-1a and NL F were still significant at p < 0.05 (Fig. 3, top). Among the N- sequences, no differences in Jh usage were observed between B-1a and BM F N- or BM F N+. B-1b cells followed the same pattern at the same level of significance. Among the B-2 cells, there was a trend for the N- sequences to be closer in Jh usage to NL F than to BM F, but these trends did not achieve statistical significance. No differences were observed among the N+ repertoires.

Dh reading frame usage also diverged from a direct fetal/adult paradigm. Dh gene segments can theoretically be read in any one of six reading frames, three by deletion and three by inversion. However, a preference for use of reading frame 1 by deletion is a near universal facet of Dh usage in all jawed vertebrates (6). This bias is greatest in the perinatal period (15). Although the three PerC B cell subsets maintained the expected preference for RF1 by deletion, use of RF2 was unexpectedly enhanced (p <
From PerC B cell CDR-H3 containing N nucleotide addition (N+). All comparisons were made to fraction F, either from 1-d liver (NL F) or BM (BM F) frame.

Top reported as the percentage of the sequenced population of DFL- and DSP-containing transcripts from each B cell population that uses the specified reading frame. Microhomology between the 3' termini of the DH gene segments facilitates RF1 rearrangement (Fig. 3) [24, 31, 37].

The global amino acid content of the CDR-H3 loops is largely dependent on N nucleotide additions

The CDR-H3 loop is biased for the use of tyrosine and against the use of highly charged or highly hydrophobic amino acids (7, 8). This bias is first established in developing BM B cells (6) and is most apparent in the perinatal liver (15). The bias for the use of tyrosine in the CDR-H3 loop was maintained in all three PerC B cell subsets when analyzed irrespective of N addition (Fig. 4, top). However, this bias was substantially diminished compared with NL F (p < 0.0001; Fig. 4, top). B-1a cells were intermediate in their use of tyrosine between NL F (p = 0.0009) and BM F (p < 0.05), whereas use of tyrosine in B-1b and B-2 cells was statistically indistinguishable from that of BM F. PerC B-1a, B-1b, and B-2 cells also used arginine (charged) and valine (hydrophobic) more frequently than did NL F (p < 0.0001; Fig. 4, top).

Although the difference in tyrosine usage was found to primarily reflect the contribution of N addition, each PerC B cell subset again demonstrated its own characteristic amino acid signature. PerC B cell sequences lacking N nucleotides from all three subsets had higher tyrosine content than that of BM F (p < 0.05) and completely lacked lysine, proline, cysteine, and phenylalanine. This matched the pattern observed in both NL F and BM F TdT−/− (Fig. 4, middle). Among the sequences lacking N nucleotides, histidine was more common in B-1a N− CDR-H3s, serine in B-1b and B-2, and valine in B-2. The increased use of histidine in B-1a reflected a preference for a specific type of V→D overlap in terminal sequence at Kabat position 95 (38). The increased use of serine in B-1b and B-2 correlated with the increased use of DFL16.1, which is enriched for serine (11). The increased use of valine in B-2 reflected the increased use of RF2 (13). Among the PerC N+ sequences, amino acid content proved similar, but not identical, to that of BM F.

One invariant amino acid was glycine, which was present at the same frequency in all three PerC subsets regardless of the presence or absence of N nucleotides. The frequency of use of glycine matched that of both WT BM F and BM F TdT−/−, but WT BM F was significantly greater than that observed in NL F (p < 0.05) (Fig. 4). These data would suggest that a specific level of glycine offers a selective advantage in an adult environment.

The average lengths of B-1a, B-1b, and B-2 CDR-H3s lie between neonatal and adult CDR-H3s

During B cell development, average CDR-H3 length is adjusted to fit an apparently preferred set point specific for each developmental checkpoint (6, 14). For example, in adult BM, the average length of BM F CDR-H3 is 0.5 codons longer than that of BM E (6). This particular transition appears to occur in the periphery because the average length of splenic T1 CDR-H3s matches that of BM E, whereas the average length of FO CDR-H3s matches that of BM F (14).

The lengths of CDR-H3 in PerC B cells in comparison with that of NL and BM cells demonstrated a progressive hierarchy with NL F << B1a < B1b << B2 << BM F (Fig. 5, left). The average CDR-H3 length of NL F B cells was 0.8 and 0.9 codons shorter than that of B-1a and B-1b, respectively (p < 0.005). The B-1a sequences averaged 0.1 codons less than that of B-1b, but this
The presence or absence of N nucleotides greatly affected CDR-H3 length. Among the N- sequences, the average lengths of NL F, B-1a, B-1b, and B-2 (\(p = 0.53\)) were statistically indistinguishable from each other. However, among the N+ sequences, the average length of all three PerC B cell sequences was easily distinguishable from that of NL F (\(p < 0.0001; \text{Fig. 5, right}\)) and, for B-1a and B-1b, from that of BM F. The average length of the B-2 N- sequences was shorter than that of BM F but did not achieve statistical significance (\(p < 0.05\)). No statistically significant differences in average length were found among the N+ sequences from B-1a, B-1b, or B2 (Fig. 5, right). Thus, the differences in average CDR-H3 length between the three PerC B cell subsets and NL F and BM F as a whole were mostly due to the sequences that contained N nucleotides.

In addition to changes in average length, we previously demonstrated that in the progression from fraction E to the intermediary populations in the spleen (T1 and FO) to recirculating fraction F, the variance in CDR-H3 length narrowed (14). This decrease in variance was greatly influenced by the progressive loss of CDR-H3s containing fewer than nine codons (6, 14). However, in the PerC, sequences containing CDR-H3s of fewer than nine codons appeared to be retained in all three of the PerC subsets (Supplemental Fig. 2), including B-2 with 9% short sequences versus only 4% for BM F (\(p < 0.05\)). This enrichment for short sequences is especially notable compared with BM F TdT-/-, which is devoid of CDR-H3s containing only five codons, whereas this length is still prevalent in B-1a and B-1b cells (Supplemental Fig. 2, center).

N nucleotide addition had a variable effect on the prevalence of short CDR-H3s. Of the B-1a CDR-H3s, one in five contained fewer than nine codons (\(p < 0.05\)); \(p < 0.01\); \(p < 0.001\); \(p < 0.0001\).
than nine codons. N addition had no effect on the prevalence of CDR-H3s with fewer than nine codons, a prevalence that matched that of NL F. However, for the B-1b subset, 1 in 4 of the N+ sequences were short, but only 1 in 10 of the N sequences contained fewer than nine codons (Supplemental Fig. 2). Similarly, short CDR-H3s were more common among B-2 N+ sequences (1 in 8) than in N+ (1 in 12).

**Enrichment for hydrophobic CDR-H3s in the PerC B cell subsets**

The average hydrophobicities of each of the B-1a, B-1b, and B-2 cell CDR-H3 repertoires were significantly more hydrophobic than NL F (p < 0.01, p < 0.01, and p < 0.0001, respectively; Fig. 6, left). The average hydrophobicity of the B-2 CDR-H3 repertoire was also significantly more hydrophobic than BM F (p < 0.05). This trend toward increased hydrophobicity was apparent among both the N+ and N sequences (Fig. 6).

As with length, the variance in average hydrophobicity of CDR-H3 also normally decreases with development (6, 14). This decrease in variance is due, in part, to the loss of sequences at the extremes (6, 14). In BALB/c BM Hardy fraction C, for example, there are a number of sequences with an average normalized Kyte–Doolittle hydrophy of 39, 40) of less than −0.700 (charged), whereas BM F sequences, with these characteristics largely no longer contribute to the repertoire (Supplemental Fig. 3 and Refs. 6, 14). This focusing of the hydrophobicity of CDR-H3 is less apparent in fetal B cells, which are more dependent on the use of germline sequence and neutral Dμ RF1 (15).

In the PerC, the hydrophobicity distributions of the repertoires of all three B cell subsets were more similar to that of NL F than BM F (Supplemental Fig. 3). In particular, the distribution of average CDR-H3 hydrophobicity for the B-2 subset demonstrated the same pattern of preservation of highly hydrophobic CDR-H3s as that of BM E and splenic T1 B cells (Supplemental Fig. 3) (14).

**Predicted CDR-H3 base and loop structure**

Given the great diversity of CDR-H3, it has proved difficult to predict definitively the structure generated by an individual sequence. However, using the H3 rules of Shirai et al. (23), it is possible to gain insight into the likely structure of the base of the CDR-H3 loop and to predict whether sequences with a kinked or extrakinked CDR-H3 base will be able to generate an intact or a broken hydrogen bond ladder. We found that the B-1a CDR-H3 repertoire was the least likely to include extrakinked bases (p < 0.02 versus BM F), whereas the B-1b CDR-H3 repertoire was the most likely to create extended bases (p < 0.05 versus BM F) (Fig. 7A). However, whereas the differences in likely base structures between the PerC B cell subsets appeared relatively minor when considered as a whole, striking differences were found among the sequences that lacked N nucleotides. The B-1a N+ CDR-H3 repertoire was devoid of extrakinked bases (p < 0.02 versus both NL F and BM F TdT−/+), whereas more than one-third of the B-1b N+ CDR-H3 repertoire contained likely extended bases with a compensatory decrease in the likelihood of generating kinked bases.

When we evaluated likely loop structures, we found that the B-1a and B-1b structures were again similar to each other in the likely distribution of intact or broken hydrogen ladders compared with NL F (p < 0.05, p < 0.01, respectively), B-2 (p < 0.001, p < 0.05, respectively), and BM F (p < 0.0001, p < 0.001, respectively), whereas the B-2 repertoire proved very similar in likely structures to BM F (Fig. 7B). No broken ladders were predicted in the N+ repertoires. Among the N+ repertoire, the NL F clearly diverged from B-1a and B-1b, which shared a similar likely distribution of intact versus broken ladders, and both of these sets diverged from the B-2 and BM F repertoires, which again shared similar likelihoods (Fig. 7). Thus among that subset of N+ sequences with kinked or extrakinked bases, the B-1a and B-1b repertoires shared a similar pattern of loop structure distributions. This and other key CDR-H3 features shared among the PerC B cell subsets are summarized in Fig. 8.

**Discussion**

The recent identification of a specific B-1 progenitor (19) has provided strong support for previous models proposing distinct developmental pathways for the peritoneal B cell subsets (18, 41, 42). In previous work, we had shown that somatic selection for or against certain categories of Ig-CRD-H3 sequence occurs during B cell development in the BM of BALB/c mice (6) and that the sequence content of CDR-H3 appears to help shape the fate of the B cells in peripheral lymphoid tissues (14). In the current study, we analyzed the expressed VH7183-containing CDR-H3 repertoire in the PerC B cell subsets and compared them with repertoires expressed in NL and adult BM. Because we were comparing equivalent repertoires, these studies allowed us to test the extent to which specific categories of CDR-H3 sequence, including those that reflect ontogenetic origin (24, 25, 31), might be associated with populating PerC B cell niches. We identified key signatures that were variably shared among the PerC B-1a, B-1b, and B-2 subsets and IgM+IgD+ B cell subsets within the NL and the adult BM with or without N addition (Fig. 8). We also identified key signatures that appeared to be specific for each individual PerC B cell subset as well as those that were shared between two or all three of these subsets.

![FIGURE 6.](http://www.jimmunol.org/)

Average CDR-H3 hydrophobicity of VH7183-containing transcripts isolated from sorted 1-d liver, PerC, and BM mature B cells. Average CDR-H3 loop hydrophobicity as assessed by a normalized Kyte–Doolittle scale (39, 40) from NL F (mature), PerC B-1a, B-1b, and B-2, and BM F (mature, recirculating) from either WT or TdT−/+ (BM F TdT−/+). Error bars depict the SE of each mean.
In a number of ways, our work confirms and extends previous observations by other investigators (24–26, 43). Our findings confirmed that the PerC B-1a N_2 repertoire is enriched for the signatures of CDR-H3 sequences present in NL, whereas the PerC B-2 N+ repertoire is enriched for adult BM sequence signatures. Our analysis extended the structural significance of these findings by demonstrating that the predicted CDR-H3 loop structures in all the N_2 transcripts from the PerC B cell subsets also share neonatal signatures, such as the absence of broken ladders. Similarly, broken ladders were present only in N+ sequences, matching the adult pattern of BM F. These observations provide strong support for the role of ontogenetic origin in controlling the composition of the Ab repertoire expressed by individual PerC B cell subsets. In particular, our findings support the now classic view that the PerC B-1a population serves as a reservoir for B cells that can produce fetal-like Igs, which reinforces the role of these cells as a source of ontogenetically regulated natural Abs (44).

Our findings also confirm that a significant subset of the B-1a repertoire includes CDR-H3s with extensive N region addition (24–26). Our extended findings that B-1a sequences containing N regions (N+) were more similar to BM F provide new support for the view that at least some B-1a cells, likely expressing CDR-H3s with N nucleotides, are derived from postnatal BM (45, 46).

Our findings confirm previous reports (26) that the B-1b population shares a number of sequence characteristics with the B-1a population. Our extended findings that the B-1b N+ repertoire also exhibits many of the features that characterize the adult BM F repertoire provide new support for the view that the B-1b population is also a mixture of the progeny of the perinatal liver and the postnatal BM (19, 42, 45).

Finally, our extended findings show that the N+ B-2 repertoire also shares many of the characteristics of the adult BM F repertoire, again providing new support for the view that B-2 cells whose Ag receptors contain N nucleotides are the product of postnatal B cell development.

However, in addition to these findings, which were to be expected if ontogenetic origin plays a key role in determining the fate of the B-1a, B-1b, and B-2 repertoires, there were several unexpected

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**FIGURE 7.** Distribution of the predicted structures of the base and the loop of CDR-H3 from V_{H}7183DJC3 transcripts isolated from sorted 1-d liver, PerC, and BM mature B cells. Structural properties were predicted according to the “H3 rules” of Shirai et al. (23) deduced from the analysis of well-determined CDR-H3 crystal structures. These rules allow prediction of structural features from the primary CDR-H3 sequence based on the location and hydrophobicity of amino acids and size of the side chain. Frequencies are reported as percentage of all sequences analyzed. A, Frequency of kinked (K~), extrakinked (K^), and extended (E) CDR-H3 bases from NL F (mature), PerC B-1a, B-1b, and B-2, and BM F (mature, recirculating) from either WT or TdT^-/- (BM F TdT^-/-) mice. B, Frequency of broken and intact hydrogen bond ladders within the CDR-H3 loop for those H chains that contain kinked or extrakinked bases depicted in A: all V_{H}7183 transcripts (top); V_{H}7183 transcripts from NL F and PerC B cell CDR-H3 without N nucleotide addition (N^-) and BM F from TdT-deficient (BM F TdT^-/-) mice (middle); V_{H}7183 transcripts from NL F, PerC B, and BM F cell CDR-H3 containing N nucleotide addition (N+) (bottom). Statistical analyzes were made comparing with PerC B cell subsets using χ² or Fisher’s exact test as appropriate. a p ≤ 0.05 compared with B-1a; b p ≤ 0.05 compared with B-1b; c p ≤ 0.05 compared with B-2.
findings that suggest that PerC-specific somatic selection is also playing a role in all three subsets. In particular, the PerC B-2 population, which is usually considered to be directly derived from the pool of conventional B cells that circulate through the blood, spleen, and BM (27), presented significantly fewer N regions than the other so-called “conventional” B cells. Also, we identified several unique sequence signatures that were shared between the two or even among all three of the PerC subsets but not with other mature perinatal or adult compartments. This latter finding suggests that all three PerC subsets are enriched for B cells that have responded to a shared Ag receptor-based selective stimulus.

We considered three possible explanations for the increased prevalence of N− sequences in the B-2 population.

First, it is possible that the B-2 cells that express CDR-H3s without N nucleotides were generated in the perinatal period and are the product of a PerC environment that permits or promotes long-term survival. This view would be consistent with the results of co-transfer studies of B220− cells sorted from both fetal liver and adult BM in the same adoptive recipients showing that B-2 cells can be derived from both sources (47). However, IL-7−/− mice, which are populated primarily by B lymphocytes of perinatal origin, show few cells with B-2 phenotype in the PerC (48). And, although a fetal origin for N− B-2 sequences is quite attractive, our analysis failed to demonstrate the same type of signature sequence similarity between the B-2 N− and NL F repertoires that was so evident for the B-1a N− sequences (Fig. 8).

Second, it is possible that whereas the current surface markers used to identify B-2 cells within the PerC also identify the conventional, recirculating, mature BM B cell pool, these PerC B-2 cells actually are either a separate B cell population for which adequate markers have not yet been identified or that these PerC B-2 cells represent a single temporal point in a continuing developmental pathway where B-2 cells can acquire a B-1b-like phenotype in the peritoneal compartment as proposed by Rothstein and colleagues (49). However, given the divergence in V_{H}7183.18 usage between B-1b and B-2 as documented by this and previous work by others (25, 26), and also the divergence in the predicted CDR-H3 base, mainly in N+ transcripts, and loop between B-1b and B-2, it seems less likely that B-2 cells represent a single temporal point in the B-1b development. Deep repertoire sequencing of the major B cell subsets from mice co-transferred with fetal liver and BM B220− cells into the same irradiated recipient may shed further light on this issue.

Third, Ag receptor-influenced selection against B cells with N region containing CDR-H3 sequences or for B cells with CDR-H3s lacking N addition might be influencing entry or survival or both (43). Such a selective process would be consistent with our current hypothesis that individual niches exert categorical selection of the B cell repertoire (14).

We recognize that all three of these possibilities are not necessarily mutually exclusive. Still, although all three PerC B cell subsets demonstrated several sequence signatures that were unique and differentiated them from other mature B cell subsets, the B-2 repertoire was the most marked of the three (Fig. 8). And, several of the sequence signatures that separated B-2 from BM F and NL F were shared with the B-1a and B-1b subsets. For example, we found a unique preference for V_{H}7183.18 gene usage among all three PerC N+ B cell subsets, which might suggest that the cells bearing BCR encoded for V_{H}7183.18 are derived from adult precursors. In support of this hypothesis, the splenic transitional 1 (T1) B cells also use V_{H}7183.18 at the same high frequency (14), suggesting that the V_{H} content also influences entry into the peritoneal compartment, as we have shown for the spleen (14). The functional meaning of the enhanced frequency of the V_{H}7183.18 gene in the PerC is unclear and may require analysis of the specificity of PerC V_{H}7183.18-containing Abs. But at least in terms of B cell development, our data point to a close relation among the splenic T1 subset and PerC B-1a N+, B-1b N+, and B-2 cells irrespective of N addition, which would be compatible with a precursor-product relationship with selection based on the composition of the Ag receptor as the final force driving cells into each individual mature compartment. Support for such a precursor-product hypothesis comes from the observation that, in the absence of a spleen, PerC B-1a cell development is inhibited (50).

Evidence of enrichment for hydrophobic CDR-H3s in B-1a N+, B-1b N+, and B-2 cells irrespective of N addition is another new finding. In BM F and the splenic follicles, the CDR-H3 loop is normally biased for the expression of neutral, hydrophilic amino acids such as tyrosine, glycine, and serine, whereas use of hydrophobic and charged amino acids is minimized (8). This bias reflects, in part, evolutionary selection of D and J_{H} sequence coupled with a preference for use of only one of the six potential
of Ag binding specificities that are uncommon or rare elsewhere that this compartment may also be able to produce a unique range of antibodies. It appears to be shaped by the H3 repertoire expressed by the B-2 compartment, which is pre-selected by the D(i)-D(i) mice and the normal number of PerC B cells presented by D(DuFS) follow the same pattern of exclusion or inclusion of specific categories of CDR-H3 hydrophobicity observed in splenic MZ and FO B cells and in the mature BM B cells from WT mice (14). Although suggestive, it remains unclear whether the low number of cells remaining in the PerC B cell compartment in D(i)-D(i) mice express the same CDR-H3 repertoire as im- mune to the D(i)-D(i) altered segment or whether there is evidence for selection of alternative clones that best fit the categorical exclu- sions in each compartment. The composition of Ig CDR-H3 repertoires in the peripheral B cell compartments of those D(i)-D(i) mice is a current focus of investigation in our laboratory.

In summary, a detailed analysis of sequences expressed by PerC B cells has shown that whereas each PerC B cell subset retains a number of CDR-H3 sequence signatures that mark their respective developmental and ontogenetic origins (54) (i.e., fetal/ adult origins or both), the final result includes signature features that are unique to the PerC. We interpret our findings to suggest that PerC B cells are the product of PerC-specific Ag receptor-mediated selective pressures, which may, in part, help regulate either entry or survival in the peritoneal compartment. PerC B-1a and B-1b cells have been credited as the source of the natural Abs, especially antibodies against these antigens.

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Disclosures

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