An Unbiased Analysis of $\text{V}_{\text{H}}$-D-$\text{J}_{\text{H}}$ Sequences from B-1a, B-1b, and Conventional B Cells$^{1,2}$

Aaron B. Kantor,* Cynthia E. Merrill,* Leonore A. Herzenberg,* and Jan L. Hillson*$$

Previous studies conclude that the repertoire of B-1a (CD5$^+$ B) cells is highly restricted. Studies here, which use FACs sorting and single-cell PCR methodology to develop an unbiased representation of the IgH repertoires of B-1a, B-1b, and conventional B cells from the peritoneal cavity, demonstrate that the B-1a cell repertoire is more diverse than previously thought. Furthermore, adult B-1a cells have significantly fewer noncened nucleotide (N) insertions than conventional B cells. However, B-1a cells are not defined by the absence of these regions, since such insertions are present in two-thirds of B-1a cell transcripts. All three B cell populations use a wide spectrum of $\text{V}_{\text{H}}$, D, and $\text{J}_{\text{H}}$ elements and display considerable diversity in complementarity-determining region 3 (CDR3). However, characteristic differences in the repertoires of all three B cell populations also exist, suggesting different selective and/or developmental forces act to shape each repertoire. The Journal of Immunology, 1997, 158: 1175-1186.

Munire B cells may be divided into at least three subsets distinguished by their surface markers and the timing, location, and pathway of development. Conventional B (B-2) cells are replenished throughout life from progenitor cells and form the bulk of circulating B lymphocytes. B-1a cells (Ly-1/CD5 B cells) arise early in ontogeny and maintain their numbers by self-replenishment. They constitute a few percent of the total B cells in the mouse and are implicated in diseases of B cell dysregulation, including leukemia and autoimmune disease. B-1b cells share many properties with B-1a cells, but can also readily develop from progenitors in adult bone marrow (1-3). Furthermore, a feedback mechanism impedes the entry of new B-1a and B-1b cells into the peripheral pool after about 6 to 8 wk of life (4, 5). The present study was designed to compare the IgH repertoire of these subsets at the molecular level with a view for providing a basis set for evaluating selective and developmental mechanisms that shape each repertoire.

By sampling in an unbiased manner, we endeavored to determine general characteristics of the repertoire, to seek characteristics that distinguish the populations, and to compare features of B-1a cells with those previously described for fetal B cells. Most of the current information about the expressed Ab repertoire has been obtained by study of hybridomas, bulk and bulk-amplified cDNA libraries, LPS-stimulated cells, and hybridization techniques. To avoid various limitations and biases presented by these

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A preliminary discussion of some of the data has been presented (65).

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5 Abbreviations used in this paper: CDR, complementarity-determining region; N-regions, nontemplated nucleotides at V-D or J-J junctions; P-regions, templated nucleotides; RT, reverse transcriptase; TdT, terminal deoxynucleotidyl transferase; RF, reading frame.

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sequences not necessarily representative of the populations studied.
In fact, by sampling repertoires in an unbiased manner, using single-cell PCR and FACS sorting, we demonstrate here the B-1a Ab repertoire can display substantial diversity.

We analyze B-1b cells as a separate group and demonstrate a different pattern of \( V_{\mu} \) family usage compared with either B-1a or conventional B cells. Specifically, B-1b cells use the J585 and Q52 families less frequently than the V\( \mu \)10 family more frequently.

We examine the CDR3 regions and the use of N region insertions. Murine fetal and neonatal B cells rarely have N region insertions in the V-D-J and D-J junctions, whereas most such junctions recovered from adults have N regions (22-26). Since B-1a cells are associated with development early in ontogeny, there is keen interest in the frequency and level of their N region insertions. Here, we interest, we evaluate cells from all \( V_{\mu} \) families as they are represented in the subpopulations and demonstrate at the single-cell level that B-1a cells in the adult use N region insertions less frequently than either B-1b or conventional B cells.

Materials and Methods

Cells, animals, and cell lines

Peritoneal washes pooled from five adult female BALB/c mice (IgH-C-3) mice at 5.5 mo of age were used in each of two separate experiments. The following cell lines (kindly provided by M. Cazenon, University of Pennsylvania, Philadelphia, PA; E. Voas, University of Illinois, Chicago, Urbana, IL; J. Goverman, University of Washington, Seattle, WA; R. single-cell PCR and FACS sorting, we deme the hectares here the B-1a Ab repertoire can display substantial diversity.

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are shown in Figure 1. Each population was first bulk sorted based on size, viability, and expression of IgM, IgD, and Ly-1. Conventional B cells are IgM<sup>+</sup>IgD<sup>+</sup>CD5<sup>+</sup>CD5<sup>-</sup>. Both B-1a and B-1b cells are IgM<sup>+</sup>IgD<sup>-</sup>CD5<sup>+</sup>; however, B-1a cells are CD5<sup>-</sup> and B-1b cells are CD5<sup>+</sup>. Left panels present the FACS profiles before sorting. The remaining panels display the reanalyses of the bulk sort for each population. After the bulk sort, the cells are sorted again at one per tube. Thus each sample is FACS purified twice.

**FIGURE 1.** Three B cell populations from the peritoneal cavity of adult BALB/c mice were sorted according to their FACS phenotype. Conventional B cells are IgM<sup>+</sup>IgD<sup>+</sup>CD5<sup>-</sup>CD5<sup>+</sup>. Both B-1a and B-1b cells are IgM<sup>+</sup>IgD<sup>-</sup>CD5<sup>+</sup>; however, B-1a cells are CD5<sup>-</sup> and B-1b cells are CD5<sup>+</sup>. Left panels present the FACS profiles before sorting. The remaining panels display the reanalyses of the bulk sort for each population. After the bulk sort, the cells are sorted again at one per tube. Thus each sample is FACS purified twice.

**B-1a, B-1b and conventional B cells have similarities in V<sub>H</sub> gene usage**

Figure 3 provides V<sub>H</sub> D, and J<sub>H</sub> frequency distributions of the data reported in Figure 2. To a first approximation, the pattern of V<sub>H</sub> gene family usage is strikingly similar among the three B cell subsets. Each subset uses a spectrum of families with frequency distributions roughly corresponding to the germline complexity of each V<sub>H</sub> family. For all three B cell populations, the J558 family, which comprises about half of all known germline genes (Ref. 29 and references therein) predominates: 30% of B-1b cells, 40% of B-1a cells, and 51% conventional B cells use J558 genes. The second largest V<sub>H</sub> family, Q52, which constitutes perhaps 10% of the total germline V<sub>H</sub> genes, is used the second most frequently in all three B cell subsets, ranging from 19% among B-1b cells, 22% among conventional B cells, to 27% among B-1a cells. Together, the J558 and Q52 families constitute 62% of all V<sub>H</sub> genes observed in our study.

This general V<sub>H</sub> family utilization pattern, favoring J558 and Q52, is similar to previous patterns using probes specific for family subsets to analyze adult spleen (32) and peritoneal B cell subsets (33). In contrast, the J558 family was observed much less frequently in the RNA hybridization analysis of LPS-stimulated BALB/c peritoneal B-1a (2-3%) and B-1b + B-2 (7-8%) cells from BALB/c mice conducted by Andrade et al. (34). While they also found that B-1a cells used a variety of V<sub>H</sub> families, the VH11 (20-25%) and Q52 (20-25%) families were most prevalent among their stimulated B-1a cells (34). No attempt was made to analyze clonal populations in that study.

**B-1a, B-1b, and conventional B cells have differences in V<sub>H</sub> gene usage**

Despite the overall similarity in general pattern, the B cell subsets do differ significantly in the representation of some V<sub>H</sub> families. They use of the J558 family is significantly lower among B-1b cells (30%) than conventional B cells (51%, p < 0.05 by the χ² statistic). The combined usage of J558 and Q52 families is also
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**FIGURE 2.** V(3')-D(4') junctions of rearranged IgH transcripts expressed in conventional B, B-1a, and B-1b cells. The solid horizontal line separates two sorting experiments. Assignment protocols are discussed in Materials and Methods. For each sequence, we report a sequence number (AA and AB represent different experiments), the V(3') family, the most 3' nucleotides of the V(3') (starting with Cys = TG), the D element, the six most 5' nucleotides of the D(4') element, the number of the V(3') element, the number of the D(4') element, the reading frames (according to Ref. 28), and the D element identification (Q = DQ); S = DSP, P = DFL, T = DST; two equivalent choices are denoted by or; an alternative assignment with different N regions is denoted with al). P nucleotides are indicated next to the germline elements. If a nucleotide can possibly be a P, it is designated as such rather than as an N. Underlined sequences can be from either of two germline elements. Here we group them with the more 5' element. Vertical lines separate multiple D elements. Only second D elements of five or more nucleotides or more are noted. There are many four-nucleotide possibilities. A total of 184 transcripts are reported, 53 for B-1a, 70 for B-1b, and 59 for B-2. Sequences designated AA and AB are from two different pools of mice, sorted on different days.
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**TABLE 1.** Continued.

Significantly less frequent for B-1b cells (49%) than for either B-1a (67%, p < 0.05) or conventional (73%, p < 0.01) B cells.

The lower usage of the J558 and Q52 families by B-1b cells must be accounted for by other Vb families. The data provide some interesting candidates, although limitations in sample size prevent absolute conclusions. The third most common family in this data set, the 3660 family (20 sequences), which constitutes about 5% of the germ line genes, occurred more frequently among B-1b cells (16%) compared with B-1a (7%) and conventional B cells (9%). Although the difference is not statistically significant. A second family, Vb10, which has been associated with anti-DNA specificity (35), occurred more frequently among B-1b cells (10%) than either B-1a (2%, p < 0.01) or conventional B cells (2%, p < 0.1). The higher use of the Vb10 family among B-1b cells was observed in both experiments (9 and 11b). The combined usage of the 3660 and Vb10 families is also significantly more frequent among B-1b cells (26%) than B-1a (9%, p < 0.05) and conventional (10%, p < 0.05) B cells and is consistent with the lower usage of J558 and Q52 by B-1b cells.

The number of sequences limits conclusions about other families. However, we note that the Vb11 and Vb12 families (four and three sequences, respectively) that have been associated with B-1
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**FIGURE 2.** (continued.)
cells specific for phosphatidylcholine (PIC liposome, BrMRBC) (20, 21, 36, 37) are found in the B-1a and B-1b subsets, but not among the conventional B cell transcripts (p < 0.1 for B-1a vs conventional B cells). This level of Vp,11 usage among B-1a cells (6%) is lower than other studies (20%) (34); however, this can be explained by a large proportion of PIC binders in BALB/c mice that use the Q52 family (K. Seidl, J. MacKenzie, L. A. Herzenberg, and A. B. Kantor, manuscript in preparation).

There is no preference for J_p-proximal Vp families in any of the cases sampled here. Previous studies with Vp family specific probes demonstrated that fetal and neonatal B cells preferentially express Vp families located J_p proximal on the chromosome (32, 38-42). In those studies, the two most J_p-proximal Vp families, Q52 and 7183, constitue 50% of the Vp usage, including very high use of the most J_p proximal gene Vp,11X. B-1a cells, which are associated with early ontologic development, might be expected to favor J_p proximal Vp genes. However, this is clearly not the case in the population sampled for the present study. We found that family usage among partitioned B-1a, B-1b, and conventional B cells is similar to that reported for adult splenic B cells (38, 41). We did not observe the 81X gene in any of the these populations.

B-1a, B-1b, and conventional B cells exhibit characteristic patterns of D and J usage

The general patterns of D family use are similar among the three B cell subsets (Figs. 2 and 3). DFL16.1 is the most commonly used single element in each subset. The use of the 10-member DSP group is most frequent among B-1a cells (58%) and least frequent among B-1b (4%) (p < 0.1). Interestingly, the DSTD4 element that contains a suboptimal 3' heptamer recombination signal sequence (43) is observed in seven sequences (4%). None are from the B-1a subset that is significantly different in this respect from conventional B cells (p < 0.05). Overall, the B-1b cells appear to use D elements in a more even pattern than the other subset, similar to the observation for Vp11 families.

Each B cell subset uses all four J_p elements. However, the B-1a cell subset is clearly distinguished from B-1b and conventional B cells by the relative use of J_p. J_p is used at a significantly higher frequency among B-1a cells (26%) than either B-1b (10%, p < 0.05) or B-2 (9%, p < 0.05) cells. Preferential J_p usage has been previously been associated with neonatal and B-1a cells in studies that focused on the J558 family (25, 31).

Adult B-1a cells have fewer N region insertions than adult B-1b and conventional B cells

N region additions have been reported to be rare in fetal and neonatal B cells and common in adult B cells (22-26). This result reflects, at least in part, differences in the VDJ recombination machinery during ontogeny, most likely at the level of terminal deoxynucleotidyl transferase (TdT) expression (13, 44-48). Since B-1a cells are associated with early ontologic development, there is a keen interest in the frequency and level of N region insertions.

**FIGURE 3.** Gene segment usage. Histograms are presented that summarize the Vp family, D element and J_p element usage for the sequences in Figure 2. y² analysis (2 x 2, using the element of interest and "all others" as the categories) indicates the following significant and nearly significant differences between populations. Vp family: J558 family, B-1b vs B-2 (p < 0.05); combined J558 + Q52 families: B-1b vs B-2 (p < 0.01); B-1a vs B-1b (p < 0.05); Vp,10 family: B-1b vs B-2 (p < 0.05), B-1a vs B-1b (p < 0.1); and Vp, 11: B-1a vs B-2 (p < 0.1).
A. V-D Junction

Percent of Cells

Conventional (n=59)  B-1a (n=55)  B-1b (n=70)

Number of N-Region Nucleotides

B. D-J Junction

Percent of Cells

Number of N-Region Nucleotides at the D-J Junction

C. Both Juncions

Number of N-region Nucleotides at the V-D Junction

FIGURE 4. Comparison of N region addition among the populations. Frequency distributions for N region nucleotides at the V_{\gamma-D} (panel A) and D_{\mu} (panel B) junctions are shown. Significance was evaluated with a nonparametric rank-order test (Mann-Whitney U), p values for the V_{\gamma-D} junction are: B-1a vs B-2, p = 0.0007; B-1a vs B-1b, p = 0.009; B-1b vs B-2, p = 0.007; B-1a vs B-2, p = 0.7 (NS); for the D_{\mu} junction, B-1a vs B-2, p = < 0.0001; B-1a vs B-1b p = 0.06 (NS); B-1b vs B-2, p = 0.03 and for the sum of the two junctions (histogram not shown): B-1a vs B-2, p = < 0.0001; B-1a vs B-1b p = 0.007; B-1b vs B-2, p = 0.2 (NS). For each cell type, there were no significant differences (p > 0.1) between the two independent experiments. The mean N regions lengths (± SD) at the V_{\gamma-D} junction are: B-1a, 1.9 ± 2.6; B-1b, 3.2 ± 2.9; B-2, 3.2 ± 2.5; and at the D_{\mu} junction: B-1a, 0.9 ± 1.6; B-1b, 1.5 ± 1.9; B-2, 2.1 ± 2.3. In panel C, the V_{\gamma-D} and D_{\mu} populations are considered simultaneously. B-1a cells lack N regions at both junctions most often. Each transcript was scored for the presence or absence of N-region nucleotides at each junction. \(\chi^2\) analysis (4 × 2, three degrees of freedom) demonstrates that the B-1a population significantly differs from the conventional (p < 0.001) and B-1b (p < 0.05) populations.

among this population. This is the first study to examine N region insertions across all V_{\mu} families as they are represented in the mouse.

We find a striking, quantitative difference in the level of N region insertions among B-1a, B-1b and conventional B cells. B-1a cells use N region insertions less frequently than either B-1b or conventional B cells. At the V_{\gamma-D} junction, B-1a cells have significantly fewer N region insertions compared with B-1b cells (p = 0.009 by nonparametric rank-order analysis of the distribution) and conventional B cells (p = 0.0001) (Fig. 4A). These differences are significant in both experiments. A similar pattern is seen at the D_{\mu} junction: we find that 71% of the B-1a cells have no N region insertions compared with 51% of B-1b cells and 34% conventional B cells (Fig. 4B). The distribution differences between B-1a vs conventional B cell (p < 0.0001) and B-1b vs conventional B cell (p = 0.03) are significant. For each B cell population, a lower level of N region insertions at the D_{\mu} junction exists compared with the V_{\gamma-D} junction.

A key question is what fraction of cells lack N regions at both junctions? This is the best indicator of B cells that might have developed in the absence of TD T activity. As indicated in Fig. 4C, fully 38% of the B-1a cells lack N region insertions at both junctions compared with 20% of B-1b cells (p < 0.05 by \(\chi^2\) statistic) and only 7% of conventional B cells (p < 0.001). Thus, by all N region parameters, B-1a cells use N region insertions the least frequently. B-1b cells use N regions more often and conventional
B cells almost always employ N regions, in 93% of the expressed transcripts. We observe the same general pattern of N region use among the B cells subpopulations for the large IJ558 and Q52 families, although the frequency of I-1 cells that have no N region insertions at either junction is lower (27%) in both IJ558 and Q52 compared with the population as a whole. The B-1a VγJ11 transcripts do not have any N regions, consistent with previous results (36, 37, 49, 50). Vγ use among the B-1a cells without N region insertions does not favor J-proximal families. We can hypothesize that these B-1a cells arose earliest in ontogeny and hence might have a distinct pattern of Vγ family usage. Instead, Vγ family usage among these cells (n = 19) is largely similar to that of B-1a cells that have N region insertions and reflect germine complexity to a first approximation.

B-1a, B-1b, and conventional B cell Vγ genes have similar CDR3 lengths

Overall, B-1a cells in the adult have as much CDR3 length diversity as conventional B cells. The CDR3 regions have very similar distribution patterns and the same average length for B-1a (11.7 ± 3.0 codons), B-1b (11.2 ± 3.4), and conventional B cells (11.6 ± 2.8) (Fig. 5A). However, transcripts that do not have N region insertions at either junction have shorter mean CDR3 lengths. Among all transcripts the mean CDR3 length is 11.5 ± 3.0 codons, among N-less transcripts (n = 39) the mean length is 10.0 ± 3.1. The majority of these transcripts (21) come from B-1a cells. B-1a cells gain CDR3 length from sources other than N nucleotides. They use the long Jγ1 element (19 potential CDR3 nucleotides) more frequently, and the short Jγ2 and Jγ3 elements (14 potential nucleotides) less frequently, than either B-2 or B-1b cells (see Fig. 3). Also, more B-1a cells have complete Jγ sequences (Fig. 5B). Transcripts with no nucleotide loss at the 5' terminal of the Jγ segment are more common for B-1a (38%) cells than either B-1b (22%; p < 0.05 by χ²) or conventional B cells (19%, p < 0.1). Nadell et al. have demonstrated that nucleotide deletion is constant throughout ontogeny in the absence or presence of TdT (51). Consequently, the differences in Jγ nucleotide loss observed here most likely reflect selective events rather than a fundamental difference in the mechanisms of rearrangement.

Joints with sequence homology occur more frequently among B-1a cells

Short stretches of sequence overlap between the coding end of Vγ and D and Jγ elements can be used in joining the gene segments (25, 52-54). Such sequences, which are underlined in Fig. 2, can be attributed to either of two germline elements. In our designation of these sequence homologies, we do not permit any mismatches or interruptions to occur. Hence this phenomenon can occur only in the absence of N region additions.

How common is nucleotide overlap among the three B cell populations? At both the VγD-D-Jγ and Jγ-D-Jγ junctions, there is a significantly higher proportion of B-1a cells that have sequence overlap (Fig. 6). Sequence homology at the D-Jγ junction is observed in almost half of the B-1a cells, but only 25% of the B-1b and 15% of the conventional B cells have overlap. At the Vγ-D junction,
22% of the B-1a, but only 9% of the B-1b and 2% of the conventional B cells have overlap. Thus, joints without N region insertions and with overlapping sequences from two germline elements are a common, but not requisite, feature of B-1a cells.

The use of sequence homology is associated more closely with the lack of N regions than with a B cell population. The comparison is best made at the D-J_{H} junction where there are a sufficient number of transcripts without N regions for each B cell subset. Among sequences without N region insertions, we find that 56% of B-1a cells, 58% of the B-1b cells, and 43% of the conventional B cells have sequence overlaps of at least one nucleotide. The lower use among conventional B cells may reflect greater "enzymatic activity" responsible for nucleotide loss, plus nucleotide addition, to give an apparent "N-less" sequence at the J_{H} segment. The use of sequence homology is associated with J_{H} preference. Among sequences with two or more nucleotides at the D-J_{H} junction, J_{H} = 2 (15 > J_{H}1 (10) > J_{H}2 (8) > J_{H}3 (3). The low use of J_{H}3 is reasonable given the lack of potential pairs at the 3' end of the D elements.

D element reading frame 1 is more prevalent among B-1a cells than B-1b or conventional B cells.

Although the D gene segments can be translated in all three reading frames (RF), RF1 is strongly favored among functional rearrangements (24, 25, 28, 55). Two factors appear to contribute to the RF1 preference. DFL1.1 and most members of the DSP family contain stop codons (1 or 2) in RF1I that must be removed during the joining process for the rearrangement to be functional and D-J_{H} junctions with DSP and DFL segments in RFII allow potential expression of the D_{H} protein (56) that may permit selection against RFI at the pre-B cell stage (57).

We observe a strong preference for RFI in all three B cell populations. However, B-1a cells clearly show the strongest preference for RFI, with > 70% compared with 60% (p < 0.05) and 64% (p < 0.1) for conventional and B-1b cells, respectively (see Table 1). Reading frame II is used the least often in all three populations. In another study, Tornberg and Holmberg analyzed bulk amplified cDNA from three V_{H} families and found that about one-third of the adult B-1b cells use reading frame II, while we find only 15% (31). The differences may be due to sampling methods.

Discussion

This study provides a comprehensive analysis of the peripheral IgH repertoire of peripheral B cell subsets. The method has several key features: 1) small numbers of highly purified and well-defined cells can be studied, permitting comparison of subsets of interest; 2) sequence is recovered from 85% of cells, leading to very little potential sampling bias; 3) no a priori assumptions need be made about the V_{H}, D, and J_{H} elements of the possible sequences; 4) the sampled repertoire more accurately reflects the distribution in the animal since the cells are not stimulated.

This approach has enabled us to discover three important features about the overall repertoire. First, the B-1a repertoire can be
Table 1. D gene segment reading frame use

<table>
<thead>
<tr>
<th>Reading Frame</th>
<th>% Conv. Cells (n = 58)</th>
<th>% B-1a Cells (n = 55)</th>
<th>% B-1b Cells (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFI</td>
<td>60</td>
<td>78</td>
<td>64</td>
</tr>
<tr>
<td>RFI*</td>
<td>17</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>RFI*2</td>
<td>23</td>
<td>13</td>
<td>21</td>
</tr>
</tbody>
</table>

*A there is a statistically significant difference in the use of RFI by B-1a cells compared to conventional B cells. *p* values, determined as a 2 x 2 matrix comparing RFI and RFI*, RFI vs. B-1a vs. B-2, < 0.05; B-1a vs. B-1b, < 0.01; B-1b vs. B-2, < 0.05.

Our data demonstrate that a solid cohort of B-1a cells (38%) lack N region insertions at both junctions. However, since 62% of transcripts have N region insertions at one or both junctions, the lack of N region insertions clearly does not define B-1a cells. B-1a cells are still markedly different from conventional B cells where 93% of the cells have IgH sequences with N region insertions. Both conventional B cells from the peritoneum and conventional B cells from the adult spleen (25, 31) almost always use N region insertions. Our N region findings are in qualitative agreement with studies using either one or three Vγ family-specific primers to amplify bcl2 CDNA (25, 31).

Our data are consistent with the idea of two developmental periods for B-1a cells. In the earlier period, rearrangement occurs in the absence of TGF and the junctions do not generally contain N regions. Data from neonatal B cells (25) and our own unpublished sequence data suggest that this first stage lasts less than 1 wk postpartum. The results presented here suggest that some of those B-1a cells that arise earliest in ontogeny maintain themselves by self-replenishment and persist into adulthood. In a later stage, B-1a cell development occurs in the presence of TGF and the junctions contain N regions more frequently. This later stage could last until only about 6 wk of life, at which time previous studies indicate that a feedback mechanism blocks new B-1a cell entry into the peripheral pool (4, 5, 14) (Watanabe, K., L. A. Herzenberg, and A. B. Kantor, manuscript in preparation).

B-1b cells show an intermediate level of N region insertions. This may indicate that some B-1b cells that constitute most of the N-less subset also develop in the first week of life. The data could also reflect limitations in our ability to resolve the B-1a and B-1b cell phenotypes by FACS. It is not likely to reflect a much later development of B-1b cells in that feedback studies with Ig-allotype heterozygous mice demonstrate that new B-1b cell entries are also prevented after about 6 wk of life.

In this report, we have focused on the functional repertoire in the periphery, which is influenced by multiple prior developmental and selective events. The different characteristics we describe for the Ab repertoires of B-1a, B-1b, and conventional B cells strongly suggest that different selective and/or developmental forces act to shape each. Only by providing an accurate picture of the repertoire at this stage of development can we eventually evaluate the interplay of these forces. The methodology introduced here can be applied to any population of B cells that can be defined by FACS-phenotype and hopefully will provide a powerful tool for further dissecting repertoire development.

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References


31. Tombo, L. C., and D. Holmberg. 1995. B-1a, B-1b and B-2 B cells display unique IgM repertoire usage from different stages of ontogeny and under different selection pressures. EMBO J. 14:6180.


