An Unbiased Analysis of V_H-D-J_H Sequences from B-1a, B-1b, and Conventional B Cells[1,2]

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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 noncoded 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 V_H, D, and J_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.**

Murine 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 techniques, we introduce and apply a method for constructing cDNA from single FACS-sorted B cells that recovers expressed Ab transcripts representative of all mouse V_H families. Others have recognized the power of the single-cell approach and applied it to DNA from the early stages of B cell developments (6), Ag-specific responses (7), and human peripheral B cells (8). Our method is best suited for rapidly analyzing repertoire without a priori assumptions about the V_H, D, and J_H elements of the possible sequences. We can analyze Ig expression from any population of B cells that can be defined phenotypically by FACS, even if the sample is very small. Furthermore, since we recover sequenceable transcripts from 85% of cells, the degree of potential bias is very small.

Studies here compare the repertoires of B-1a, B-1b, and conventional B cells isolated from the adult peritoneum. By harvesting all three populations from the same location, any differences that are found can be attributed to the populations themselves rather than anatomical location. In this paper we evaluate some general features of the V_H repertoire and the details of the complimentary-determining region 3 (CDR3)[5] where the V_H, D, and J_H elements are joined.

The potential diversity of these Ab repertoires derives from the multiplicity of encoded V_H, D, and J_H elements; from variability in their sites of joining (9–12); from addition of nontemplated nucleotides at their junctions, termed N regions (13); and from templated nucleotides, termed P sequences (14). Although these mechanisms can potentially generate more than 10^{12} different Ag-binding sites (15, 16), the number of possible different Ag-binding sites present in the mouse at any given time is limited to the total number of B cells in the circulation, 2×10^8.

Several investigations suggest that B-1a cells favor a smaller portion of this potential diversity than conventional B cells. The B-1a IgH repertoire has been observed to be largely restricted to V_H regions specific for self and bacterial Ag, most notably phosphatidylcholine (17–21). However, these studies use methods such as LPS stimulation and hybridoma production, that may recover

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[2] 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 junctions; P sequences, 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_H family usage compared with either B-1a or conventional B cells. Specifically, B-1b cells use the J558 and Q52 families less frequently and the V_H10 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 at the V-D 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 evaluate cells from all V_H 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/cHz (IgH-C^a) mice at 5.5 mo of age were used in each of two separate experiments. The following cell lines (kindly provided by M. Cancro, University of Pennsylvania, Philadelphia, PA; E. Voss, University of Illinois, Champaign, Urbana, IL; J. Goverman, University of Washington, Seattle, WA; R. Hardy, Fox Chase Cancer Center, Philadelphia, PA; and S. Clarke, University of North Carolina, Chapel Hill, NC) were used to test the V_H primers: J558 (J558 family), Nab2 (3609 family), B12.12E11H3.G9 (3609 family), 4-4-20 (J606 family), 3A4 (Vgam3.8 family); B7.14D1.B5.A7 (Vgam3.8 family), MOPC315 (36-60 family), F12-75-13 (X-24 family), S107 (T15 family), 18-2-3 (Q52 family), P3x+78 (7183 family), 2C8 (V_H11 family), CH27LX.1FG (V_H12 family).

FACS analysis

FACS methods and reagents have been described previously (3). Some changes were made for sorting for construction of cDNA from single cells. After staining, cells (2.5×10^8/ml) are incubated with 10 ng/ml RNase A (Boehringer Mannheim, Indianapolis, IN) for 30 min on ice and then diluted 10-fold (2.5×10^7 cells/ml, 1 ng/ml RNase) for sorting. The instrument fluidics system is washed with 0.1 M NaOH for 5 min to remove extraneous RNA and 70% ethanol for 5 min to sterilize. Sheath fluid is deficient RPMI 1640, with 10 mM HEPES and 0.5 ng/ml RNase.

The B cell populations are bulk-sorted first, reanalyzed, and sorted again to deposit single cells. The two-step process ensures purity; moreover, since the cells are at a concentration of 2×10^5/ml for the second sort, there is very little chance of a doublet landing in the final tube. Polystyrene beads (2.2 μm, 3×10^6/ml; Spherotech, Libertyville, IL), which can be readily distinguished from lymphocytes, are added to a sample of cells as a negative control. One cell or bead per 0.5-ml microcentrifuge tube containing 4 μl of lysis buffer (0.5× PBS; 10 mM DTT; 2 U/μl placental RNase inhibitor, and 0.2 U/μl Inhibit-Ace, 5 Prime→3 Prime, Boulder, CO) is deposited by FACS. Tubes are kept on ice before sorting and on a cold stage during the sort. For the production of cDNA from single cells, the voltage to the deflection plates and aspirator position are adjusted so that unselected cells and sheath fluid are removed at an angle while selected cells are sorted vertically. Tubes are aligned so that the cell lands directly into the 4 μl of lysis buffer. Four to six cells are processed (sorted, spun, frozen on dry ice) at a time. Samples can be stored at −70°C before preparation of cDNA for at least 1 year.

Sample deposition, cDNA construction, and PCR

The single-cell PCR methodology is described in detail in a separate paper (27). Key aspects are presented here. Samples are kept on dry ice while primer mix is added. Each added 7-μl aliquot contains 1 μl of random hexamer (stock at 300 ng/μl), 1.0 μl of 10% Nonidet P-40 (Boehringer Mannheim), 1 U of Inhibit-ACE (1–2 μl; concentration from supplier varies) and RNase-free water (BioWhittaker, Walkersville, MD). After the primer mix is added to all tubes, samples are placed in a thermocycler that has been preheated to 37°C, heated to 65°C for 1 min, and then cooled to 10°C for at least 3 min. Samples are removed one tube at a time and 14 μl of reverse transcriptase (RT) mix is added immediately (final cDNA vol, 25 μl). The RT mix consists of 5 μl of 5× RT buffer, 1 μl of stock solution that is 25 mM for all four dNTPs, 2 μl of 100 mM DTT, RNase-free water sufficient to bring the final volume to 14 μl, 1.0 μl of rRNAsin (10 U/μl, Life Technologies, Grand Island, NY), 1 U of Inhibit-Ace (1–2 μl), and 1.5 μl RT mix (200 U/μl, Life Technologies, Superscript II). Solution is kept at room temperature for 10 to 15 min to promote annealing and then at 37°C for 30 min, then heated to 90°C for 6 min to destroy enzyme, and then cooled to 4°C. The cDNA solution may be aliquoted and stored at −20°C until ready for amplification.

The target for the analysis is functional V_HDJ_H-μ transcripts. cDNA is amplified by PCR between a promiscuous 5′ V_H primer and a Cμ primer (primers were prepared at the Stanford Protein and Nucleic Acid Facility). The cDNA (one-tenth to one-half of the sample) is added to a primer mix consisting of 1× Taq buffer, 2 mM $MgCl_2$, 100 ng of both the upstream (MsV_HE=GG*GAATTC*GAGGTGCAGCTGCAGGAGTCTGG) and downstream primers (MsCμE ATGGCCACC*GAATTC*TTATCAGA) and an appropriate amount of RNase-free water. The primers can incorporate *Eco*RI (italicized) sites. Final sample volume is 98 μl. Samples are heated to 96°C for 3 min to unfold the DNA, then cooled to 50°C. Two microliters of Taq mix (0.4 μl of Taq at 5 U/μl from Life Technologies or other source, 1.0 μl of dNTP stock, 25 mM concentrations of each) and 0.6 μl of 1× Taq buffer is added to 2 to 4 samples at a time, layered with mineral oil, and then returned to the thermocycler (50°C). Samples are amplified for 35 cycles: 97°C for 30 s, 50°C for 30 s, 72°C for 30 s. Samples are purified with QiaQuick PCR spin columns (Qiagen, Chatsworth, CA) using 50 μl of 10 mM Tris, pH 7.2, for the final elution. EDTA is avoided because it can interfere with the Taq sequencing.

The second amplification, which is semi-nested, uses an internal constant region primer (m13 MsCμN = **TGTAAAACGACGGCCAGT** CATTTGGGAAGGACTGA) and the same promiscuous V-region primer. We have used an internal promiscuous V_H primer (MsV_HN *GG GAATTC*TGGACGAGACTTGGTGCAGC) for part of this study; however, we have stopped using this primer because V_H information (the first 7 codons) is lost. An aliquot (5–8 μl) of the purified first round PCR product is added to a primer mix that is same as above except for the primers, heated to 96°C for 3 min, and cooled to 50°C. Taq mix (same as above) is added and the samples amplified as above. The primers for the second amplification can incorporate *Eco*RI (italicized) sites for cloning or M13 sequence (bold type) for Taq cycle sequencing.

Sequencing

Positive samples are purified with QiaQuick PCR spin columns (Qiagen) using 50 μl of 10 mM Tris, pH 7.2, for the final elution. PCR products are sequenced directly using dye-labeled primer chemistry (Applied Biosystems, Foster City, CA) using the Prism kit designed for the M13 sequence. We also have the option to amplify the first or second round product with m13rMsV_HN (**CAGGAAACAGCTATGACCT**GGACG CTTGGTGCAG) and sequence in the reverse direction.

Sequence assignments and data analysis

Sequences are first aligned with the best match to one of four J_H genes and a V_H gene from a germline database (A. B. Kantor, J. MacKenzie, J. L. Hillson, and L. A. Herzenberg, manuscript in preparation). If the actual germline gene has not been sequenced to the recombination signal sequence, the closest match is used to identify the 3′ end of the V_H. Next the longest D elements matches are assigned. The D element reading frame is given for the longest D match, according to the nomenclature of Ichihara et al. (28). A second D element is assigned only if there is a five nucleotide (or greater) match. There are many possible four-nucleotide matches that are not shown. All possible P elements are assigned (14). The remaining nucleotides are then assigned to the N nucleotides. Frequency distributions are compared using the Mann-Whitney *U* nonparametric rank-order test, which is resistant to outliers in either of the groups being compared, using Statview software (Abacus, Berkeley, CA). Nominal parameters such as gene family usage are compared using the χ^2 test. All combinations of family usage were compared in 2 × 2 contingency tables (e.g., J558 or not J558 for B-1a vs B-2). Significant ($p < 0.05$) and some nearly significant ($p < 0.1$) differences are presented in the results.

Results

FACS sorting and single-cell PCR efficiently recovers IgH transcripts derived from diverse V genes from all B cell subsets

We sorted B-1a, B-1b and conventional B cells from the adult BALB/c peritoneum. The phenotype of these cells and reanalyses

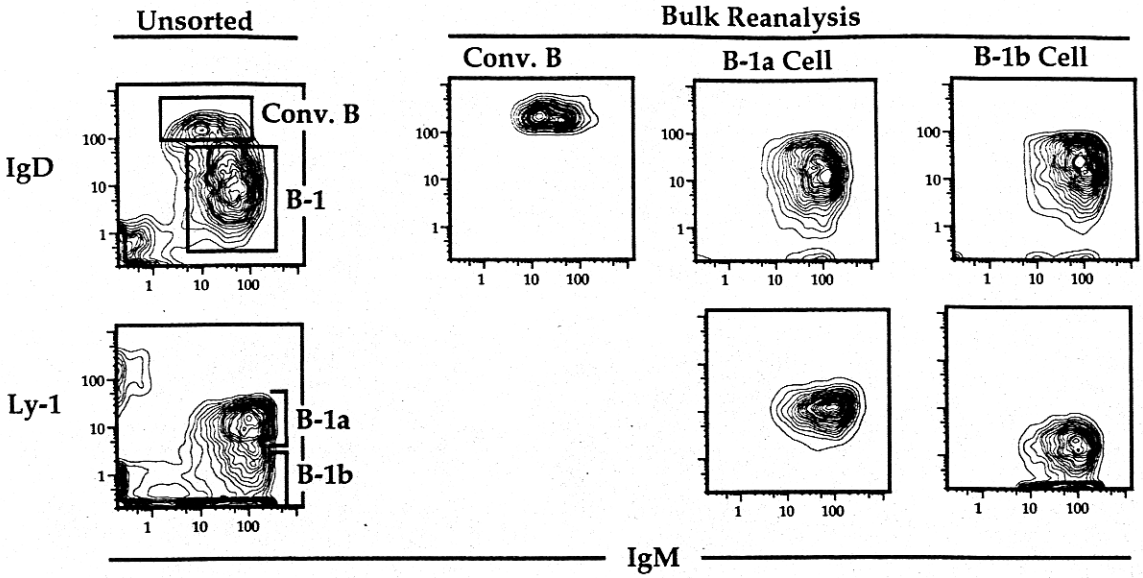

FIGURE 1. Three B cell populations from the peritoneal cavity of adult BALB/c mice were sorted according to their FACS phenotype. Conventional B cells are $IgM^{low}IgD^{high}CD5^-$. Both B-1a and B-1b cells are $IgM^{high}IgD^{low}$; however, B-1a cells are $CD5^+$ and B-1b cells are $CD5^-$. *Left 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.

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 $IgM^{low}IgD^{high}CD5^-$. Both B-1a and B-1b cells are $IgM^{high}IgD^{low}$; however, B-1a cells are $CD5^+$ and B-1b cells are $CD5^-$ (Ref. 2 and references cited therein). Following reanalysis, single cells were sorted again, according to phenotype, directly into the lysis solution and snap frozen for future analysis. The two-step sorting process ensures purity and the absence of a doublet landing in the final tube.

The mouse heavy chain locus consists of about 200 V_H gene segments that are classified into 14 families according to their nucleotide sequence similarities (29). We designed a single, promiscuous V_H-region primer to amplify all V_H genes. The effectiveness of the primer pair, which is designed to amplify only V_HDJ_H-μ transcripts, was tested against known murine V_H genes. Messenger RNA was prepared from cell lines representative of 11 V_H families (V_H1/J558, V_H2/Q52, V_H3/3660, V_H4/X24, V_H5/7183, V_H6/J606, V_H7/S107, V_H8/3609, V_H9/VGAM3/8, V_H11, and V_H12) and reverse transcribed into cDNA. V_H genes from all of these families were successfully amplified in this way (data not shown). In the present study, a sequenceable transcript was recovered from 85% of single cells attempted for a total of 184 sequences. As shown in Figure 2, 13 of the 14 known murine V_H families are represented in the data set. The only family not represented, V_H8 (3609), has been recovered from single cells in other data sets (K. Seidl et al., manuscript in preparation).

The 184 sequences shown in Figure 2 are quite diverse. Each represents a unique rearrangement, as indicated by the unique CDR3 region. In the course of the present study, only one sequence was independently recovered (AB190, J558 family) from two separate B-1a cells. We cannot determine whether this represents sampling of two cells from a single clone or sampling of independent, identical rearrangements. However, clearly there was no evidence of dominant B-1a cell clones in this data set, as has been observed in other studies (17, 30, 31). There were also no duplicates from either the B-1b or conventional B cells. Only single functional μ transcripts were obtained in this data set.

B-1a, B-1b and conventional B cells have similarities in V_H gene usage

Figure 3 provides V_H, D, and J_H frequency distributions of the data reported in Figure 2. To a first approximation, the pattern of V_H 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_H 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_H family, Q52, which constitutes perhaps 10% of the total germline V_H 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_H genes observed in our study.

This general V_H 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_H 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_H gene usage

Despite the overall similarity in general pattern, the B cell subsets do differ significantly in the representation of some V_H 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 χ^2 statistic). The combined usage of J558 and Q52 families is also

A **Conventional B Cells**

CELL	Fam	V H(3')	P	N	P	D	P	N	P	JH (5')	JH	J-	RF	D
AA018	7183	TGTGCAAGAG		GA		TACTACGGTAGTAG		GTGG		GCTATG	4	8	1	F16.1
AA121	J558	TGTGCAAGA		GGG		GATTACGAC				TACTAT	4	2	1	P2.2
AA122	J558	TGTGCAAGA	TC	GGG		GTATGGTAACT				GGTTTG	3	1	1	P2.8
AA124	7183	TGTGCAAG		CCCTG		ATGATTACG				GGTACT	1	5	1	P2.1
AA126	J558	TGTGCAAGA	TC	AGACTTAGG		GGTACGAC				ATGCTA	4	6	3	P
AA127	J558	TGTGCAAGA		AGCGCCCGGG		AGGTACGAC		AGGG	GT	ACTACT	2	0	3	P+P
AA128	Q52	TGTTCCAGAGA		AC		TTACTACGGTAGT		CTCG	T	ATTACT	4	0	2	F16.1
AA130	J558	TGTGCAAGA		GG		CGGG		GCCAGACCGG		GGTACT	1	5	2	T/alt T
AA137	3660	TGTTCAAG		GGAAANCCAGC		GGTTACTAC		GAG		CTATGG	4	9	3	P2.9
AA143	J558	TGTGCAAGA	T	GGG		CTACGGTAGT|GTCGTAC				TACTTT	2	0	2	F16.1+P2.10
AA148	J558	TGTACAAGA		GGGG		ATGATTAC		AC	GG	CCTGGT	3	0	1	P2.2
AA151	J558	TGTGCAAGA		GGGGG		AACTACGGCT		T		CCTGGT	3	0	2	F16.2
AA152	J558	TGTGCAAGA		GGGG		ACTAC		TGCC		ACTGGG	3	12	1	P/F
AA154	Q52	TGTGCCA		AAGGGA		TTTATTACTACGGTAGTAGCT		C		TTACTA	4	1	1	F16.1
AA155	3660	TGTGCAAGA				TGGG		CGGC		CTACTG	2	10	3	Q
AA157	DNA4	TGTGTGAGA		AG		AGG		A		GACTAC	2	8	1	P
AA163	J558	TGTGCAAGA		A		ATGATTACGAC	G	AGAATCC		GTTTGC	3	4	1	DSP2.2
AA165	Q52	TGTGCCAGAG		GGTAC		TATTACTACGGTAGTA		CGTACGC		GTTTGC	3	4	1	F16.1 (5'P?)
AA168	J558	TGTGCAAGA		CG		CC				ATGCTA	4	6	-	?
AA169	J558	TGTGCAAGA	T	GGA		ATAGG		C	AGT	ACTACT	2	0	1	P2.9/11
AA172	J606	TGTACCAG		GG		ACTACGGTAG		GGG		CTTTGA	2	4	1	F16.1
AA173	J558	TGTGCAAGA	TC	CG		GATTAC		CGTTTT		CCTGGT	3	0		P2.2
AA177	Q52	TGTGCCAGAGA		GGGA		GATGGT			G	CCTGGT	3	0	1	P2.9
AA178	J558	TGTGCCGG		G		GATTACG		GGGT		CTGGTT	3	1	1	P2.2
AA180	Q52	TGTGCCAGAG		GA	AGA	TCTACTATGATTACG		CT		CTATGC	4	4	1	P2.2
AB322	J558	TGTGCAAGA		AGTC		GGGAC		C		TTGACT	2	6	3	Q Alt=T
AB324	J558	TGTGCAA		AG		TTATTACT		GGG		TTACTA	4	1	3	F16.1
AB325	J558	TGTGCAAGA		AGAAGCAG		ACTACGGCT		CCC		CTATGC	4	4	3	F16.2
AB327	J558	TGTAAAAGA	TC	C		CTGGG		CCTT		TTTGCT	3	5	2	Q
AB328	J558	TGTACAAGA	T	TCCCC		TACGAC				TGGGGC	2	12	1	P
AB331	Q52	TGTGCCAGAA	T	ACT		CTACGGTAGTAGCTAC|GAC				TGGTTT	3	1	3	F16.1+P
AB333	J558	TGTGGAAG		GG		CAGCTCGGG		G		TTACTA	4	1	1	T
AB334	Q52	TGTNCCAGAAA	T			TATGGTGATTACGAC|G|GGGAC				TATGCT	4	3	1	P+Q
AB336	S107	TGTGCAAGAGA	T	TCG		GATGGTTACTAC		A		ACTGGT	1	2	1	P2.9
AB337	S107	TGTGCAAGAGATA	TA			ACGGTAGTAGCTAC		GTA		CTACTG	1	0	2	F16.1
AB339	J558	TGTGCAAGA	TC	GA		TATA		CCCC	T	ACTACT	2	0	1	P
AB342	3660	TGTGCAAGA	TC	CTT		CTATAGGTACGAC	G	GAGGC		TACTTT	2	2	1	P
AB344	Q52	TGTGCCA		CCTCT		AACTGGG				CTTACT	3	8	3	Q
AB345	J558	TGTGCAAGA		GGGGGA		GATGGTT		CAC		TTGCTT	3	6	1	P2.9
AB346	J606	TGTACCAGG		CCGG		CCTACTATGGTAACTAC	GT	TCT		GTTTGC	3	4	1	P2.7
AB347	3660	TGTGCAAGA		AAACCT		TCTACTATGGTAAC		CTCT		GGGCCA	2	16	2	P
AB348	J558	TGTGCCAGA				TCTACTATGATTACGAC	G	GGG		GCTATG	4	8	2	P2.2
AB350	Q52	TGTGCCA		AAGGGG		GATTACGAC	GT	TC		CTATGC	4	4	3	P2.2
AB351	Q52	TGTGCAAGA	T	TAGATCC		GTATGGTAACTAC				TATGCT	4	2	1	P
AB353	Q52	TGTNCCAGAGA	T			TACTACGGTAGTA				ACTTCG	1	8	1	F16.1
AB354	J558	TGTGCAAGA		GG		GTATGGT		GC		TGCTTA	3	7	1	P2.8
AB355	7183	TGTGCAAG		GGG		ACTGGGAC		G		CTTTGA	2	4	2	Q
AB356	Q52	TGTGCCAGA		G		TCTACTATAGGTACG		G		CTTTGA	2	4	1	P
AB357	3660	TGTGCAAGA		GGGGG		CTATAGGTACGAC		A		CCTGGT	3	0	1	P
AB358	J558	TGTGCAAGA	TCT			TGGGAC				TACTGG	2	8	3	Q
AB359	J606	TGTACCA				ATAGGTAC			T	ACTACT	2	0	1	P
AB362	Q52	TGTGCCAGAGA				CTACGGTAGTAGCTAC	G	AAG		ACTATG	4	3	1	F16.1
AB363	7183	TGTGCAAG		GCC		CTACGGTAGTAGCTAC				TGGTTT	3	1	1	F16.1
AB365	J558	TGTGCAAGA		AGGA		TCTACTATGATTACGAC	G			ATGCTA	4	6	1	P 2.1
AB366	J558	TGTGCAAG		GA		AGCTCGGGCTAC				ACTATG	4	3	2	T
AB367	J558	TGTGCAAGA	T	GG		GATGGTTACTAC		C		TTGCTT	3	6	1	P2.9
AB368	SM7	TGTGCT		CA		TAGCT				CCTGGT	3	0	3	F16.1 (or T)
AB369	J558	TGTACAAGA		G		ACTATAGGTACGA		G		TACTAT	4	2	1	P
AB370	J558	TGTGCAAGA	TC	AGGG		GATTACGA				TGCTAT	4	6	1	P2.2

FIGURE 2. V_H-D-J_H 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 an identifier number (AA and AB represent different experiments), the V_H family, the most 3' nucleotides of the V_H (starting with Cys = TGT), the D element, the six most 5' nucleotides of the J_H, the number of the J_H element, the number of J_H nucleotides lost (J-), the D element reading frame (according to Ref. 28), and the D element identification (Q = DQ, S = DSP, F = DFL, T = DST; two equivalent choices are denoted by or; an alternative assignment with different N regions is denoted with alt). 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, 55 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.

B **B-1a Cells**

CELL	Fam	V H(3')	P	N	P	D	P	N	P	JH (5')	JH	J-	RF	D
AA001x	S107	TGTGCAAGACA				CGAC				TACTGG	2	8	1	P
AA003x	J558	TGTGCAAGA				GGGAC|C|ATTACTACGGTAG		A		TGACTA	2	7	2	Q+F
AA004x	Q52	TGTGCCAGAG				TTTATTACTACGGTAGTAGCTCTA				TGGACT	4	9	1	16.1
AA007X	J558	TGTACAAGA				TGGTTACT		GAGT		TACTTT	2	2	3	P2.9
AA008X	Q52	TGTGCCAGAGA		A		TATGGTAACTAC				TGGTAC	1	0	1	P
AA010X	J558	TGTGCAA				CTGG				TGACTA	2	7	1	Q
AA014	7183	TGTTCAAGACA		TGAAG		ATGGTAACTAC		CCT		TGGTAC	1	4	1	P2.1
AA015	J558	TGTGCAAGA		GCCCC		CTATGGTA|AC|TACGG				GTTTGC	3	3	1	P2.1 + F
AA017	J558	TGTGCAAGA				TTAC				TTGATT	2	6	1	P2.2
AA018x	J558	TGTGCAAGA				TATGGTTACGA				TTACTA	4	0	1	P
AA023	Q52	TGTGCCAGAG		C		TTACTACGGTAGTAGCTAC				TGGTTT	3	2	1	F16.1
AA025	J558	TGTGCAAGA	T	GGGAG		ACTACGGTAGTAGC		CCT		CTACTG	1	0	2	F16.1
AA026	SM7	TGTGCTAGA		G		ACTATGATTACGAC	G			ATGCTA	4	6	1	P2.2
AA027	J558	TGTGCAAGA		G		ATGATTACGAC	G	CCATTTC		GTTTGC	3	4	1	P2.2
AA028	J606	TGTAC				CAACTGG				GCTTAC	3	8	3	Q
AA029	Q52	TGTGCCAGAGA	TCT		AA	TTTATTACTACGGTAGTAGCTAC		A		ATTACT	4	0	2	F16.1
AA030	Q52	TGTGCCAGAGA				CTAT				TACTGG	3	11	1	P
AA032	SM7	TGTGCTA				ACTGGGAC				TGGTAC	1	2	3	Q
AA033	Q52	TGTGCCAGAGA	T			TATGGTAACTTC	G	TG		TACTAT	4	2	1	P
AA034	J558	TGTGCAAGA	TC	CT		ACTATGGTAACTAC				TTTGAC	2	0	1	P2.7
AA035	3660	TGTGCAAGA		GAAGGAGGG		TATTACTACGGTAGTA				ACTGGT	1	2	1	F16.1
AA037	J558	TGTGCAAGA				ACTGGGA				ACTATG	4	3	1	Q
AA038	J558	TGTACAAGA		AAGAAGAA		TTCATTAC		AA		TTTGCT	3	5	3	F16.2
AA039	Q52	TGTGCCAGA		GATCGGGG		CTACGGTAGTAGC		CCT		TACTGG	1	1	1	F16.1
AB180	Q52	TGCGCCAG		GCTGTCCCC		TTATTACTACGGTAGTAGCTA		TG		GGTACT	1	5	1	F16.1
AB183	J558	TGTGCAAG		GGGG		GGTT				TTGCTT	3	5	1	P
AB187	J558	TGTGCAAGA		GAAG		ACTACGGCTAC|CTATA				TTACTA	4	0	1	F16.2 + P
AB189	VH11	TGTATGAGATA	TA			ATGGTTACTAC				TGGTAC	1	0	1	P
AB190	J558	TGTGCAAGA				CGA			T	ATTACT	4	0	3	P
AB191	Q52	TGTGCCAGAGA				TGGGAC|T|TGGTACG|G|CCTATA				TTACTA	4	0	1	Q*+P+P
AB192	VH11	TGTATGAGATA				TGATGGTTACTAC				TGGTAC	1	0	1	P2.9
AB193	Q52	TGTGCCAGA				TATTACTACGGTAGTAGCTAC				TATGCT	4	2	1	F16.1
AB196	VH11	TGTATGAGATA				CGGTAGTAGCTAC				TGGTAC	1	0	1	F16.1
AB197	J558	TGTGGAAGA	T	T		TTT				TTACTA	4	1	2	F16.1
AB198	3660	TGTGCAAGAT		GGGG		CTACGGTAGTAGCTAC	G	GG		GCTATG	4	8	1	F16.1
AB199	7183	TGTGCAAGACA				TGGTTACGAC				TATGCT	4	3	1	P
AB200	3660	TGTGCAAGATA				TTACTACGGTAGTAGCTAC				TACTGG	2	9	1	16.1
AB216	J558	TGTGCAAG		GGGG		GGTT				TTGCTT	3	5	1	P
AB217	3660	TGTGCAAGA				TATAGGTAC				TACTTT	2	0	1	2.11
AB218	7183	TGTGCAAGA		GGCC		ACTATGATTACGAC				TACTTT	2	0	1	P2.2
AB220	Q52	TGTGCCAGAG				CCTACTATAGGTACGAT				TACTAT	4	0	1	P2.11
AB223	J558	TGTGCAAGA	T	TTGCC		GGTTA				CTACTG	1	0	1	P2.9
AB224	VH12	TGTGCAGGAGACAG		TA		ATGGTTACT|T|TTACT		TTG		ACTACT	2	0	1	P2.9+2.9
AB225	J558	TGTGCAAGA				GGTAACTAC	G		G	CTACTG	1	0	1	P2.8
AB227	J558	TGTGCAAG		GCA		CCT		C		TACTGG	3	11	3	P
AB228	Q52	TGTGCCAGA		G		ACGGTAGTAGCTAC				TTTGAC	2	1	1	F16.1
AB231	J558	TGTGGAAGA	T	G		CTACGG|GG|AGTAT		TC		CTATGC	4	4	3	F16.2+P2.8
AB232	Q52	TGTGCCAGAG		CCC	GA	TCTACTATGATTACGAC		AG		ACTATG	4	3	2	P2.2
AB233	Q52	TGTGCCAGAGA	T	GGAGGA		TACTACGGTAGTAGCTAC		TTT		TGGTAC	1	4	1	F16.1
AB234	J558	TGTGCAAGA	TC	AG		ACTATAGGT		CTC		ATTACT	4	0	1	P
AB235	DNA4	TGTGTGAGA		C		ATGATTACGA				TGCTAT	4	6	1	P
AB236	X24	TGTGCAAGACC		CC		TCTATGATGGTTACTAC	GT			CTGGTT	3	1	1	P2.9
AB237	7183	TGTGCAAG		GG		ATTACTACGGTAGTAGCTAC				TGGTAC	1	0	1	F16.1
AB238	Q52	TGTGCCAGAGA	T			G				GGTTTG	3	3	1	P2.9
AB240	J558	TGTGCAAGA	TC	AG		ACTATAGGT		CTC		ATTACT	4	0	1	P

FIGURE 2. (*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 V_H 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 germline 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, V_H10, which has been associated with anti-DNA specificity (35), occurred more frequently among B-1b cells (10%) than either B-1a (2%, $p < 0.1$) or conventional B cells (2%, $p < 0.1$). The higher use of the V_H10 family among B-1b cells was observed in both experiments (9 and 11%). The combined usage of the 3660 and V_H10 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 use of J558 and Q52 by B-1b cells.

The number of sequences limits conclusions about other families. However, we note that the V_H11 and V_H12 families (four and three sequences, respectively) that have been associated with B-1

C **B-1b Cells**

CELL	Fam	V H(3')	P	N	P	D	P	N	P	JH (5')	JH	J-	RF	D
AA003	DNA4	TGTGTGAGAGA	T			AACTGGGAC	G	GGG		GGTTTG	3	3	3	Q
AA004	Q52	TGTGCC		NAACA		TGGGAC		AGGG		TACTAT	4	2	1	Q
AA007	3660	TGTGCAA		C		CAACTGGGAC	G	GG		TATGCT	4	5	3	Q
AA009	DNA4	TGTGTGAGA		CAGGG		TACGGCTAC			AT	ATTACT	4	0	2	F16.2
AA010	Q52	TGTGCCAGAGA		GGCA		TATTACTACGGTAGTAGC		CC		TTACTA	4	1	1	F16.1
AA011	3660	TGTGC				TTACTACGGTAGTAGCTT				TGACTA	2	4	1	F16.1
AA012	J558	TGTGCAAGA		GG		TTATTACTACGGTAGT		TGGG		ATGCTA	4	6	1	F16.1
AA061	Q52	TGTGCCAGAG		TTC		ACAGCTCGGGCT				CTTACT	3	9	3	T
AA063	3660	TGTGCAAGAT		GGA		ATGATGGT		CCC		GACTAC	2	8	1	P2.9
AA064	J558	TGTGCA		GGC	A	TCTACTATGATTACGAC	G	AA		GCTTAC	3	8	1	P2.2
AA065	3660	TGTGCAAGAT		GGCG		CTACGGTAGT		GAG		GCTTAC	3	8	1	F16.1
AA066	DNA4	TGTGTGAGA		CAGN		GCTAC|T|GTAGTAGCTAC				TATGCT	4	2	1	F16.1+F16.2
AA067	3660	TGTGCAAGAT		GG		TACTACGGCTAC|GGGA|CGGGCTAC				TACTTT	2	0	1	FL16.2+DST
AA068	3660	TGTGCAAGATA				CTATGATGGTTACTAC				TTTGAC	2	0	1	P2.9
AA070	J558	TGTGCAAGA		C		ACGG		GT		CTATGG	4	9	1	F16.1
AA091	Q52	TGTGCCA		AAG		ACT		CA		TTTGCT	3	5	2	P/F
AA093	J558	TGTGCAAGA	TC			AGACAGCTCGCTAC				GGTTTG	3	3	3	T
AA095	J558	TGTGC				TGGTTACTA		TTG		CTATGC	4	4	1	P2.9
AA096	S107	TGTGCAAGAGAT		TTGG		GATGGTTA		A		GGTTTG	3	3	3	P2.9
AA097	3609N	TGTT				AGTAACT		GG		GACTAC	2	8	1	P2.10/11
AA098	Q52	TGTGCCAGAGA	TC	GGGG		GGGC|TAC|TATGGTAACTAC	G			ACTGGT	1	2	1	T+P
AA099	Q52	TGTGCCA		CCC		TCTACTATGGTAACT		C	AT	ATTACT	4	0	1	P2.1
AA100	J558	TGTGCAAGA		GGGGGGAT		TATGATTACGA				TGGACT	4	11	1	P2.2
AA101	Q52	TGTGCCAGA		GGGTCCTATTG		CTACGGTAGTAGCTAC				TTTGAC	2	1	1	F16.1
AA103	Vh12	TGTGCAGGAGACAGA				TATGGTTAC				TGGTAC	1	1	1	P2.3/6
AA104	Q52	TGTGCCA		AAGAGG		GGGA		TCAGGCC		TTTGCT	3	5	3	Q
AA105	X24	TGTGCAAGA		CCG		GATGGT		AACCC		CTATGC	4	4	1	P2.9
AA112	Vh12	TGTGCAGGAGAC				TACTACGGCTAC				TGGTAC	1	0	1	F16.2
AA113	7183	TGTGCAAG		CTGGC		GATGGTTACTAC		AGGA		CTGGTT	3	1	3	P2.9
AA114	Gam38	TGTGCAAGA	TCTT	TGGGCTT		TGGTTACT		TCCCT		TTTGCT	3	5	1	P2.9
AA115	J558	TGTACA		CCC		CTATGGT				CTTACT	3	9	3	P
AA116	J558	TGTGCAAGA	TC	CT		CTACGGCTAC	GT	C		CTACTT	2	1	2	F16.2
AA118	J558	TGTGCAAGA		GGG	A	TTTATTACTACGGTAGTAGC		CC		CTTTGA	2	4	1	F16.1
AA119	Q52	TGTGCCAGAGA	TC	GAGGGGGC	A	TTTATTACTACGGTAGTAGC				GATGTC	1	12	1	F16.1
AA120	x24	TGTGCAAGACC	GG	ATGA		GTACG		GCC		CTATGG	4	9	1	P
AB244	DNA4	TGTGTGAGA				CTACTATGGTA				TTACTA	4	0	3	P
AB245	DNA4	TGTGTGAGAG		TGGGGA		CCTACTATA		CCAGGG		ACTGGG	3	12	1	P2.11
AB246	J558	TGTGCAAG		TGGGA		GGGAC				TGGTAC	1	2	3	Q
AB248	7183	TGTGCAAGA				ACTGGG				TTTGCT	3	3	1	Q
AB250	J558	TGTGCAAG		GGC		TGGT				TTGCTT	3	5	1	P(altQ,S)
AB251	3660	TGTGCAAGAT		GGG		ACTACGGTAGTAGCTAC				TTTGAT	2	1	1	F16.1
AB252	J558	TGTACAAGA		GAGGGGG		TCTACTATGAT		C		TTGACT	2	6	1	P2.2
AB253	S107	TGTGCAAGAGATA				ACCA		CG		ACTGGT	1	2	-	P'
AB254	J558	TGTGCAAGA				TCTACTATGATTAC				CTATGC	4	4	2	P2.2
AB256	J558	TGTGCAAGA		G		ATGGTAACTAC			G	CCTGGT	3	0	1	P
AB257	J606	TGTACCAGGGAT		A		ACTACGGCTAC	G			GGTTTG	3	3	1	F16.2
AB258	J558	TGTGCAAGA		GAGGCCT	A	TTCATTACTACGGCT		CC		TGGACT	4	12	3	F16.2
AB261	S107	TGTGCAAGAGAT				GGTAACTAC				TTTGAC	2	0	1	P
AB262	J558	TGTACAAGA		TCAGAGGGGGGT		GGAC		TCATGGG		CTACTG	2	10	2	Q/?
AB263	3660	TGTGCAAGA		G		ATTACTACGGTAGNTA|CTACG				CCTGGT	3	0	1	F16.1+F16.2
AB265	Q52	TGTGCCAGAGA		GG		TGGGAC	G	AGAT		TACTTT	2	2	2	Q
AB268	J558	TGTGGAAG		GG		AGGTACG		GGGG		TACTTT	2	2	3	P
AB269	3660	TGTGCAAGAT		TG		GGG				GACTAC	4	14	-	?
AB272	Q52	TGTGCCAGAAA				CTGG				TTTGCT	3	1	3	Q
AB274	DNA4	TGTGTGAGAG				GTTACGAC				TATGCT	4	3	1	P
AB275	J558	TGTGCAAGA		CGG		CGGG		GGA		ATGCTA	4	6	3	T
AB276	S107	TGTGCAAGAGA		CCGCGG		CGGTAGTAGCT		T		CTACTT	2	1	1	F16.1
AB277	Q52	TGTGCCAGAGA		GGG		GGTAG				ACTTTG	2	3	2	F16.1
AB278	Vh11	TGTATGAGATA	T	CACCGAAA		GTCGTAC				TACTTT	2	0	1	P
AB280	J606	TGTACCAG		GA		ATGGTTACTAC				TGGTTT	3	1	1	P2.9
AB282	3660	TGTGCAAGA		GGA	A	TCTACTATGATT		CTC		ACTTTG	2	3	1	P2.2
AB285	Q52	TGTGCCAGAAA	T	CGGGCT		TTTATTACTACGGTAGTAGCTAC	G	AG		CTACTT	2	1	2	F16.1
AB286	J558	TGTGCAAGA		G		ATGGTAACTAC	G			CCTGGT	3	1	1	P2.1
AB287	J558	TGTGCAAGA		GGAAGG		ACTACGGCTAC	G			TATGCT	4	5	2	F16.2
AB289	3660	TGTGCaAGA				G				TTGACT	2	6	-	?
AB290	DNA4	TGTGTGAGAG		CCAGA		GGG				TATGCT	4	5	-	?
AB291	J558	TGTGCAAGA	TC	GAG		GGGAC		AC	G	CCTGGT	3	0	2	Q
AB292	S107	TGTGCAAGAGAT				TACTACGGTAGTAGCTAC				TGGTAC	1	0	1	F16.1
AB293	J558	TGTGNAAGA	TGT			GG				CTTTGA	2	4	1	DQ
AB294	7183	TGTGCAAGACA		GAGGGGGTCG		GGTAACTAC	G	AGAAA	G	CCTGGT	3	0	1	P

FIGURE 2. (*continued*).

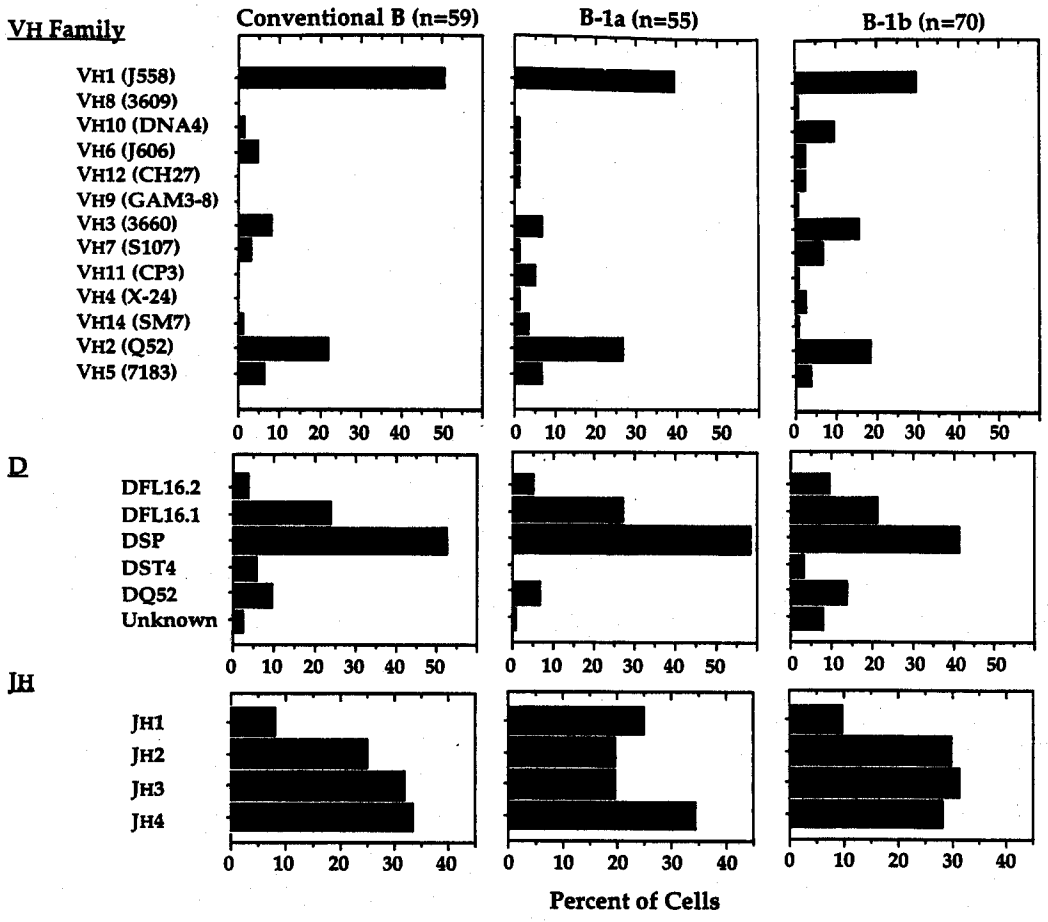

FIGURE 3. Gene segment usage. Histograms are presented that summarize the V_H family, D element and J_H element usage for the sequences in Figure 2. χ^2 analysis (2 × 2, using the element of interest and "all others" as the categories) indicates the following significant and nearly significant differences between populations. V_H 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 ($p < 0.05$); V_H10 family: B-1b vs B-2 ($p < 0.05$), B-1a vs B-1b ($p < 0.1$); and V_H11: B-1a vs B-2 ($p < 0.1$); J elements: J_H1 B-1a vs B-1b ($p < 0.05$) and B-1a vs B-2 ($p < 0.05$).

cells specific for phosphatidylcholine (PtC 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 V_H11 usage among B-1a cells (6%) is lower than other studies (20%) (34); however, this can be explained by a large proportion of PtC 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_H-proximal V_H families in any of the populations sampled here. Previous studies with V_H family specific probes demonstrated that fetal and neonatal B cells preferentially express V_H families located J_H proximal on the chromosome (32, 38–42) In those studies, the two most J_H-proximal V_H families, Q52 and 7183, constitute 50% of the V_H usage, including very high use of the most J_H proximal gene V_H81X. B-1a cells, which are associated with early ontologic development, might be expected to favor J_H proximal V_H genes. However, this is clearly not the case in the population sampled for the present study. We found that family usage among peritoneal 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 (41%) cells ($p < 0.1$). Interestingly, the DST4 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 V_H families.

Each B cell subset uses all four J_H elements. However, the B-1a cell subset is clearly distinguished from B-1b and conventional B cells by the relative use of J_H1. J_H1 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_H1 usage has been previously been associated with neonatal B cells 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

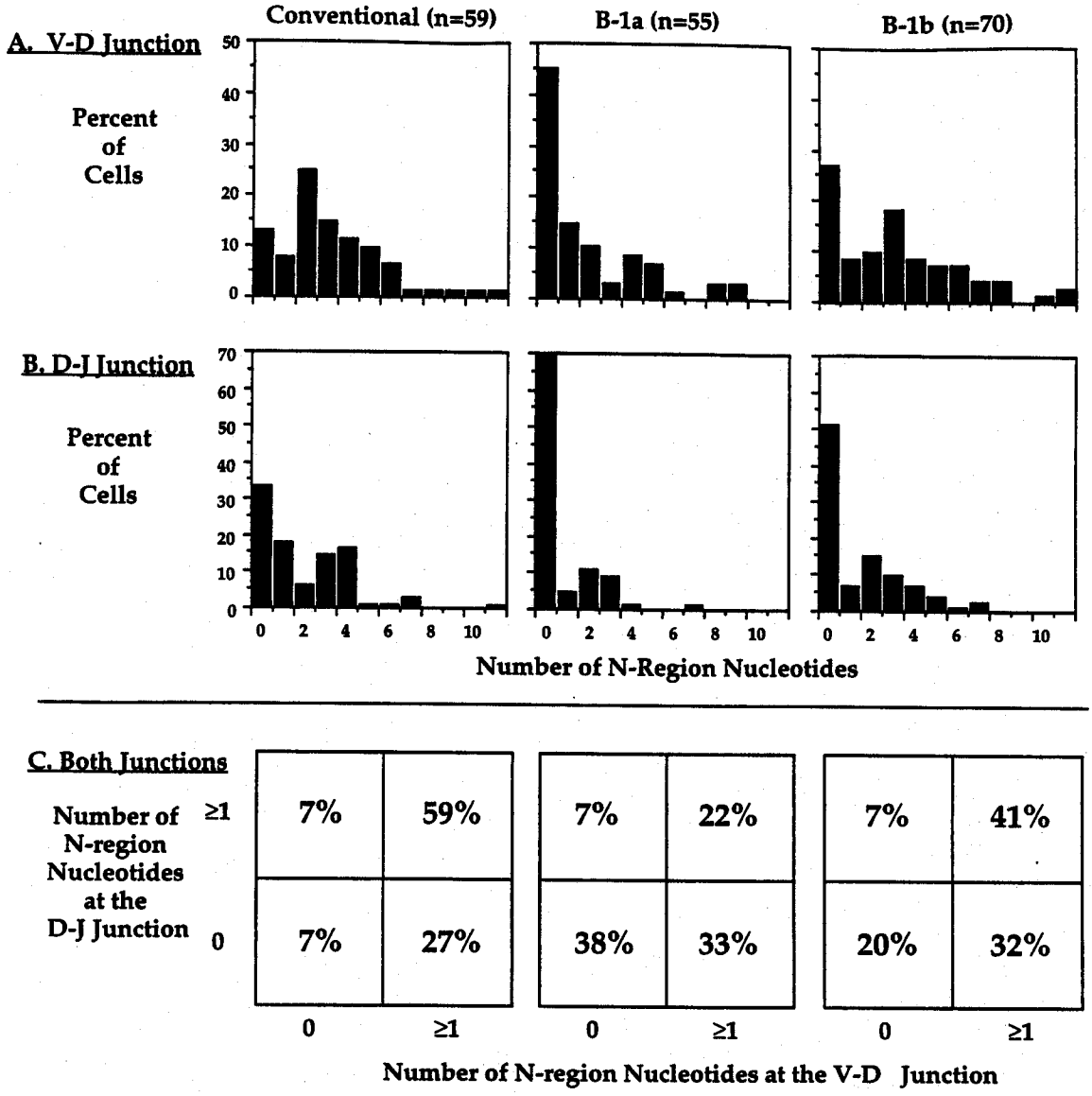

FIGURE 4. Comparison of N region addition among the populations. Frequency distributions for N region nucleotides at the V_H-D (*panel A*) and D-J_H (*panel B*) junctions are shown. Significance was evaluated with a nonparametric rank-order test (Mann-Whitney *U*). *p* values for the V_H-D junction are: B-1a vs B-2, $p = 0.0007$; B-1a vs B-1b, $p = 0.009$; B-1b vs B-2, $p = 0.7$ (NS); for the D-J_H 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_H-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-J_H junction: B-1a, 0.9 ± 1.6; B-1b, 1.5 ± 1.9; B-2, 2.1 ± 2.3. In *panel C* the V_H-D and D-J_H junctions 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. χ^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_H 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_H-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. 4*A*). These differences are significant in both experiments. A similar pattern is seen at the D-J_H 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. 4*B*). 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-J_H junction exists compared with the V_H-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 TdT activity. As indicated in Fig. 4*C*, 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 χ^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

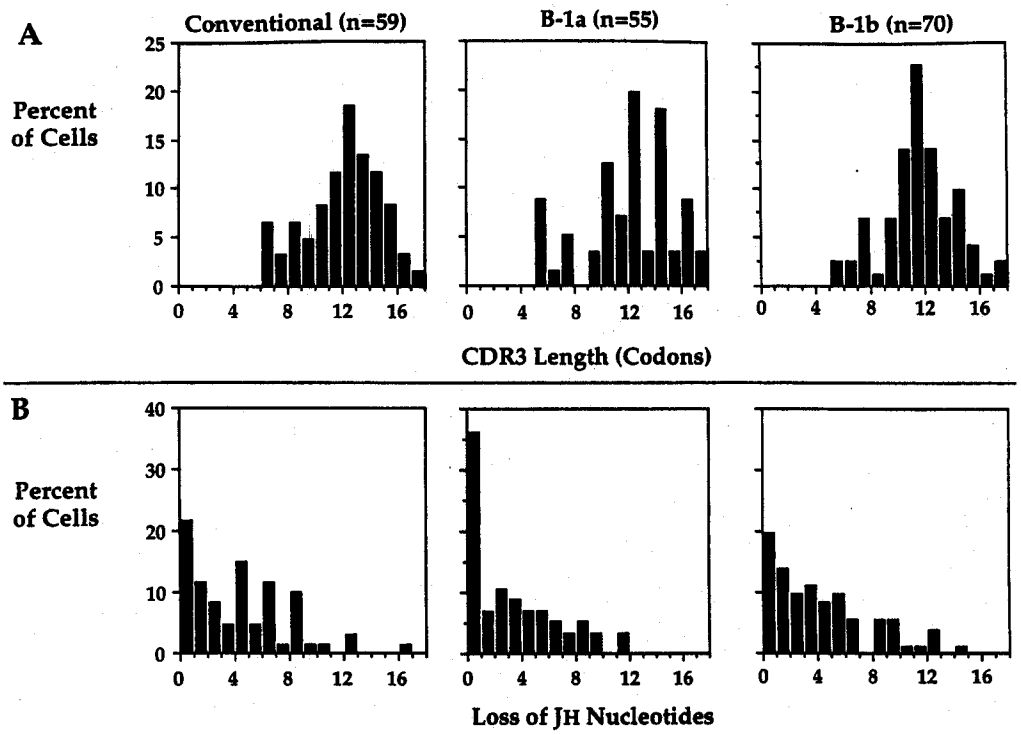

FIGURE 5. *A*, CDR3 lengths. The frequency distribution CDR3 lengths is plotted for each cell type. CDR3 was measured between the TGT (Cys at position 92) of the V_H to the TGG (Trp at codon 103) in the J_H. This includes two more codons from the V_H than is often used by others. There is no significant difference in the CDR3 length distributions among the three cell types: B-1a vs B-2, $p = 0.89$; B-1a vs B-1b, $p = 0.17$; B-1b vs B-2, $p = 0.25$ (NS). The mean CDR3 regions lengths (± SD) are: B-1a, 11.7 ± 3.4; B-1b, 11.6 ± 2.8; and B-2, 11.6 ± 2.7. *B*, J_{HH} nucleotide loss. The frequency distribution for the loss of 5′ nucleotide from the J_H segment is plotted for each cell type. More B-1a cells have complete J_H sequences (38%) than either B-1b (22%, $p < 0.05$ by χ^2) or conventional B cells (19%, $p < 0.1$). However, differences in the complete distributions by nonparametric rank-order test are not significant (Mann-Whitney *U* test *p* values are: B-1a vs B-2, $p = 0.11$; B-1a vs B-1b, $p = 0.12$; B-1b vs B-2, $p = 0.8$. The numbers of J_H nucleotides lost (± SD) are: B-1a, 3.0 ± 3.2; B-1b, 3.9 ± 3.6; and B-2, 3.9 ± 3.6.

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 J558 and Q52 families, although the frequency of B-1a cells that have no N region insertions at either junction is lower (27% in both J558 and Q52) compared with the population as a whole. The B-1a V_H11 transcripts do not have any N regions, consistent with previous results (36, 37, 49, 50).

V_H 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 V_H family usage. Instead, V_H family use among these cells ($n = 19$) is largely similar to that of B-1a cells that have N region insertions and reflect germline complexity to a first approximation.

B-1a, B-1b, and conventional B cell V_H 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. 5*A*). 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 J_H1 element (19 potential CDR3 nucleotides) more frequently, and the short J_H2 and J_H3 elements (14 potential nucleotides) less frequently, than either B-2 or B-1b cells (see Fig. 3). Also, more B-1a cells have complete J_H sequences (Fig. 5*B*). Transcripts with no nucleotide loss at the 5′ terminal of the J_H segment are more common for B-1a (38%) cells than either B-1b (22%, $p < 0.05$ by χ^2) or conventional B cells (19%, $p < 0.1$). Nadel et al. have demonstrated that nucleotide deletion is constant throughout ontogeny in the absence or presence of TdT (51). Consequently, the differences in J_H 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 V_H and D and D and J_H elements can be used in joining the gene segments (25, 52–54). Such sequences, which are underlined in Figure 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 V_H-D and D-J_H junctions, there is a significantly higher proportion of B-1a cells that have sequence overlap (Fig. 6). Sequence homology at the D-J_H junction is observed in almost half of the B-1a cells, but only 26% of the B-1b and 15% of the conventional B cells have overlap. At the V_H-D junction,

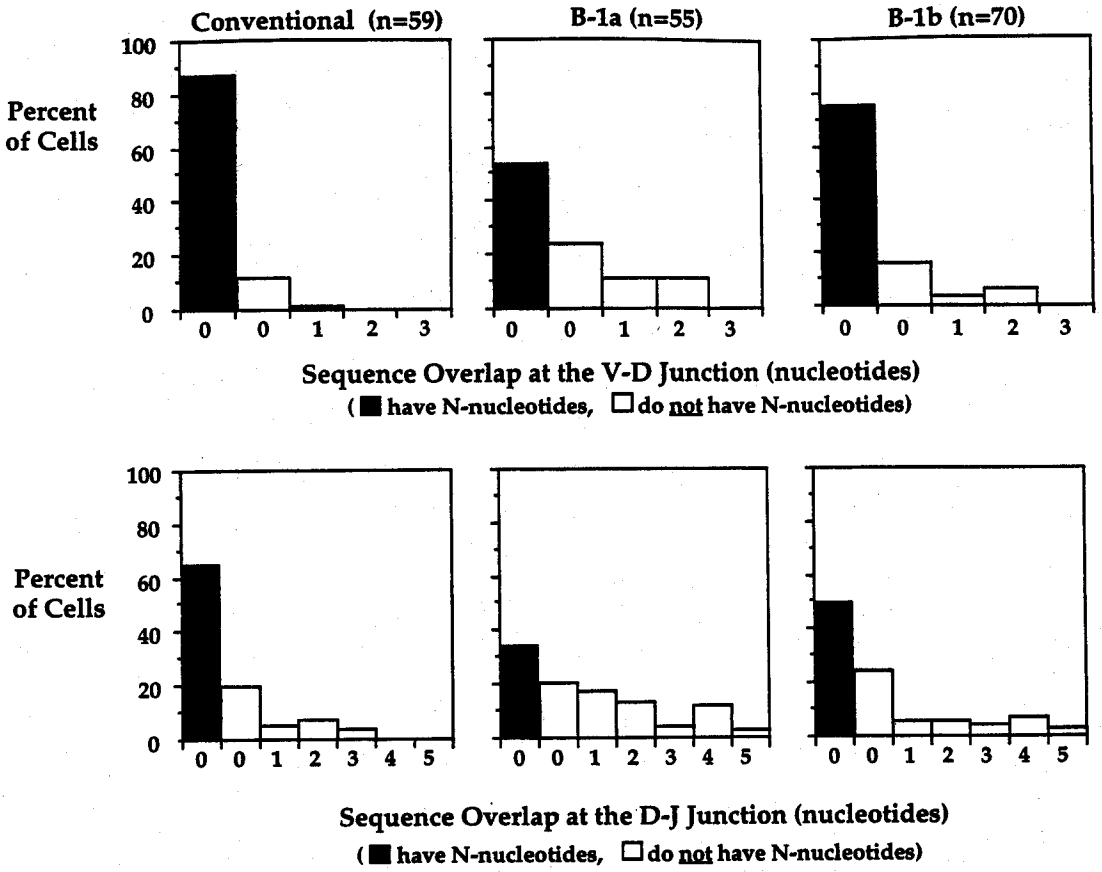

FIGURE 6. Sequence homology. The percentage of cells displaying sequence overlap at the V_H-D (*upper panel*) and D-J_H (*lower panel*) junctions is shown. All transcripts are included. The sequences that have N nucleotide additions are indicated in black. There is a statistically significant difference in the use of sequence homology by B-1a cells compared with B-1b and conventional cells. Mann-Whitney *U* test *p* values at the D-J_H junction are: B-1a vs B-2, $p = 0.0004$; B-1a vs B-1b, $p = 0.051$; B-1b vs B-2, $p = 0.1$ (NS); and at the V_H-D junction: B-1a vs B-2, $p = 0.0007$; B-1a vs B-1b, $p = 0.042$; B-1b vs B-2, $p = 0.08$ (NS).

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 of lack of N regions than with a B cell population. The comparison is best made at the D-J_H junction were there are a sufficient number of transcripts without N regions for each B cell subset. Among sequences without N region insertions, we find that 66% 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 junction, J_H2 (15) > J_H1 (10) > J_H4 (8) > J_H3 (3). The low use of J_H3 is reasonable given the lack of potential pairs at the 3′ end of the D elements.

D element reading frame I 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), RFI is strongly favored among functional rearrangements (24, 25, 28, 55). Two factors appear to contribute to the RFI preference. DFL16.1 and most members of the DSP family contain stop codons (1 or 2) in RFIII 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 Dμ protein (56) that may permit selection against RFII at the pro-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 78% compared with 60% ($p < 0.05$) and 64% ($p < 0.1$) for conventional and B-1b cells, respectively (see Table I). 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 peritoneal 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 I. *D gene segment reading frame use*[a]

Reading Frame	% Conventional Cells (n = 58)	% B-1a Cells (n = 55)	% B-1b Cells (n = 66)
RFI	60	78	64
RFII	17	9	15
RFIII	23	13	21

[a] There is a statistically significant difference in the use of RFI by B-1a cells compared with conventional B cells. χ^2 values, determined as a 2 × 2 matrix comparing RFI and not RFI (RFII + RFIII) are B-1a vs B-2, < 0.05; B-1a vs B-1b, < 0.01; B-1b vs B-2, > 0.5.

substantially more diverse than has previously reported. In fact, all three B cell populations show many unique rearrangements, using a variety of V_H families and CDR3 regions. Second, B-1b cells have a distinct pattern of V_H family usage compared with either B1-a or conventional B cells. Third, adult B-1a cells use N regions insertions least frequently; however, the majority of B-1a transcripts do use them.

In contrast to these findings, previous studies have associated B-1a cells with a limited repertoire, marked by characteristic specificities for self and particular bacterial Ags (18, 58–61). Our study demonstrates that the B-1a repertoire can be more diverse than previously reported. One explanation for the difference is that B-1a cells undergo clonal expansion. When present, clonal populations can overemphasize the extent of repertoire restrictions, as observed in some studies (17, 30, 31, 49, 62, 63). The B-1a and B-1b cells in our study showed no evidence of clones by either FACS or sequence analysis. All 55 B-1a cell sequences shown in Figure 1 are unique rearrangements. Only one B-1a sequence arose from two separate cells. The B-1a cells used 11 of 14 V_H families, all 4 D families, all 4 J_H elements, and a range of CDR3 lengths. Thus, this subset exhibits a good deal of diversity. Similarly, the B-1a J558 repertoire in young, unmanipulated mice appears to be quite diverse (64). We predict that the repertoire of "nonclonal B cells" in mice with B-chronic lymphocytic leukemia-like clones also exhibits substantial diversity.

Despite this diverse set of rearrangements, B-1a cells still exhibit characteristic features. They use J_H1 more frequently than either conventional or B-1b cells and their D elements favor RFI even more strongly than B-1b or conventional B cells. B-1a and B-1b cells, but not conventional B cells, use V_H11 and V_H12. Further analysis of characteristic V_H gene elements associated with the B-1a repertoire will be presented elsewhere (Kantor A. B., J. MacKenzie, J. L. Hillson, and L. A. Herzenberg, manuscript in preparation)

The new V_H family data suggest that the small B-1b population has differences from both B-1a and conventional B cells. The J558 and Q52 families are underutilized and the 3660 and V_H10 families over-represented among these cells compared with the B-1a and conventional B cells. V_H10 was initially associated with anti-DNA specificity and it would be interesting if this is a feature of B-1b cells.

Several groups have demonstrated that fetal and neonatal V_H-D and D-J_H junctions often lack N region insertions, whereas most such junctions recovered from adults have N regions (22–26). As a consequence of the absence of N region insertions early in ontogeny, rearrangement of certain V_H-D-J_H gene segments are potentially favored, i.e., those with short sequence homologies (25, 54). Together, these rearrangement mechanisms potentially restrict the early B cell repertoire and thus may have a disproportionate effect on the Ig produced by B-1a cells.

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 V_H family-specific primers to amplify bulk 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 TdT 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 TdT 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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