Natural Occurrence and Origin of Somatically Mutated Memory B Cells in Mice

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Summary

While most murine peripheral B cells express germline-encoded antibodies of classes M and D (\(\mu^+\delta^+\) cells), small numbers of memory B cells expressing somatically mutated immunoglobulin G antibodies are generated upon T cell-dependent immunization. Analyzing the antibody repertoire of the \(\mu^-\delta^-\) B cell pool in unimmunized mice, we show that these cells express somatically mutated \(V_n\) genes and that most of these genes derive from a set of germline \(V_n\) genes dominantly expressed by \(\mu^+\delta^+\) B cells. Thus, class-switched memory B cells are generated in the absence of intentional immunization, presumably in response to environmental antigens. These cells are either recruited from \(\mu^+\delta^+\) B cells or selected from newly arising B cells in parallel to the latter, by the same antigens.

The hallmark of acquired humoral immunity is the generation of memory in response to T cell–dependent antigens. This process is known to involve the generation of a special subset of antibody-producing lymphocytes, so-called memory B cells that respond to antigenic challenge by the production of large amounts of high-affinity antibodies (for review, see reference 1).

Memory B cells arise from precursor B cells that are triggered by antigen and helper T cells into extensive proliferation in the microenvironment of the germinal center, a histologically defined substructure of peripheral lymphoid organs (2, 3). In the course of proliferation, somatic point mutations are introduced into the rearranged antibody V region genes of the cells at a high rate and in a stepwise manner, and cells expressing antibodies with a high affinity for the antigen are selected to become long-lived, recirculating memory cells (4–8).

Whereas the majority of peripheral B cells in the mouse express IgM and IgD antibodies on their surface (\(\mu^+\delta^+\) cells), most B cells recruited into germinal centers after primary stimulation initially bear surface IgM but no detectable surface IgD (\(\mu^-\delta^-\) cells; reference 9). In responses to proteins or hapten-protein conjugates, most of these cells subsequently switch from the expression of IgM and IgD to that of other antibody isotypes (10–12; in the response to certain bacterial antigens, class switching appears to occur only in a fraction of the memory cells [13; for review, see reference 1]), either before or after the onset of somatic hypermutation (14). These cells can then be isolated as antigen-binding, \(\mu^-\delta^-\) B cells. It became clear on the basis of such experiments that the memory cells generated in response to a particular antigen represent only a minor fraction (0.02–0.1%) of the total B cell pool (8, 10, 15).

One might extrapolate from these results, and the fact that the total fraction of \(\mu^-\delta^-\) cells in the peripheral B cell population of an adult, not intentionally immunized mouse amounts to a few percent (1–3% in the spleen), that in ontogeny, mice “naturally” accumulate memory B cells specific for a large number of antigens. However, such an extrapolation implies that \(\mu^-\delta^-\) B cells indeed largely represent memory B cells, a notion for which there is no experimental evidence, except that the \(\mu^-\delta^-\) cell population resembles memory B cells generated upon intentional immunization in a variety of surface markers (10).

Therefore, in an attempt to identify and characterize the memory B cell compartment in nonmanipulated mice, we have sequenced rearranged antibody H chain V region genes (\(V_n\), \(D_n\), \(J_n\) genes) from \(\mu^-\delta^-\) B cells isolated from such animals by flow cytometry. A similar analysis of \(\mu^+\delta^+\) B cells had previously shown that the vast majority of these cells produce germline-encoded antibodies and that they dominantly express a restricted set of \(V_n\) genes (16). In the present study we find dominance of the same set of germline \(V_n\) genes in \(\mu^-\delta^-\) B cells, but in most cases the genes are modified by somatic point mutation whose overall pattern is similar to that observed in memory B cells induced by intentional immunization.

Materials and Methods

Mice. All experiments were performed with female C57BL/6 mice kept under conventional conditions. The mice were either 12 wk, 15 wk, or 10 mo old. The 15-wk- and 10-mo-old mice were bred in our own animal facility, whereas the 12-wk-old mice were obtained at the age of 8 wk from Bomholtgard (Ry, Denmark) and kept until use in our own facility.

Isolation of \(\mu^-\delta^-\) B Cells. Fluorescence staining of the cells was
performed as described previously (8) Briefly, spleen cells of three mice at the age of 15 wk (exp. 1), 12 wk (exp. 2), and 10 mo (exp. 3), respectively, were pooled and depleted of erythrocytes by treatment with 0.8% NH4Cl. The cells were stained with the biotinylated antibodies MB86 (anti-IgM; 17), 4/4D7 (anti-IgD; gift of Dr. T. Tokuhisha, Kobe, Japan), HO13-14 (anti-Thyl.2; 18), and streptavidin-FITC (Boehringer Mannheim, Germany). Thy1.2-IgM - IgD - cells were enriched to 85-95% using a magnetic cell sorter (19). The enriched cells were stained with the anti-B220 antibody RA3.62B2 (20) coupled to PE and the B220 - IgM - IgD - cells were isolated using a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). The size of the μ - δ - B cell populations ranged from 2 to 6% of all B cells without a clear age correlation. The lymphocyte population was gated according to forward scatter and orthogonal scatter as described earlier (21). In each experiment, 4-5 x 10^5 cells were sorted. In exp. 1, the cells were 74%, in exp. 2, 2% and in exp. 3, 96% pure. The contaminating cells were mainly B220 - μ - δ - cells in exp. 1, whereas in exp. 2 and 3 B220 - μ - δ - and B220 - μ - δ - cells were present at an equal frequency. Since it is known that plasma cells express only low levels of the B220 molecule on the cell surface (22), we expected that the sorted cells did not contain such cells. To verify this point, 5 x 10^4 (exp. 1) or 2-2.5 x 10^5 sorted cells (exp. 2 and 3) were transferred to slides, fixed in 70% ethanol, and stained in the cytoplasm with a goat anti-mouse IgG antibody coupled to FITC (Southern Biotechnology Associates, Birmingham, AL). No IgG + plasma cells were detected among the sorted cells.

Preparation of cDNA Libraries and V, Gene Amplification. Preparation of total cellular RNA as well as first-strand cDNA synthesis were performed as described previously (23). Briefly, RNA was isolated from the sorted cells (exp. 1, 4.5 x 10^5 cells; exp. 2 and 3, 2-2.5 x 10^6 cells) by direct phenol extraction. From the total RNA, first-strand cDNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) using a C'y primer specific for a sequence in the first exon of C3,1 b. The primer sequence used was 5'-CCCTTG-ACCAGGCACTCC-3' (amino acid positions 151-146).

For amplification of rearranged V, genes, we tailed the cDNA by poly(dG) and amplified 10% of the cDNA by PCR using a poly(dC) primer and a C'y primer containing a HindIII or EcoRI and BamHI site at the 5' end, respectively (see also reference 23). PCR amplification was performed for 35 cycles using a thermal cycler (Eriex Corp., Inc., San Diego, CA) under conditions described previously (23). The primer sequences were as follows: poly(dC), 5'-GAAAGCTTCCCCCCGCCCGCG-3'; C'y, 5'-CAGAAGTCGGATTCGCAATTC-3' (positions 133-128) (restriction site is underlined). The temperature profile of the amplification was 94°C for 1.5 min and 72°C for 2.5 min. For construction of cDNA libraries, the PCR product was cut with NcoI/EcoRI, size fractionated, and cloned into a pUC19 vector that contains an additional cloning cassette derived from the plasmid vector pGEM-57 (Promega Biotec, Madison, WI) (kind gift of S. Jung, Institute for Genetics, Köln).

PCR Amplification of Genomic DNA. Genomic DNA was prepared from C57Bl/6 liver cells as described by Maniatis et al. (28). The V, genes V186.2 and V145 were amplified by 35 cycles of PCR from genomic liver DNA equivalent to 5 x 10^6 cells under the same conditions described already for the V186.2 gene amplification from cDNA (see previous section). The primers used for amplification were a primer hybridizing 5' to the leader exon of V186.2 (see above) and a primer hybridizing to the nucleotide sequence in amino acid positions 74-80 of the V186.2 and V145 V, genes. The sequence of the latter primer containing an Xbal site is as follows: 5'-GGTGCAGAGCTGAGCTGACAGC-3' (amino acid positions 74-80) (restriction site is underlined). The PCR product was cut with NcoI/XbaI, gel purified, and cloned into a pTZ19R vector.

Bacterial Transformation, Colony Hybridization, and DNA Sequence Analysis. DH5α bacteria were transformed by electroporation (gene pulse; Bio-Rad Laboratories, Munich, Germany). To identify clones of the J558 V, gene family, a 254-bp HindII/PstI fragment of the V186.2 gene (27) was used as a probe (kind gift of Dr. H. Gu, Institute for Genetics, Köln). After hybridization with the 32P-labeled probe, the filters were washed at a maximum stringency of 4 x SSC at 65°C. Colonies were picked irrespectively of the intensity of the hybridization signal. Bacterial colonies containing a rearranged V186.2 gene were screened with an oligonucleotide specific for amino acid positions 71-76 of the V186.2 gene (kind gift of Dr. U. Weiss, Institute for Genetics, Köln). The oligonucleotide sequence is as follows: 5'-CTGGAGGGTTGCTCTC-3'. Colony hybridization with the 32P-labeled oligonucleotide was performed as described (29).

V, gene sequences were obtained by direct plasmid sequencing using a Sequenase™ kit (U.S. Biochemical Corp., Cleveland, OH).

Calculation of the Mutation Frequency. To calculate the mutation frequency, the number of mutations in the mutated genes analyzed was divided by the number of nucleotides sequenced. Germline genes were not considered for the calculation. All calculations are based on the frequencies of nucleotide exchanges in the V, and J, segments. Nucleotide replacements at the V,DR4 junctions were not scored as mutations. Two nucleotide exchanges present in one codon were scored independently, and replacement mutations were considered to have occurred first.

V, Gene Assignment. The sequences of the J558 V, genes isolated from μ - B cells were compared with those of known members of the J558 V, gene family from the Igh-b allotype (see reference 16) (see Fig. 3). Complete identity was found only in one case. Among the 17 mutated V, genes, we identified one V, gene (26F.1 in Fig. 1), which could either be a previously unknown germine V, gene or represent the germine gene vwp32 carrying a PCR-induced mutation in amino acid position 38 (GAG to AAG). This exchange leads to a sequence that is found in this codon in 57 of 67 known J558 V, genes in the IghA haplotype (16). Among the other mutated V, genes, we identified six (3C.2B, 2F.7A, 16C.1, 2C.2A, 13B.2A, 10C.2A, and 11C.2B in Fig. 3) that have exchanges in positions that are conserved among at least 63 of the 67 members of the J558 family (positions 39, 57, 62, 82, 88, and 90). In addition, positions 31 and 65, which are recurrently mutated in V186.2 (see Figs. 1 and 2), are mutated in eight
of the J558 V\textsubscript{\textalpha} genes depicted in Fig. 3 (13F.2B, 43F.1, 3D.2A, 16C.1, 2C.2A, 28F.2B, 7A.1, and 35F.2B). These positions were proposed to be mutational hotspots for somatic hypermutation (30). In the two J558 V\textsubscript{\textalpha} genes that do not carry mutations in conserved positions or mutational hotspots (47F.2A and 14C.2B), the exchanges that distinguish them from the most homologous germline genes are not seen in any other of the known germline genes. Thus, 16 of the 17 mutated J558 V\textsubscript{\textalpha} genes isolated from \textalpha-B cells are likely to represent somatic mutants of known V\textsubscript{\textalpha} genes rather than previously unknown germline V\textsubscript{\textalpha} genes.

Results

Do \mu-\delta- B Cells Express Somatically Mutated Antibodies? This question can be answered by the molecular cloning of rearrangements of a known V\textalpha gene from \mu-\delta- B cells and subsequent sequence analysis. We chose for our analysis the V\textalpha gene V186.2, which belongs to the J558 V\textsubscript{\textalpha} gene family and is dominantly expressed in the T cell-dependent antibody response to the hapten (4-hydroxy-3-nitro-phenyl)acetyl (NP)\textsuperscript{1} (31-33) and strongly somatically mutated in NP-specific memory B cells (7, 30, 34). We also know from our previous work that most rearranged V\textsubscript{\textalpha} genes, including V186.2 expressed by peripheral B cells, are devoid of somatic mutations irrespective of whether the animal is immunized or not (16, 30, 34). Given this situation, it would be particularly significant if V186.2 genes expressed by the small \mu-\delta- B cell subset from unimmunized mice turned out to be somatically mutated.

Somatic Mutants of V186.2 Can Be Unambiguously Identified. Comparison of V186.2 with the other known J558 germline V\textalpha genes in the IgH\textsuperscript{b} haplotype reveals that at amino acid positions 74 and 75, V186.2 shares its sequence with only two other members of the family, the V\textalpha genes V145 and V6 (16, 27). To establish that this together with a specific gene amplification protocol allows the unambiguous identification of somatic mutants of V186.2, we performed PCR amplification on genomic liver DNA from C57BL/6 mice, using a pair of primers that hybridize to the CCCTCC sequence around positions 74 and 75 on the one hand, and to a region 5' of the leader exon of V186.2 and V145 on the other (see Materials and Methods). With these two primers the V\textalpha genes V186.2 and V145 should be amplified, but not gene V6, since the primer hybridizing 5' to the leader exon has two mismatches to this region in the V6 gene (27). Sequence analysis of the cloned PCR products would reveal whether in the germline of C57BL/6 mice V\textalpha genes other than V186.2 and V145 exist that can be amplified in the presence of the two primers.

Of the 21 genes sequenced, 9 were identified as V186.2 and 12 as V145 (which differs from V186.2 only at amino acid positions 22 and 91), indicating that these genes are present in the genome either as single copy genes or at an equal copy number (data not shown). In 4,668 bp sequenced we found three exchanges to the respective germline gene, corresponding to a mutation frequency of 1/1,556. This is a misincorporation rate of 3.7 x 10\textsuperscript{-5} and corresponds well to previously determined PCR mutation frequencies in similar amplification experiments (16, 30). All of the exchanges were A to G exchanges, the most frequent error of Taq polymerase (35).

These data show that the V\textalpha genes V186.2 and V145 carry a unique sequence pattern 5' of the leader exon and at positions 74 and 75. Gene amplification from cDNA with a constant (C) region primer and the primer 5' of the V186.2 leader exon followed by selection by an oligonucleotide complementary to the V186.2 sequence around positions 74 and 75 should therefore allow the selective isolation of rearranged V186.2 and V145 genes and most of their somatically mutated progeny. This protocol was used for the analysis of V\textalpha gene mutation in \mu-\delta- B cells. We chose a C region primer specific for the \gamma1, \gamma2a, and \gamma2b H chain C region genes in order to avoid amplification from contaminating \mu-\delta- B cells.

Most V186.2 Genes Expressed by Splenic \mu-\delta- B Cells Are Somatically Mutated. When rearranged V\textalpha genes of \mu-\delta- B cells isolated from the spleens of C57BL/6 mice at the age of 12 wk, 15 wk, and 10 mo were amplified under the conditions described above and subsequently sequenced, we obtained the results depicted in Fig. 1. All sequences shared with the V186.2 and V145 germline genes the CCCTCC sequence at positions 74 and 75, but at position 22 they all resemble V186.2 rather than V145. On the basis of the results described in the previous section, this identifies the V\textalpha gene sequences in Fig. 1 as progeny of the V186.2 germline gene; progeny of V145 was not detected in accord with the notion that V145 is a pseudogene (27).

Most of the sequences in Fig. 1 (34/45; 76%) differ from the V186.2 germline sequence by somatic point mutations whose frequency ranges from 1 to 20 per sequence and that distribute over the \gamma1, \gamma2a, and \gamma2b isotypes (Fig. 1 and Table 1). Overall, the mutation frequency is 1 in 52 bp (Table 2), i.e., 30-fold higher than the frequency of point mutations generated by Taq polymerase under similar conditions (see previous section). Thus, the majority (97%) of the mutations present in the 45 V186.2 genes in Fig. 1 must have been generated in vivo.

Clonal Expansion of Memory Cells in the \mu-\delta- B Cell Compartment. Among the sequences obtained from 12- and 15-wk-old mice (Fig. 1, a and b), but not those from 10-mo-old animals (Fig. 1 c), we find substantial numbers of clonally related sequences (six sets with two to four members), which because of their unique, shared V\textalpha-D\textalpha-J\textalpha rearrangement are likely to originate from single B cell clones (Table 1). Significantly, the average mutation frequency in these sequences is almost three times lower than in the others (1/110 vs. 1/40) and there is a strong overrepresentation of silent over replacement mutations in both framework regions (FRs) and CDRs (Table 2). These molecular features are typical for the early phase of memory B cell development after T cell–dependent immunization when clonal expansion of B cells undergoing hypermutation and class switching occurs in germinal centers.

\textsuperscript{1} Abbreviations used in this paper: NP, (4-hydroxy-3-nitro-phenyl) acetyl; FR, framework region.
CDR1

CDR2

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but selection of high affinity mutants has not yet set in (5, 34, 36). The clonally related sequences shown in Fig. 1, a and b, therefore, likely represent B cells at that stage of differentiation. The fact that we find these cells in young, but not in old, mice could indicate that aged mice have built up a stable memory B cell pool in response to environmental antigens.

The Pattern of Mutation in Naturally Occurring V186.2 Mutants Is Similar, but Distinct from That Generated by Intentional Immunization with a Hapten. The pattern of mutation exhibits striking similarities between the naturally occurring antibody mutants and mutants intentionally generated by immunization with the NP hapten. The distribution of the frequency of point mutations per gene is very similar in the two cases (Fig. 2a). In addition, the naturally occurring V186.2 mutants exhibit a strong underrepresentation of replacement mutations in their FRs (Table 2), as it is found as a rule in somatically mutated V region genes generated in response to intentional immunization with defined antigens (for review, see reference 1). Finally, as in the mutants generated by immunization with NP, the mutations in the naturally occurring V186.2 mutants cluster in the CDRs, with a possible third cluster in FR3 (positions 76-82a). There are indications that the amino acids encoded by codons 76 as well as 79-83 (numbering according to Kabat et al. [37]) may occasionally be involved in contacting antigens in addition to the classical CDRs (38, 39) (Fig. 2b).

It is notable, however, that there are also distinct differ-
Table 1. Somatically Mutated J558 \( \mu^- \delta^- \) Genes Isolated from \( \mu^- \delta^- \) B Cells

<table>
<thead>
<tr>
<th>V(_\mu) genes</th>
<th>Class</th>
<th>Sequences</th>
<th>Mutants</th>
<th>Clonally related genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>V186.2*</td>
<td>( \gamma 1 )</td>
<td>4</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \gamma 2a )</td>
<td>9</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \gamma 2b )</td>
<td>32</td>
<td>24</td>
<td>19*</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>45</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>J558 V(_\mu) family#</td>
<td>( \gamma 1 )</td>
<td>4</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \gamma 2a )</td>
<td>7</td>
<td>6</td>
<td>2|</td>
</tr>
<tr>
<td></td>
<td>( \gamma 2b )</td>
<td>7</td>
<td>7</td>
<td>2|</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>18</td>
<td>17</td>
<td>4</td>
</tr>
</tbody>
</table>

* See Fig. 1.
\# Six sets of V\(_\mu\) genes with two to four members.
\# See Fig. 3.
\| One set with two members.

ences in the distribution of mutations in V186.2 genes recovered from NP-immunized animals as compared with V186.2 mutants from unimmunized mice (Fig. 2 b). Whereas in nonintentionally immunized mice mutations in the V186.2 gene are equally well distributed in CDR1 and CDR2, in NP-immunized mice mutations are predominantly located in CDR1. Most strikingly, the key mutation conferring high affinity to the NP hapten, a tryptophan to leucine exchange in position 33 (7, 40), commonly seen in anti-NP antibodies (30, 32, 33), is absent in the natural mutants (Figs. 1 and 2 b).

Taken together, these results suggest that while the an-

tigens that induce and select the naturally occurring antibody mutants in the \( \mu^- \delta^- \) compartment of normal mice are not known, the rules of mutant generation and selection are the same as those identified through the analysis of model immune responses.

The Antibody Repertoire of \( \mu^- \delta^- \) B Cells Is Dominated by Somatically Mutated Progeny of the Germline V\(_\mu\) Dominantly Expressed by \( \mu^- \delta^- \) B Cells. We had previously analyzed the antibody repertoire expressed by peripheral \( \mu^- \delta^- \) cells at the level of individual V\(_\mu\) genes of the J558 family, the largest V\(_\mu\) gene family in the mouse. This analysis had shown that in contrast to their progenitors (pre-B cells) in the bone marrow, \( \mu^- \delta^- \) cells are strongly selected for the dominant expression of a restricted set of germline V\(_\mu\) genes (16).

Is there also dominant usage of certain V\(_\mu\) genes in the
μ-δ- population? To answer this question, total RNA was isolated from μ-δ- B cells of 15-wk-old C57BL/6 mice, Cγ-specific cDNA synthesized and tailed with dGTP. Rearranged Vμ genes were subsequently amplified by PCR using a poly(C) primer and a primer specific for the H chain C region genes γ1, γ2a, and γ2b. Clones containing Vμ genes of the J558 family were identified by a family-specific probe (16), and the VμDμJμ genes of 21 clones were sequenced.

The sequences were aligned to the most homologous known germline Vμ genes of the IgH haplotype (16), as shown in Figure 3. Sequence of rearranged Vμ genes of the J558 Vμ gene family derived from splenic μ-δ- B cells from 15-wk-old, unmanipulated mice. In the Jμ regions, we find 12 mutations distributed over 7 sequences (not shown). For further description, see legend to Fig. 1.
Fig. 3. Only a single Vn sequence (37F.2a) perfectly matches that of a known germline Vn gene (CH10). 17 sequences differ from the most homologous known germline genes by 1-11 nucleotides (Fig. 3 and Table 1). Three sequences were excluded from the analysis because they presumably represent hybrid genes artificially generated in the course of gene amplification (41) (the 5' and 3' part of these sequences resemble different germline genes most closely [data not shown]).

Having established that most V186.2 genes expressed by μ−δ− B cells are somatically mutated (see above) and that a perfect match to a known germline Vn gene is obtained in 75-80% of the cases when Vn genes are randomly isolated from pre-B cells (16; D. Tarlinton, unpublished data [for μ−δ+ B cells the percentage is even higher because these cells are selected for the expression of certain known germline genes]), we can assume that most of the Vn genes in Fig. 3 are indeed somatic mutants of the germline Vn genes to which they are aligned. (The higher frequency of somatic mutants in the present collection as compared with the V186.2 progeny [Fig. 1] could be due to a counterselection of V186.2 mutants because of the identification of V186.2 progeny through oligonucleotide hybridization. In addition, we cannot exclude that a few sequences in Fig. 3 represent hitherto undiscovered germline genes.) Making this assumption, we find in this collection of genes an overall mutation frequency of 1/69 bp: very close to the mutation frequency calculated for the naturally occurring V186.2 mutants (1/52; Table 2). We also note that the range of mutations per Vn gene is similar to that seen in the V186.2 mutants (1-11 as compared with 1-20 in the V186.2 gene), and that in the FRs there is strong counterselection against replacement mutations (Table 2).

The most striking result, however, is seen when we compare the repertoire of Vn genes expressed by μ−δ− B cells with that expressed by splenic μ−δ+ B cells and pre-B cells (16). As the μ−δ− B cells, μ−δ− B cells dominantly express J558 genes of the V186.2 and V3 subgroups, and in some cases the very same Vn genes appear repeatedly in the two groups (V23, 593.3, CH10 in Fig. 4), although in the μ−δ− B cells in somatically mutated form.

Discussion

A Compartment of T Cell–dependent Memory B Cells in Non-manipulated Mice. In T cell–dependent model immune responses, memory B cells have been identified as resting cells that are generated in germinal centers through proliferation accompanied by hypermutation of rearranged antibody V region genes and selection of high affinity mutants (5, 8, 10, 11, 15, 30, 36). Most of these cells have switched antibody isotype (μ−δ cells; in response to certain antigens, IgM-expressing memory B cells have also been identified [see Introduction]) and express as their characteristic molecular marker somatically mutated antibody V regions selected for proliferation, hypermutation

Figure 4. Schematic representation of Vn gene utilization among the J558 Vn gene family in pre-B cells, μ−δ− B cells, and μ−δ− B cells. The data for pre-B cells and μ−δ− B cells are taken from Gu et al. (16). Individual Vn genes expressed by the cells are represented as circles and positioned according to their homology to each other (16). Clonally related genes (marked with an asterisk in Fig. 3) are represented only once. Germline Vn genes are depicted as open circles, mutated Vn genes by filled circles. The subfamilies of the J558 Vn gene family are indicated on the left. All genes that were identified more than once in this and the previous study (16) are designated by a particular character (A, V23; B, 165.1; C, 593.3; D, 24.8; E, V3; F, CH10; G, 2.2; H, vpw32; I, VMU3.2; J, BULK11; K, 205.12; L, 10B10S; M, V186.2; N, CIH4; O, 2.5).

Figure 5. Generation of the peripheral B cell pool through cellular selection. a and b denote possible pathways of selection. For further explanation of this hypothetical scheme see the text.
high affinity antigen binding. Somatic mutation distinguishes these cells from the bulk of the peripheral (\(\mu^\delta\)) B cells, the vast majority of which express germline-encoded antibodies (16, 30).

The present data indicate that memory B cells of this type arise in the mouse also in the absence of intentional immunization, and that, indeed, the population of \(\mu^\delta\) B cells in the animal largely consists of such cells. This is also supported by experiments conducted in the course of a previous study (8) in which we analyzed the proliferative behavior of PE-specific \(\mu^\delta\) memory B cells. We found that within the first 5 wk after immunization most of these cells incorporated the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into their DNA at high rate in vivo. However, at later stages proliferative activity dropped to very low levels (0.4–10% 20 wk after immunization, after a 1–3-wk labeling period), and the cells persisted over long periods of time in a resting state (8). In the same animals we had also determined (but had not included in our previous publication) the extent of BrdU labeling in the total \(\mu^\delta\) population. This was consistently below 15% after labeling periods ranging from 1 to 3 wk, except early after immunization when apparently antigen-activated \(\mu^\delta\) cells represented a major fraction of the total \(\mu^\delta\) pool. Thus, in an unmanipulated adult mouse the vast majority of the \(\mu^\delta\) cells are long-lived cells as one would expect for mature memory B cells (8).

We consider it likely that the somatic antibody mutants that populate most of the \(\mu^\delta\) B cell compartment have arisen in T cell–dependent responses to environmental antigens. We base this argument on the observations that most other peripheral B cells express germline-encoded antibodies (see above), that the process of hypermutation is induced upon intentional T cell–dependent immunization (5, 6, 34, 42), and that the naturally occurring mutants express a pattern of mutation similar to the one observed in intentionally induced mutants (see Results). We therefore conclude that the generation of T cell–dependent memory based on V gene hypermutation and selection of high affinity binding sites is a physiological property of the immune system in its interaction with the environment. This interaction is likely to reflect responses against environmental pathogens rather than self-antigens: in specific pathogen-free mice, the frequency of \(\mu^\delta\) cells is reduced to 1–3% of the total splenic B cell population compared with the 2–6% seen in the animals used in the present study (our own observations), and in the spleen of germfree C57BL/6 mice such cells are below the level of detection (\(\leq0.1\%\); D. Tarlinton, personal communication). The mice used in the present experiment were raised under conventional conditions and are most likely exposed to a broad range of potentially pathogenic bacteria and viruses. Thus, we have occasionally detected in sera of animals from our colony antibodies against mouse hepatitis virus.

Concerning the isotype distribution among the \(\mu^\delta\) B cells, it was surprising that most V186.2-expressing, naturally occurring B cells produce IgG2b antibodies, whereas the dominance of this isotype is not seen in B cells expressing other \(V_n\) genes of the J558 family (see Table 1). Since the nature of the immunizing antigen seems largely to determine the isotype distribution in the antibody response (reviewed in reference 43), it is likely that the particular (unknown) antigen(s) involved in the activation of the V186.2-expressing cells promoted class switching to IgG2b, by a mechanism that is presently not understood.

The size of the \(\mu^\delta\) memory compartment amounts to approximately 2–6% of the total peripheral B cell pool, i.e., a few million B cells in an adult mouse raised in a conventional animal facility. Given that the memory cells generated in response to a particular antigen appear to represent only 0.35–7% of the total \(\mu^\delta\) B cells (8, 10, 15), an adult mouse could harbor memory to a fairly large, though limited number of antigens. It remains to be explored how the size of this cellular compartment (kept remarkably constant from the age of 10 wk to 10 mo) is controlled, and to which extent persisting antigen is required for the survival of the cells (3, 44). It is noteworthy in this context that the mutation frequency in rearranged V186.2 genes expressed by \(\mu^\delta\) cells does not differ significantly between young and old mice (Fig. 1 and Table 2). This could reflect a substantial flux of cells through this cellular compartment over time such that the majority of the cells would not go through consecutive rounds of mutation as they might do upon intentional hyperimmunization (45).

Although somatic \(V_n\) gene mutants predominate in the \(\mu^\delta\) compartment, some \(\mu^\delta\) B cells express rearranged germline \(V_n\) genes. In the case of the V186.2 gene, in which such genes could be unambiguously identified, the germline sequences amounted to 24% (11 of 45 sequences; Fig. 1). A similar phenomenon has been described earlier in cases of intentionally induced immune responses (30, 34, 45), but in these cases, it was not determined whether the unmutated genes were expressed in IgM antibodies or antibodies of other isotypes. It is not clear whether cells expressing unmutated \(V_n\) genes in IgG antibodies appear in the \(\mu^\delta\) compartment because they had been recently recruited by an antigenic stimulus into the pathway of memory cell generation or into a primary response where they would not yet have reached the plasma cell stage. The cells would have already undergone isotype switching, which is known to occur in both pathways and to be independent of somatic hypermutation (14). However, the presence of such cells could also indicate that mature, long-lived memory cells can occasionally be generated without undergoing somatic hypermutation, perhaps, if their receptors display high affinity for antigen from the beginning. This important problem could be further approached by determining whether the cells expressing germline \(V_n\) genes are enriched in the small fraction of proliferating cells in the \(\mu^\delta\) compartment, as it would be predicted if they represent newly activated cells.

**Antibody Repertoire and Cellular Origin of T Cell–dependent Memory B Cells.** As shown by the previous analysis of Gu et al. (16) and the present results, the \(\mu^\delta\) B cell population and the bulk of the peripheral (\(\mu^\delta\)) B cells exhibit a striking similarity in \(V_n\) gene usage (Fig. 5): both cell populations are selected for the predominant usage of certain
V_{n} genes of the J558 V_{n} family. In $\mu^{\delta^{-}}$ cells, these genes are expressed as encoded in the germline; in the $\mu^{\delta^{-}}$ B cell population, they are modified by somatic hypermutation.

Together with data indicating that $\mu^{\delta^{+}}$ B cells can be precursors of germinal center cells (46), this result suggests a scheme of peripheral B cell selection, as depicted in Fig. 5. In this hypothetical picture, the V region repertoire of $\mu^{\delta^{-}}$ memory B cells is selected in parallel with, or derived from, the repertoire expressed in the $\mu^{\delta^{+}}$ population, or both. Parallel selection (a in Fig. 5) would imply that both populations are selected by the same (environmental) antigens. All cells would require the presence of antigen to persist as long-lived cells, a concept supported by experimental evidence for memory B cells (3, 44). T cell help would be required to drive cells into the $\mu^{\delta^{-}}$ rather than the $\mu^{\delta^{+}}$ compartment, but not for the persistence of mature memory cells (47). In the case of sequential selection of the cells (b in Fig. 5), i.e., first into the compartment of long-lived $\mu^{\delta^{+}}$ cells and from there into the $\mu^{\delta^{-}}$ compartment, the second step again depends on T cell help, whereas the selection of $\mu^{\delta^{+}}$ cells may not require T cells. Sequential selection is known to occur since $\mu^{\delta^{+}}$ cells can function as precursors of germinal center cells (46). This could simply represent a special case of parallel selection in the sense that the same antigens are involved in the selection of $\mu^{\delta^{-}}$ and $\mu^{\delta^{+}}$ cells. Alternatively, the selection of $\mu^{\delta^{+}}$ cells may be driven by other antigens than those selecting $\mu^{\delta^{-}}$ cells. Self-antigens like antibody idiotypes come to mind in this context (48, 49).

The scheme of B cell selection depicted in Fig. 5 deals with "conventional" B cells and does not address the problem of selection in the B-1 cell (previously called CD5 B cell) compartment (16, 50). It also does not specifically address mechanisms of negative selection that are known to shape the peripheral B cell receptor repertoire (51). Rather, it emphasizes that the peripheral B cell pool consists largely of antigen-selected, long-lived cells instead of "naive" cells with a rapid turnover, as suggested by Freitas et al. (52); and that somatic memory may be expressed at two distinct, but interconnected, levels in the B cell system, through the selection of germline specificities in the $\mu^{\delta^{+}}$ compartment and of high affinity somatic mutants of these same antibodies in the compartment of classical, T cell–dependent $\mu^{\delta^{-}}$ memory cells. The extent to which cells are recruited into this latter compartment may simply depend on the ability of a given antigens stimulus to recruit T cell help, if one does not want to resort to Linton et al.'s (53) proposal of differential lineage commitment.

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