Most Peripheral B Cells in Mice Are Ligand Selected

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Summary

Using amplified cDNA and genomic libraries, we have analyzed the V_H gene repertoire of pre-B cells and various B cell subsets of conventional mice at the level of V_H genes belonging to the J558 V_H gene family. The sequence data were evaluated on the basis of a newly established list of 67 J558 V_H genes that comprise approximately two-thirds of the J558 V_H genes of the murine IgH^b haplotype. The results of the analysis demonstrate that V_H gene utilization in pre-B cells, although biased to some extent by B cell autonomous V_H gene selection, scatters over the whole range of J558 V_H genes present in the germline. In contrast, in mature, peripheral B cells comprising long-lived $\mu^+\delta^{high}$ B cells as well as Ly-1 B cells, small overlapping sets of germline V_H genes are dominantly expressed. The data indicate that the recruitment of newly generated B cells into the long-lived peripheral B cell pool is mediated through positive selection by internal and/or external antigens. Because of the absence of immunoglobulin class switching and somatic hypermutation, this process is different from the selection of memory B cells in T cell–dependent immune responses.

Peripheral B cells in the immune system are usually classified as either naive or memory B cells, based on their antigenic experience. Naive B cells represent B cells that have not been selected by antigen and are commonly thought to account for the majority of mature, peripheral B cells in the animal. In this compartment, a high flexibility in antigen recognition is ensured by a continuous supply of newly generated B cells from the bone marrow (for review, see reference 1), thereby providing a broad repertoire of antibody specificities. Memory B cells, on the other hand, are generally defined as B cells that have been selected in T cell–dependent immune responses for high affinity binding of their target antigen, express somatically mutated antibody variable (V) region genes, and persist in the immune system over long periods of time (2–5). While these cells account only for a minor fraction of the total B cell pool, we have recently demonstrated (6), in agreement with earlier data obtained by others (7–10), that in adult mice more than two thirds of peripheral B cells represent long-lived cells, which clearly differ in their phenotype from classical memory B cells. The function of these long-lived surface (s)μ^+sδ^{high} B cells is unknown. In the present experiments, we have analyzed the antibody repertoire of these cells at the level of Ig V gene usage.

Over the last years the V gene repertoire of B lineage cells has been explored repeatedly, concentrating on the utilization of the V gene families of the Ig heavy chain locus. These analyses consistently demonstrated that pre-B cells in the bone marrow exhibit predominant expression of V_H gene families

located at the 3' end of the V_H gene cluster in the genome, perhaps because of preferential rearrangement of V_H genes close to the D locus (11, 12). In contrast, in peripheral B cells, V_H gene family utilization roughly corresponds to the complexity of these families in the germline (13–16). Although the mechanism of this "randomization" of V_H gene family usage is elusive, it is likely to involve cellular selection of newly generated B cells (12) and may well coincide with the formation of the stable, peripheral B cell pool. Such a selection could result in a broad utilization of V_H genes, the common interpretation of randomization. However, it is equally possible that randomization reflects a highly restricted V_H gene usage, assuming, as it seems reasonable, that the selected genes distribute over the entire V_H gene cluster. In agreement with the latter hypothesis, studies on the V_H gene repertoire of the Ly-1 B cell subset (reviewed in references 17 and 18) demonstrated that V gene expression may be strongly selected at the level of individual V genes, while this is not necessarily apparent at the level of V gene family utilization (19–22).

With regard to this finding, we decided to analyze the representation of individual V_H genes (instead of V_H gene families) in the various peripheral B cell subsets. In this analysis, we focussed on the relative representation of V_H genes belonging to the J558 V_H gene family, which is the largest V_H gene family and comprises about half of all V_H genes in the IgH locus of the b allotype (23). To identify V_H genes expressed in the various B lineage populations, cells of each subset were isolated by FACS®, amplified cDNA libraries or

genomic libraries were prepared from these cells using specific primers, and cloned V_HDJ_H rearrangements containing V_H genes of the J558 family were sequenced. These sequences were compared with each other and with J558 V_H genes of the IgH[b] locus described in the literature. On the basis of these data, we established a list of all presumptive IgH[b] J558 germline genes known to date, ordered according to the homology of the genes to each other. This list provided the basis for the evaluation of the sequence data derived from the amplified libraries of pre B cells, peripheral $s\mu^+s\delta^{high}$ B cells, as well as Ly-1 B cells.

Materials and Methods

Mice. All experiments were performed with CB.20 mice that were bred in our animal facility.

FACS®. A detailed description of the isolation of the cell populations analyzed in this study and of the reagents used for fluorescence staining has been given by Gu et al. (24). Briefly, pre-B cells were obtained from a pool of spleen cells derived from five 2-d-old mice and from bone marrow cells from pools of three 4-mo-old mice, and were sorted as $B220^{dull}\mu^-$ cells. From the same pool of bone marrow cells, we also sorted $B220^{bright}\delta^+$ B cells as a source of long-lived bone marrow–derived B cells. Splenic $\mu^+\delta^{high}$ B cells were isolated from pools of three 4-mo-old mice. Conventional B cells from the spleens of two 4-wk-old mice were sorted as $B220^+Ly-1^-$ cells. Ly-1$^+$ B cells were isolated from spleen cells of 15 4-d-old mice or from pooled peritoneal cells derived from 10 4-wk-old mice as $B220^+Ly-1^+$ cells. To control for plasma cell contamination, 2–5 × 10^4 cells from each sorted cell population were transferred to slides, fixed in 95% ethanol and 5% acetic acid, and stained in the cytoplasma with anti-IgM FITC. No IgM$^+$ plasma cells were detected among the sorted cells, indicating that possible contamination with plasma cells was <2–5 × 10^{-5}.

Preparation of Amplified cDNA Libraries. The protocols of RNA isolation, V_H gene amplification, and cDNA library construction have been described previously (24). Briefly, total cellular RNA was isolated from 10^4 to 3 × 10^5 sorted cells by a direct phenol extraction method, and first-strand cDNA was synthesized using a primer specific for the μ constant region (Cμ1; see also reference 19). The cDNA was tailed with poly(dG) and amplified using a poly(dC) and a Cμ2 primer containing a HindIII or BamHI site at the 5' end, respectively. PCR was performed for 30–35 cycles using a thermal cycler (Techne Ltd., Cambridge, UK). For construction of cDNA libraries, the PCR product was cut with HindIII/BamHI and cloned into a pTZ19R vector (Pharmacia Fine Chemicals, Piscataway, NJ).

Preparation of Genomic Libraries. High molecular weight DNA was prepared from FACS® (Becton Dickinson & Co., Mountain View, CA) purified cells as previously described (21). Genomic V_H rearrangements involving members of the 186.2 and V3 subfamilies and J_H4 were then amplified by 40 cycles of PCR from genomic DNA equivalent to 5 × 10^4 cells using a thermal cycler (Ericomp Inc., San Diego, CA). The primers for the amplification were as follows: 186.2/V3 5' - CAG GTC CAA CTG CAG CAG - 3' and J_H4 5' - ACG GAT CCG GTG ACT GAG GTT CCT - 3'. The temperature profile of the amplification was 94°C for 1.5 min and 72°C for 2.5 min. The subsequent sequence analysis showed that only 186.2/V3 subfamily members were amplified under these conditions. The PCR product was then digested with PstI and BamHI, gel purified, and cloned into the pTZ19 vector (Pharmacia Fine Chemicals). Sequences were determined directly

from plasmid DNA using the Sequenase™ kit (U.S. Biochemical Corp., Cleveland, OH).

Colony Hybridization and DNA Sequencing. To identify V_HDJ_H-containing clones, a mixed J_H probe (see reference 24) was used for colony screening. Hybridization was performed according to Winter et al. (25). For detection of V_H genes belonging to the 7183 or the J558 V_H gene family, a 220-bp EcoRI/SacI fragment of the VH81X gene (26) or a 254-bp HinfI/PstI fragment of the V186.2 gene (27) were used as probes. V_H gene sequences were obtained by direct plasmid sequencing using the Sequenase™ kit (U.S. Biochemical Corp.).

Results

Homology Plot of J558 V_H Gene Sequences. In the course of the present work, a series of J558 V_H genes were identified that either had or had not been described before. To simplify the analysis of these data, we compared all the available J558 V_H gene sequences to each other and set up a list of the J558 germline or presumptive germline genes known to date (Fig. 1). Germline genes were defined as genes that had either been directly sequenced from clones of unrearranged germline DNA or genes that had been repeatedly isolated as rearranged genes in at least two independent experiments. Such genes are marked by an asterisk in Fig. 1. Presumptive germline genes were defined as rearranged V_H genes that were isolated (either by direct mRNA sequencing or as cDNA clones) from unstimulated B lineage cells or from B cells that had been polyclonally activated (e.g., by bacterial LPS). This appears justified since resting $\mu^+\delta^+$ B cells or polyclonally activated B cells were shown to express germline V genes (28, 29). On this basis, a total of 67 J558 V_H genes from the IgH[b] allotype could be used to establish a homology plot in which the percentage of homology between individual DNA sequences was plotted as dots of decreasing size in units of 2% ranging from 100% to 82% (Fig. 1; see also Dildrop [30]). The sequences were arranged in such a way that sequences with the highest homology are positioned next to each other. The sequence of the V186.2 germline gene (27) was arbitrarily put on top of the homology plot. As can be noticed from the triangular patterns formed in the dot matrix, the IgH[b] J558 V_H gene family harbors groups of sequences that are more closely related to each other than to neighboring sequences. In total, seven such subfamilies can be identified and are designated as the V186.2, V3, G4D11, VMU3.2, 2.9, 205.12, and V130 subfamilies, respectively. Eight V_H genes that do not fall into any of these subgroups appear in the middle of the homology plot. Although the relative size of the individual subgroups may change in the future with the identification of additional J558 V_H genes, the data suggest that the 205.12 subfamily is the largest subfamily, followed by the V186.2 and V3 subfamilies.

Preparation of cDNA Libraries from Various B Lineage Subsets. For preparation of cDNA libraries, 1–5 × 10^5 pre-B cells, mature $\mu^+\delta^{high}$ B cells, or Ly-1 B cells were isolated from CB.20 mice of various ages by FACS® as described in Materials and Methods. Part of the cells were used for reanalysis to determine the purity of the sorted cell populations. In general, the cells were 93–98% pure, except for the Ly-1
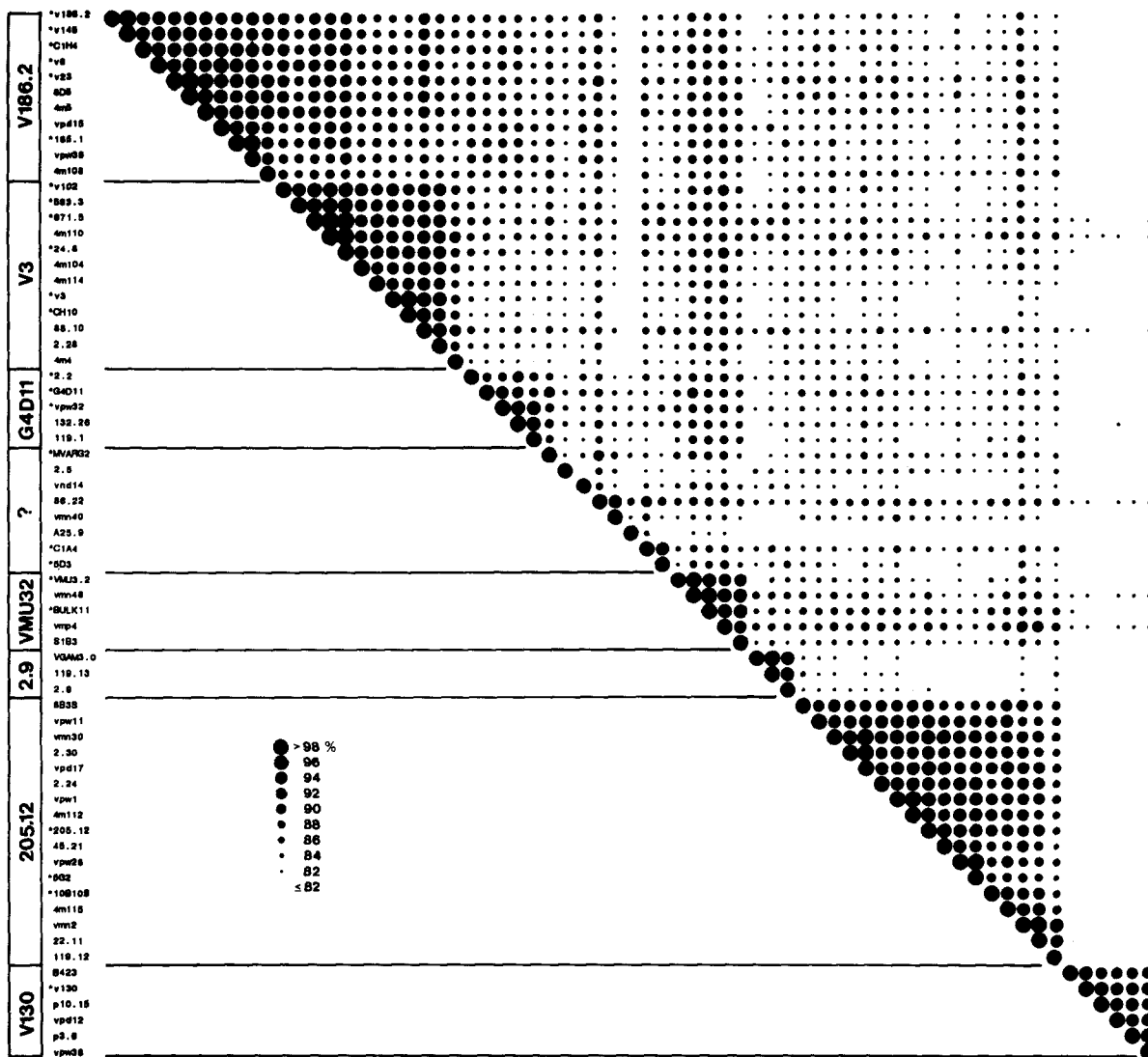
Figure 1. Homology plot of J558 V_H gene sequences. The dot matrix has been created on the basis of 67 J558 V_H gene sequences that were compared to each other at the level of their nucleotide sequences. The sequences were classified into germline genes (marked by an asterisk) and presumptive germline genes as defined in the text. In addition, three mutated genes (A 25.9, p10.15, and p3.6) were exceptionally included in the list because the corresponding germline genes would otherwise not have been represented. This can be deduced from the finding that the consensus sequence of each of these mutated genes and related genes isolated in the same experiments is clearly different from all other genes represented in the list. All genes identified in the present study were sequenced from their 3' ends up to at least position 30 according to the numbering system of Kabat et al. (53) so that all complementarity determining regions (CDRs) are included in the sequences. The sequences taken from the literature are as follows: v186.2, v145, v6, v23, v102, v3 (27); 165.1, 593.3, 671.5, 24.8, 88.10, 86.22 (36); 8B3S, 10B10S (19); 132.2, 119.1, 119.13, 22.11, 119.12, v130 (54); CH10 (20); VMU3.2, VGAM3.0 (55); 45.21 (56); p10.15, p3.6 (57); B423 (58); 8D5, 5D3, 5G2 (59); 205.12 (60); MVARG2 (61); A25.9 (62); C1H4, G4D11, C1A4, BULK11, S1B3 (R. Dildrop, unpublished data). The sequences of the V_H genes presented in this figure and the corresponding references are available via bitnet from NETSERV@EMBL:BITNET under the accession number DS6748. The list will be updated as new germline genes are identified. The computer program used to create the homology plot and the updated list of V_H gene sequences can be obtained from the authors upon request.

B cell population of 4-d-old mice, where only 75% of the sorted cells fell into the desired fluorescence window. The sorted cells were essentially free of plasma cells (see Materials and Methods). Total cellular RNA was isolated from 10^4 to 3×10^5 cells of each subset and was used for preparation of the amplified cDNA libraries. cDNA clones containing V_H genes of the J558 family were identified by hybridization to a J558-specific probe. From each library 6–15 individual clones were randomly picked and sequenced.

The sequences are depicted in Figs. 2–6. They are aligned to the V186.2 germline gene and are grouped into the seven subfamilies as defined in Fig. 1. Shown are only those codons where nucleotide differences between the respective sequences and the V186.2 gene were found. Some of the sequences differed by a single or, in one case, two nucleotides from their presumptive germline counterparts. These nucleotide exchanges probably represent artifacts due to the PCR amplification. They are marked with open circles in the figures

Figure 2. V_HDJ_H region sequences of cDNA clones derived from neonatal pre-B cells. The sequences are compared to the V186.2 germline gene (27), the Dfl16.1 D segment, and the 5' region of J_H1. Only those codons of the V186.2 V_H gene that differ from the sequences below are shown. Sequence ambiguities are indicated by an X, a dash indicates identity, and dots represent missing nucleotides. Circled nucleotides represent point mutations that were probably introduced during PCR amplification. Codons are numbered according to Kabat et al. (53). If the V_H sequences are identical to known germline genes, the names of the respective germline genes are indicated on the left.

CDR1

CDR2

CDR3

Figure 3. V_nDH_H sequences of cDNA clones derived from pre-B cells of adult bone marrow. For further description, see legend to Fig. 2.

CDR1 and CDR2

```
GLVH    cDNA         CDR1                                          CDR2
        clones
        28  30  33  34  35  37  43  44  48  49   50  52  53  54  55  56   58  60  61  65  69  71  72  74
v186.2  ACC ACC TGG ATG CAC GTG CGA GGC ATT GGA  AGG GAT AAT AGT GGT GGT  AAG AAT GAG AGC CTG GTA GAC CCC

24.8   vmp24  --- --- --- --- --- --- -A- --- --C ---  GA- --- TC- GA- A-- TA-  --C --- C-A G-- T-- --- --- T--

       vmp17  AT --A A-- -C- -AG --A --- -C- --- T-- GGA --- --- AA- T-C --- --- G-- --- -C- --A T--
       vmp5   A -AT --- --- --- GAT T-C GGA G-- --- TA- --C --- --- G-- --- -C- --- T--

BULK11 vmp14  -GT --- --- A-- --- AAG --T --- --- --- CA- T-- GGA GA- --- -A- --C -X -XA G-- --- -C- --- T--
BULK11 vmp16  A -GT --- --- A-- --- AAG --T --- --- --- CA- T-- GGA GA- --- -A- --C --C -GA G-- --- -C- --- T--
       vmp4   A -GT --- --- A-- --- AAG --T --- --- --- CA- T-- GGA GA- --- -A- -CC --C C-- GXX T-- --- --- T--
```

CDR3

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GLVH    cDNA                    Df116.1 TTTATTACTACGGTAGTAGCTAC  C TAC TGG TAC TTC GAT GTC  JH1
        clones                  Dsp2.3         TC-----T---TACGA-            AC --- --T --C TA-  JH2
v186.2  77  82  83  88  89  91  93  94  Dsp2.5  TC-----T----ACTA-            CC -GG --T -C- TA-  JH3
        ACA CTC ACA GCG GTC TAT GCA AGA DQ52    CA---GG-AC       AT --- -AT GCT A-G --C TA-  JH4

24.8   vmp24  --- --- --- --- --- --C --- ---  CGAAA--T---------------CC          T --C TA-  JH2

       vmp17  --T --- --- --T --- -TC --- ---  --C-A---------------CC       - --- --- --- ---  JH1
       vmp5   --- T-- --- --C A-- --C --T ---  TCGGGGGG-A------A-GGGGG        C --- --T --C TA-  JH2

BULK11 vmp14  --- --- --C --- --- -TC --- ---  AGGGGG-A-TACGAA                -GG --T -C- TA-  JH3
BULK11 vmp16  --- --- --C --- --- -TC --- --.  GGATA                         -- --- --- --- ---  JH1
       vmp4   --- --- --C --- --- -TC --- --.  GGATA                         -- --- --- --- ---  JH1
```

Figure 4. V_HDJ_H sequences of cDNA clones derived from bone marrow δ^+ B cells. For further description, see legend to Fig. 2.

and are not further discussed in the text. In general, the frequency of point mutations generated by PCR amplification was found to be in the order of 1 in 1,000 nucleotides, corresponding to a mutation rate of 3.3×10^{-5} per nucleotide per round of amplification (31). Throughout the analysis, V_H genes differing in at least three nucleotides from each other were classified as separate genes.

In this paper, we focus on the analysis of the V_H genes expressed in the different B lineage subsets. A detailed analysis of the V_HDJ_H joining regions (CDR3) of the same sequences with respect to N region insertion, J_H usage, and nucleotide homologies at the joining ends has been presented elsewhere (24).

Expression of J558 V_H Genes in Pre-B Cells Scatters Over All J558 Subfamilies. For all of the cDNA libraries analyzed in this study, we determined the ratio of cDNA clones containing V_H genes of either the J558 or the 7183 V_H gene family (representing the most 3' V_H gene family in the V_H gene cluster). In general, these data showed that the overrepresentation of V_H genes of the 7183 family in pre-B cells compared to peripheral B cells (see above) was also observed in our amplified cDNA libraries. Thus, in agreement with earlier work (11, 12), the libraries of the pre-B cell population isolated from newborn and adult mice contained only slightly more J558 V_H genes than V_H genes of the 7183 family (Table 1). Our main interest, however, lay in the representation of individual J558 V_H genes. To address this question, 13 cDNA clones derived from pre-B cells of 2-d-old mice and 15 clones derived from pre-B cells of adult mice were randomly picked from the clones hybridizing to the J558-specific probe and were sequenced. The sequences of the V_HDJ_H regions of these clones are depicted in Figs. 2 and 3. A schematic representation of the V_H genes identified in these clones with regard to their distribution over the J558 subfamilies is given in Fig. 7. In this figure, each V_H gene is represented by a circle that is positioned according to the location of the respective V_H gene in the homology plot shown in Fig. 1. All genes that were identified more than once within this study are designated by a particular character (A–L).

It is apparent from Figs. 2, 3, and 7 that in the pre-B cell population of both neonatal and adult mice, V_H genes of all J558 subfamilies are expressed and appear to be evenly distributed, except for a slight overrepresentation of the 205.12 subfamily. Within this subfamily we found that two genes were repeatedly expressed. The 205.12 V_H gene itself (K in Fig. 7; 32, 33) was detected twice among the 13 cDNA clones of neonatal pre-B cells and once among those of adult pre-B cells. The V_H gene 10B10S (L in Fig. 7; 19) was isolated once from both pre-B cell populations. There was no obvious differences in terms of V_H gene usage between pre-B cells of neonatal and adult mice.

Splenic $\mu^+\delta^{high}$ Cells are Roughly Selected for Expression of V_H Genes Belonging to the V186.2 and V3 Subfamilies. As shown previously, the majority of conventional B cells in the

Table 1. *Representation of J558 and 7183 V_H Family Genes in cDNA Libraries*

cDNA libraries		No. of positive colonies*		
		J558	7183	J558/7183
Pre-B cell	BM (4 mo)‡	31	16	2
	SP (4 d)	30	26	1.2
$\mu^+\delta^{hi}$ B cell	BM (4 mo)	67	10	6.7
	SP (4 mo)	234	39	6
Ly-1$^+$ B cell	SP (4 d)	120	160	0.8
	PC (4 wk)	36	35	1
Ly-1$^-$ B cell	SP (4 wk)	25	4	6

* No. of colonies hybridizing to a J558 or 7183 V_H gene family–specific probe.
‡ The various B cell subsets were isolated by FACS® from either bone marrow (BM), spleen (SP), or peritoneal cells (PC) from 4-d, 4-wk, or 4-mo-old mice.

Figure 5. $V_H D J_H$ sequences of cDNA clones derived from splenic $\mu^+ \delta^{high}$ B cells of adult mice. cDNA clones were obtained from two independent experiments as indicated on the left. For further description, see legend to Fig. 2.

Figure 6. $V_H D J_H$ sequences of cDNA clones derived from Ly-1 B cells of 4-d- and 4-wk-old mice. For further description, see legend to Fig. 2.
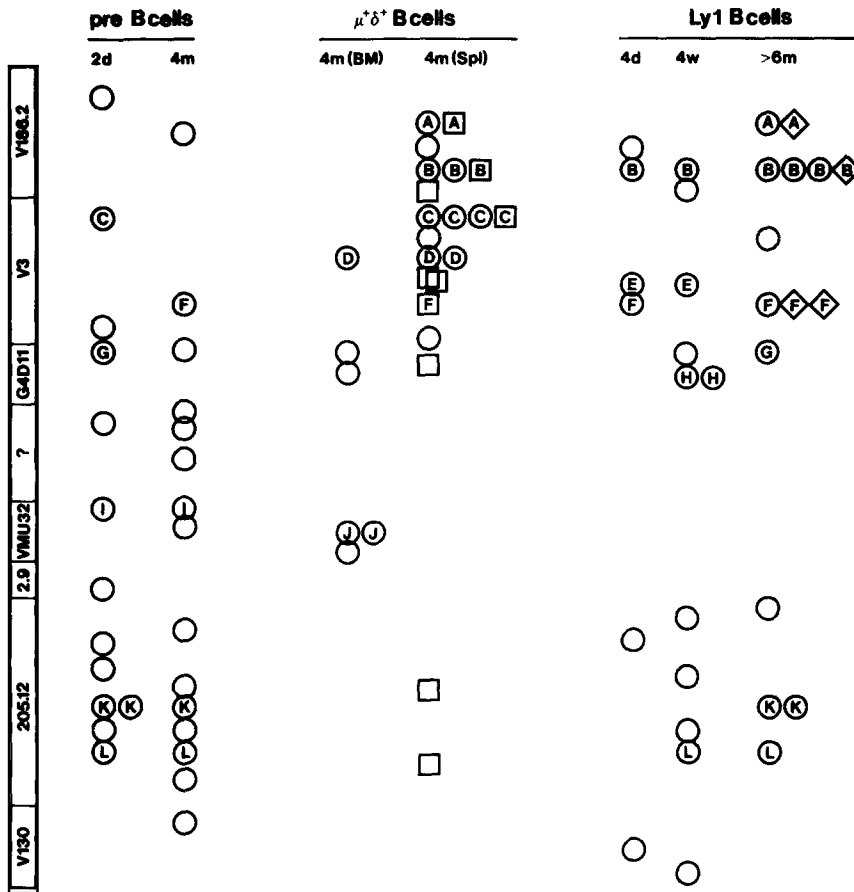
Figure 7. Schematic representation of V_H gene utilization in pre-B cells, $\mu^+\delta^+$ B cells, and Ly-1 B cells. Individual V_H genes are represented as circles, squares, and diamonds, which are positioned according to the location of the respective genes in the homology plot shown in Fig. 1. In the case of $\mu^+\delta^+$ B cells from adult spleen, V_H genes identified in Exp. 1 are represented as circles and those identified in Exp. 2 as squares. V_H gene sequences expressed in Ly 1 B cells of aged mice were taken from the literature (19, 20). In this case, circles represent V_H genes expressed in Ly-1 B cell hybridomas and diamonds represent those expressed in Ly-1 B cell lymphomas. All genes that were identified more than once within this study 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).

peripheral immune system represent long-lived, resting cells (6–10). These cells are characterized by a low to intermediate expression of sμ and high expression of sδ, and can thus be distinguished from newly generated δ^- B cells as well as Ly-1 B cells that express high amounts of sμ and low amounts of sδ (6, 34, 35). $\mu^+\delta^{high}$ B cells account for 80–90% of splenic B cells and ~30% of bone marrow B cells (35). To analyze the V_H gene utilization within this B cell subset, amplified cDNA libraries were prepared from pools of bone marrow or splenic $\mu^+\delta^{high}$ B cells derived from three mice each. In all of these libraries, about six times more V_H genes of the J558 than of the 7183 V_H gene family could be detected (Table 1), in agreement with previous findings that the representation of V_H genes in peripheral B cells roughly corresponds to the complexity of these families in the genome (13–16). From the library of bone marrow B cells, V_HDJ_H regions of six cDNA clones containing J558 V_H genes were sequenced and are shown in Fig. 4. In the case of splenic $\mu^+\delta^{high}$ B cells, cDNA libraries were prepared in independent experiments from which 11 (exp. 1) or 10 (exp. 2) clones were sequenced, respectively (Fig. 5). The V_H genes identified in these clones are schematically depicted in Fig. 7.

Inspection of the sequences reveals that the J558 V_H genes expressed in splenic B cells are largely restricted to the V186.2 and V3 subfamilies (Figs. 4 and 7). Only 10% of the sequences fall into the 205.12 subfamily, compared with 43% in the pre-B cell population. The V130, 2.9, and VMU3.2 subfamilies as well as the "unclassified" genes are not represented at all.

Furthermore, some genes belonging to the V186.2 and V3 subfamilies were found repeatedly expressed in the $\mu^+\delta^{high}$ B cell pool. Thus, the 593.3 V_H gene (C in Fig. 7; 36) was isolated from as many as 4 out of 21 cDNA clones, V_H 165.1 (B in Fig. 7; 36) was identified in three, V23 (A in Fig. 7; 27) in two, and V_H 24.8 (D in Fig. 7; 36) in another two independent cases. It is of interest that two of these genes, V23 (A) and 165.1 (B), as well as CH10 (F in Fig. 7; 20), which was identified in one of the splenic cDNA clones, are also preferentially expressed in the Ly-1 B cell population (19, 20, 37, see also below).

In the case of bone marrow $\mu^+\delta^{high}$ B cells, the limited number of cDNA clones analyzed allows only a preliminary evaluation. In one of the six cDNA clones, V_H 24.8 (D) was found, which we had also identified in two of the splenic cDNA clones. Apart from this, the cDNA clones derived from bone marrow B cells contained J558 V_H genes that were not found in any of the other populations analyzed. In two clones the same V_H gene, namely BULK11 (J in Fig. 7; R. Dildrop, unpublished data) belonging to the 2.9 subfamily was found. These data suggest that the pattern of V_H genes expressed in bone marrow $\mu^+\delta^{high}$ B cells might be different from that of splenic B cells.

The data presented in this and the previous section demonstrate that V_H gene expression in the long-lived peripheral B cell pool is highly biased and clearly differs from the broad distribution of J558 V_H genes that are rearranged in the pre-B cell population.

Biased V_H Gene Expression in Ly-1 B Cells Is Evident Already Early in Ontogeny. In earlier experiments analyzing the V gene repertoire of Ly-1 B cells, it was shown that in Ly-1 B cells that had been propagated in vivo over long periods of time V gene expression was strongly biased towards the expression of particular V_H and V_κ genes (19–21, 38). In the present study, we were interested to see whether this biased V gene expression can also be observed at early stages of ontogeny. For this purpose, amplified cDNA libraries were prepared from Ly-1 B cells derived from the spleen of 4-d-old and from the peritoneum of 4-wk-old CB.20 mice. In the case of these libraries, the ratio of J558/7183 V_H gene expression was 0.8 and 1, respectively (Table 1). This corresponds to the J558/7183 ratios found in pre-B cells and neonatal B cells but is clearly different from a ratio of 6 in conventional B cells of 4-wk-old mice (Table 1; see also below). With regard to the utilization of individual V_H genes, six J558 V_H gene-containing cDNA clones from neonatal Ly-1 B cells, as well as 11 clones from Ly-1 B cells of 4-wk-old animals, were randomly picked and sequenced as shown in Fig. 6. A schematic representation of theses V_H genes and the J558 V_H genes isolated in previous experiments from Ly-1 B cell hybridomas and lymphomas is given in Fig. 7 (V_H genes expressed in the lymphomas are shown as diamonds).

The V_H genes isolated from all of the different Ly-1 B cell populations fall into two clusters, one comprising the V186.2, V3, and G4D11 subfamilies, and the other the 205.12 and V130 subfamilies. In none of the populations were V_H genes belonging to the 2.9 and VMU3.2 subgroups nor any of the unclassified genes found. This corresponds to what had been seen in the splenic $\mu^+\delta^{high}$ B cell population (Fig. 7). Likewise, some of the genes that were found preferentially expressed in the conventional, $\mu^+\delta^{high}$ B cell population were also repeatedly isolated from Ly-1 B cells at various stages of ontogeny. This is true for V23 (*A*), CH10 (*F*), and, especially, 165.1 (*B*), which was found three times in the conventional and six times in the Ly-1 B cell population (Fig. 7).
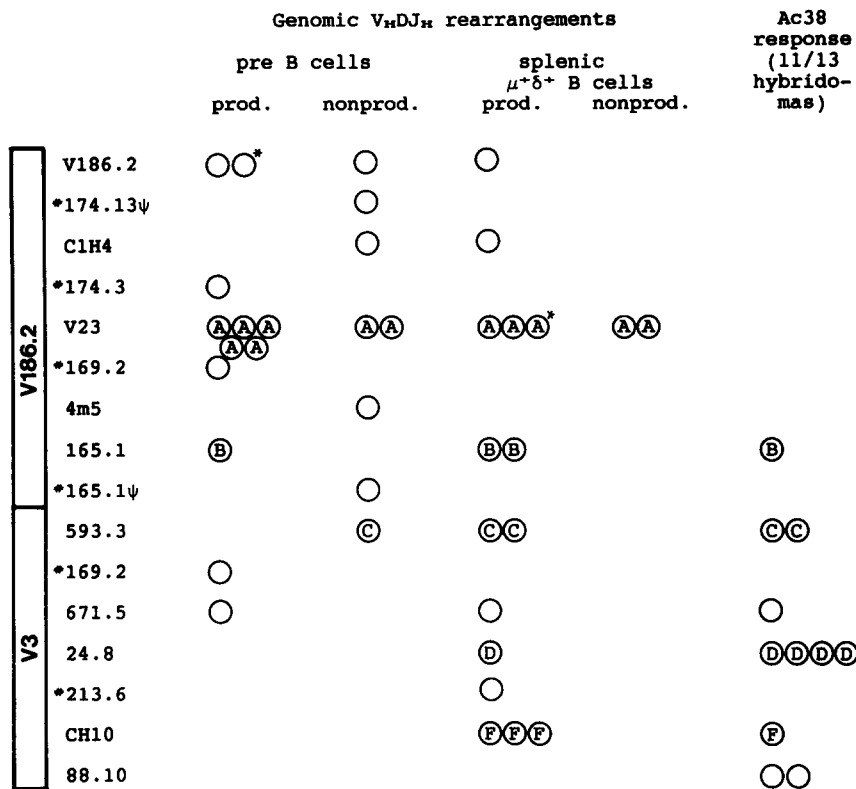
In contrast to the similarity of the conventional and Ly-1 B cell populations with respect to the preferential expression of V_H genes belonging to the V186.2 and V3 subfamilies, Ly-1 B cells differ from $\mu^+\delta^{high}$ B cells in the expression of the V_H genes belonging to the 205.12 and V130 subfamilies. Thus, similar to what had been found in the pre-B cell population, the 205.12 V_H gene itself (*K*) as well as the V_H gene 10B10S (*L*) were repeatedly isolated from Ly-1 (but not conventional) B cells at different stages of ontogeny (Fig. 7). Together with the finding that, at least in 4-wk-old mice, the ratio of J558/7183 V_H gene expression is much lower in Ly-1 B cells compared with conventional B cells (Table 1), these data imply that some of the cells expressing V region genes that are preferentially expressed in pre-B cells are maintained in the Ly-1 B cell population at high frequency, whereas they are underrepresented in the conventional, $\mu^+\delta^{high}$ B cell pool.

The Biased V_H Gene Usage of $\mu^+\delta^{high}$ B Cells Can Also Be Demonstrated Using Genomic Libraries. The data presented so far indicate that certain J558 V_H genes belonging to the V3 and V186.2 subfamilies are predominantly expressed in both splenic $\mu^+\delta^{high}$ B cells and Ly-1 B cells. This bias could either result from cellular selection within the peripheral B cell pool or reflect high transcriptional activity of these particular V_H genes compared with other J558 V_H genes. To distinguish between these two possibilities, we set up a genomic amplification protocol that allowed us to compare V_H gene utilization in the different B cell subsets at the level of DNA instead of mRNA. For this purpose, splenic $\mu^+\delta^{high}$ B cells and bone marrow pre-B cells were isolated by FACS®, and genomic DNA obtained from these cells was amplified using a J_H primer at the 3' end and a primer specific for J558 V_H genes of the V3 and V186.2 subfamilies at the 5' end. In this way, we were able to amplify at least 22 different J558 V_H genes belonging to the V3 and V186.2 subfamilies. If the biased V_H gene expression seen in splenic $\mu^+\delta^{high}$ B cells is due to cellular selection, then the same bias should also be noticed at the level of genomic $V_H DJ_H$ rearrangements. Indeed, out of a total of 17 clones analyzed from a genomic library of splenic $\mu^+\delta^{high}$ B cells, 13 contained a V_H gene of the group of five genes (*A*, *B*, *C*, *D* and *F* in Fig. 7) that had been most frequently isolated from the cDNA libraries of mature B cells (Fig. 8). In contrast, a diverse set of V_H genes belonging to the V186.2 and V3 subfamilies was identified among the genomic $V_H DJ_H$ rearrangements of pre-B cells, except for a striking overrepresentation of the V23 gene (*A* in Fig. 8). Since this gene was highly overrepresented in both productive and nonproductive rearrangements of pre-B as well as B cells, it appears to be preferentially rearranged compared with other V_H genes of the V186.2 and V3 subfamilies or, alternatively, present in multiple copies in the germline.

Discussion

The V_H Repertoire of B Lineage Cells In Vivo Can Be Efficiently and Reliably Analyzed Using PCR Technology. In this paper, we present an analysis of the V_H gene repertoire of various B lineage subsets in mice, looking at the representation of individual V_H genes. With the help of the PCR, it was possible to directly isolate $V_H DJ_H$ rearrangements from small numbers of B lineage cells without the requirement of cellular selection through in vitro activation or cell fusion. However, it is conceivable that in spite of the use of constant region primers the amplification procedure itself leads to a bias in V_H gene representation, if the different V_H genes would be amplified and detected with varying efficiency. The result of our analysis makes this possibility unlikely for the following reasons: (*a*) we repeatedly isolated a broad spectrum of J558 V_H genes from pre-B cells showing that V_H segments from any point on the homology plot could be amplified and detected; (*b*) similar results were obtained using cDNA libraries and genomic libraries; (*c*) the ratio of each tissue type of rearranged J558 to 7183 V_H segments that we observed was in excellent agreement with the values obtained by other groups without the use of PCR; and (*d*) V_H genes

Genomic V_H DJ_H rearrangements

		pre B cells		splenic $\mu^+\delta^+$ B cells		Ac38 response (11/13 hybrido-mas)
		prod.	nonprod.	prod.	nonprod.	
V186.2	V186.2	OO*	O	O		
	*174.13ψ		O			
	C1H4		O	O		
	*174.3	O				
	V23	Ⓐ Ⓐ Ⓐ Ⓐ Ⓐ	Ⓐ Ⓐ	Ⓐ Ⓐ Ⓐ*	Ⓐ Ⓐ	
	*169.2	Ⓐ Ⓐ O				
	4m5		O			
	165.1	Ⓑ		Ⓑ Ⓑ		Ⓑ
	*165.1ψ		O			
V3	593.3		Ⓒ	Ⓒ Ⓒ		Ⓒ Ⓒ
	*169.2	O				
	671.5	O		O		O
	24.8			Ⓓ		Ⓓ Ⓓ Ⓓ Ⓓ
	*213.6			O		
	CH10			Ⓕ Ⓕ Ⓕ		Ⓕ
	88.10					OO

Figure 8. Frequency of J558 V_H genes belonging to the V186.2 and V3 subfamilies in genomic V_H DJ_H rearrangements of pre-B cells and splenic $\mu^+\delta^+$ B cells (*left*) and in the Ac38 response (*right*). In the case of genomic V_H DJ_H rearrangements, productively and nonproducctively rearranged V_H genes are listed separately. Pseudogenes are marked (ψ). Genes that had already been repeatedly isolated in the cDNA libraries are designated by the same characters as in Fig. 7. V_H genes that are not included in the homology plot (Fig. 1) are marked (#). (*) The respective genes differed in two nucleotides from their presumptive germline counterparts. In the case of the Ac38 response (36), the productively rearranged V_H genes in 11 out of a total of 13 hybridomas analyzed are schematically depicted. The other two hybridomas expressed J558 V_H genes that did not belong to the V186.2 or V3 subfamily.

identical to the ones preferentially isolated from the amplified libraries of mature B cells had also been repeatedly isolated from B cell hybridomas or lymphomas by direct mRNA sequencing or use of conventional cDNA cloning (19, 20, 36; below; see also Fig. 7). For these reasons, we consider the present cloning technique an efficient and reliable way of studying the V gene repertoire of B lineage cells.

The Germline of the Mouse Contains ∼100 Functional J558 V_H Genes. Previous estimates on the germline of the mouse were either in the range of 100–200 genes (23, 39) or in the order of 1,000 genes (40). In mice of the IgH^b haplotype, approximately half of all V_H genes belong to the J558 V_H gene family (23). The list of 67 different J558 V_H genes shown in Fig. 1 was established on the basis of the sequences obtained from the amplified cDNA libraries and of sequences that had been described previously in the literature. The sequences derived from the genomic libraries (Fig. 8) were obtained independently of the sequences used to establish the homology plot. Looking at the V_H genes isolated from the genomic libraries, it is therefore possible to estimate the number of functional J558 V_H genes in the germline by comparing the fraction of known genes (i.e., genes already contained in the list) to that of unknown genes. Such a calculation is only valid if the respective V_H genes are isolated from unselected V_H DJ_H rearrangements, which we consider to be true in a first approximation for the sequences derived from the pre-B cell library. In this set of sequences, 3 of 12 different functional J558 V_H genes were not contained in the J558 V_H gene list. This suggests that ∼25% of the func-

tional J558 V_H genes are not represented in the present list and that the actual size of the J558 V_H gene family in the IgH^b haplotype, excluding pseudogenes, is in the order of 100 genes. It cannot be excluded that the J558 family contains additional genes that are rearranged at a much lower frequency. However, it seems more likely that the estimate of Livant et al. (40) includes V_H gene–related nonfunctional sequences and/or points to a difference between the IgH^b and the IgH^a haplotype.

$\mu^+\delta^{high}$ Peripheral B Cells as well as Ly-1 B Cells Express a Strongly Selected V_H Gene Repertoire. Previous studies on the V_H gene repertoire of B lineage cells have shown a random representation of the different V_H gene families in peripheral B cells of adult mice compared with preferential expression of V_H gene families located of the 3' end of the V_H gene locus in pre-B cells and neonatal B cells (11, 12). This is in line with data from the present study, in which we also observed a lower ratio of J558 to 7183 V_H genes in pre-B cells (J558/7183, ≤2) compared with mature, splenic B cells (J558/7183, ≥6) (Table 1). The only indication for a shift in V_H gene family usage in long-lived compared with short-lived peripheral B cells was given by Freitas et al. (11), who reported that B cells enriched for long-lived cells by treatment with hydroxyurea in vivo almost exclusively expressed V_H genes belonging to the J558 V_H gene family. As a general point, the result of the present analysis shows that studies on the V gene repertoire of B lineage cells based on the utilization of V_H gene families only can be misleading in the sense that random family usage may not correspond to random

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usage of V_H genes. Thus, in spite of a "random" utilization of V_H gene families in mature, peripheral B cells, V_H gene expression in this B cell population appears to be highly biased towards the expression of a limited set of V_H genes (Fig. 7). Pre-B cells, on the other hand, show a more diverse representation of J558 V_H genes, with the exception of the V23 and the 205.12 genes, which appear to be overrepresented at this stage of B cell development (see below and Figs. 7 and 8).

Comparing the cellular selection seen in the conventional B cell population to that in the Ly-1 B cell subset, both similarities and differences emerge. Thus, some of the V_H genes predominantly expressed in the Ly-1 B cell subset are also preferentially expressed in long-lived conventional cells. This is in contrast to our previous expectation that the predominant expression of particular V_H genes is typical of Ly-1 B cells only (19). On the other hand, V_H genes of the 205.12 subfamily are more frequent in the Ly-1 B cell population than in the $\mu^+\delta^{high}$ B cell pool. In addition, it has been demonstrated by several groups that V_H genes of the V_H11 and V_H12 families encoding antibody specificities against bromelain-treated red blood cells are almost exclusively expressed in Ly-1 B cells (22, 41, 42). The same appears to be true for $V_H DJ_H$ rearrangements encoding antibodies with antiphosphorylcholine specificity bearing the T15 idiotype (43). It should also be noted that selective expression of particular J558 V_H genes can already be observed in Ly-1 B cells of 4-d-old mice, at a time when the stable pool of conventional B cells has not yet been built up (see reference 6). Taken together, these findings imply that Ly-1 B cells and conventional B cells are both selected for the expression of particular V region genes, but that this selection may vary with different organ location, activation requirements, and growth properties of the cells.

Ligand-dependent vs. B Cell Autonomous Selection in the Generation of the Peripheral V_H Gene Repertoire. In principle, two different mechanisms can influence V_H gene usage in B lineage cells. One is B cell autonomous overexpression of certain V_H genes as a consequence of either preferential rearrangement or of the presence of multiple copies of a particular gene in the germline; the other is receptor-mediated selection of B lineage cells that already possess functional $V_H DJ_H$ rearrangements. B cell autonomous overexpression is likely to account for the predominant expression of D-proximal V_H genes in the pre-B cell population. Similarly, the overrepresentation of the V23 gene in both productive and nonproductive rearrangements indicates that this gene is either preferentially rearranged or present in multiple copies in the genome. The fact that V23 was not among the five V186 and V3 subfamily members observed in the two pre-B cell cDNA libraries is somewhat puzzling, given its apparent overrepresentation at the genomic level. We do not have an explanation for this, but it could possibly be due to the fluctuations inherent in small sample sizes. In the case of the 205.12 gene, B cell autonomous overexpression is likely but has not been formally demonstrated since we did not analyze the frequency of nonproductive rearrangements of this gene. On the other hand,

the majority of the V_H genes dominantly expressed in splenic $\mu^+\delta^{high}$ B cells were not found to be overrepresented at the pre-B cell level. The data therefore indicate that peripheral B cells expressing these genes are selected on the basis of their antigen receptor V region.

A clue to the mechanism of this ligand-dependent selection comes from an experiment in which the V_H gene usage of B cells involved in an immune response against a particular antiidiotypic antibody called Ac38 was studied (36). The special feature of this immune response is that it is restricted to B cells bearing λ light chains but appears to be largely independent of the V_H gene used (32). Significantly, the V_H genes expressed in hybridomas isolated from this immune response show the same pattern of selection as observed in the splenic B cells analyzed in the present study. Thus, out of 13 clonally independent hybridomas analyzed, one expressed $V_H165.1$ (B), two $V_H593.3$ (C), four $V_H24.8$ (D), and one $V_H CH10$ (F) (Fig. 8), indicating that the V_H gene usage in the Ac38 response reflects the overall V_H gene usage in the peripheral B cell population. Since 95% of peripheral B cells, including $\mu^+\delta^{high}$ cells, express κ and not λ light chains, this finding implies that the selection of B cells expressing these particular V_H genes is independent of the light chain used.

On the basis of this result, one may speculate that the selection of the cells occurs already at the pre-B cell stage when the heavy chain first comes to the surface in association with a surrogate light chain (44, 45). However, since in our analysis the V_H gene repertoire of pre-B cells was clearly different from that of the peripheral B cells, we consider this possibility unlikely. We propose that selection takes place at the stage of newly generated B cells during the process of entry into the stable peripheral B cell pool. If this is true, ligands responsible for the selection of the newly generated B cells must bind to the heavy chain V region independently of the light chain. There is evidence in the literature that certain antigen binding specificities can be assigned to individual V_H or V_L domains (46–48), a principle that may be of key importance for the evolutionary selection of V_H and V_L genes in the germline. In the case of TCRs, so-called superantigens have been described that bind to a particular V_β region independent of the V_α chain (49). The ligands responsible for selection of B cells into the peripheral B cell pool could act in a comparable way. The similarity between the Ac38 response (which was obtained in C57BL/6 mice) and the present data (obtained in the IgH^b congenic strain CB.20) demonstrates in addition that this selection is not influenced by polymorphism at the MHC. We want to point out, however, that light chain–independent B cell selection cannot operate exclusively since clear cases of selection of certain V_H-V_L combinations have been observed in the Ly-1 B cell compartment (19, 20).

What Function Do the Selected B Cells Fulfil in the Immune System and What Is the Nature of the Selecting Ligands? The question on the nature of the selecting ligands is connected to the question whether selection acts in a positive or negative way. Are the selected B cells able to participate in an immune response or are they perhaps functionally inactivated

similar to the anergic B cells described by Goodnow et al. (50)? The immune response against Ac38 described above strongly indicates that the cells are functionally active. We consider it unlikely that the selected cells represent the survivors of a negative selection process since this would mean that the majority of V_H genes in the germline encode for autoreactive specificities that would usually be depleted from the pool of newly generated B cells and therefore could not participate in an immune response. Thus, the selective V_H gene expression seen in the peripheral B cell pool is in all likelihood the result of a positive selection process that could either be mediated through recognition of foreign antigens or of internal ligands such as antiidiotypic antibodies (51). If the selection is induced by foreign antigens, it is well possible that in environments different from our animal facility other V_H genes than the ones predominantly expressed in the peripheral B cells of our mice would be selected. Current experiments with mice from other sources aim at clarifying this point.

Positive selection as described above differs from selection of classical memory B cells in the course of T cell–dependent immune responses since the splenic $\mu^+\delta^{high}$ B cells do not express somatically mutated antibody V regions and have not undergone Ig class switching. MacLennan and Gray (52) have speculated that in the initial phase of a T cell–dependent immune response naive B cells are recruited into the long-lived B cell pool through recognition of antigen presented on interdigitating cells in extrafollicular areas of secondary lymphoid organs. Alternatively, peripheral B cells could be selected independently of an ongoing immune response in a T cell–independent fashion. Whatever the way of positive selection is, the long-lived $\mu^+\delta^{high}$ B cells in adult mice probably do not represent naive B cells but rather B cells that have already been selected on the basis of their antibody V region. We therefore conclude that two different types of "memory B cells" are present in the mouse: one type are classical memory B cells, selected for high affinity binding in T cell–dependent immune responses and representing only a minor fraction of the total B cell pool. The other are long-lived $\mu^+\delta^{high}$ B cells that express germline-encoded specificities and account for the bulk of the peripheral B cell pool. Antigens present in the animal in low concentration and/or in a T cell–independent form may select these latter cells.

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