Variable Region Gene Analysis of B Cell Subsets Derived from a 4-year-old Child: Somatically Mutated Memory B Cells Accumulate in the Peripheral Blood Already at Young Age

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Summary

Tonsillar germinal center and immunoglobulin M+ (IgM+)IgD+ B cells as well as peripheral blood (PB) CD5+ and CD5− (conventional) B cells from a 4-yr-old child were isolated and nucleotide sequences of expressed Ig heavy chain variable regions encoded by V.4 gene family members were determined from amplified cDNA. Whereas both tonsillar IgM+IgD+ cells and the majority of IgM-expressing CD5+ and CD5− PB B cells showed no or little somatic mutation, tonsillar germinal center (GC) B cells and IgG-expressing PB B cells carried a high load of somatic mutations in their V region genes. This suggests that somatically mutated memory B cells which have switched isotype accumulate in the PB already at young age. Their frequency seems to increase with age. On the other hand, the antibody repertoire of tonsillar IgM+IgD+ B cells and the majority of IgM-expressing PB B cells is determined by germline-encoded specificities and by generation of variability in the complementary determining region III through VH-D,-J recombination. A fraction of IgM-bearing PB B cells carries somatically mutated V region genes and probably represents GC-derived B cells which have left the GC at an early stage of the GC reaction without undergoing isotype switching. 10 V.4 germline genes were found to be expressed. Three gene segments were overrepresented in the sequence collection (35 of 50 clones): V.4.21 (30%), V71-4 (20%), and 3D279D (20%). It appears that most potentially functional V.4 germline genes are expressed in peripheral B cells. Some members of this V. gene family are clearly overrepresented over others.

Immunoglobulin heavy chain V region genes expressed by B lymphocytes are generated by assembling Vn, Dn/Dn, irregular recombination signal (DIR), and Jn gene segments during B cell development in the bone marrow (1, 2). The descendants of newly formed B cells in the bone marrow, IgM+IgD+ “naive” B cells, mostly express unmutated V region genes (3, 4) and are considered precursors of germinal center B cells (GCC)1 that participate in T cell–dependent immune responses to exogenous antigens (5, 6). In the course of a germinal center (GC) reaction, B cells hypermutate their V region genes (7–9). Mutants that exhibit a higher affinity to their target antigen are selected (8, 10) and are subsequently released into the periphery as long-lived memory B cells most of which have switched isotype (11).

Over the last years, many studies in humans focused on rearranged V region genes in malignant and autoimmune B cells. Only recently has the normal B cell repertoire become a subject of investigation. The analysis of secondary immune responses to defined antigens showed that IgG-expressing (memory) B cells carry somatically mutared V region genes (12, 13). Also, the majority of isotype-switched B cells derived from the peripheral blood (PB) of adults show a high level of somatic mutation (4, 14), mirroring the situation in the mouse spleen (15).

At the time of birth, IgM-bearing B cells from cord blood carry virtually unmutated V region genes (16, 17). In contrast, a portion of IgM-expressing PB B cells derived from adults express mutared V region genes (16, 18, 19). These cells most likely represent IgM+IgD− B cells which constitute a minor fraction of IgM-expressing PB B cells (4). Results obtained by Ebeling et al. (20) suggest that the vast majority of tonsillar B lymphocytes express somatically mutated antibodies. However, mantle zone B cells (which surround GCs and most of which are IgD positive) derived from lymph nodes carry V regions that show no or little somatic mutation (9).

Most of the previous studies focused on the analysis of only a single B cell subpopulation and were performed on tissue or blood samples derived from donors of various ages. There-
fore, we sought to characterize B cell subsets differing in terms of cellular maturation derived from a single individual at the level of rearranged V genes. This allowed us to compare the extent of somatic hypermutation in V region genes as well as V gene usage between subpopulations. The analysis comprised naive IgM+IgD+ B cells, GCC, and IgG- (memory) as well as IgM-expressing PB B cells. PB B cells were separated into conventional (CD5- and CD5+ B cells, the latter representing the majority (about 70%) of PB lymphocytes in young children, their percentage declining in the course of life (20-30% in the adult) (21).

As an additional point, we investigated whether GCC and GC-derived (memory) B cell subsets derived from a child already show the characteristics of the same subsets in the adult, namely a high load of somatic mutations in their rearranged V genes. Results obtained by Mortari et al. (22) suggest that the hypermutation mechanism is already active at birth. The analysis concentrated on rearranged V region genes of the V 4.4 gene family (23) that has been extensively characterized in recent years (24-30) and seems to consist of about 10-12 genes/haploid genome (26, 29, 30). Members of the V 4.4 gene family have been found expressed in both mutated and unmutated form. V 4.4 gene segments were found to be rearranged in various B cell subsets. Overall, the V 4.4 family seems to be suitable to analyze B cell subsets for somatic mutation.

Materials and Methods

Cell Preparation. Tonsils obtained from a routine tonsillectomy of a 4-yr-old child and PB from the same patient were provided by the Hals-Nasen-Ohren-Klinik of the Cologne University Hospital. The child suffered from recurrent tonsillitis and was otherwise healthy. Tonsillar cell suspensions were obtained by mincing the tissue in ice-cold culture medium (RPMI-1640; GIBCO BRL, Düsseldorf, Germany) and pressing it through a stainless steel sieve. Mononuclear cells were isolated by Ficoll (Histopaque 1077; Sigma, Deisenhofen, Germany) gradient centrifugation performed at 4°C. PBMC were isolated by Ficoll gradient centrifugation of heparinized blood at room temperature.

mAbs and Lectins. The following mAbs were used: anti-CD20 (Leu16) (IgG1), either as FITC or as PE conjugate, and biotinylated anti-CD5 (Leu1) (IgG2a), obtained from Becton Dickinson & Co. (Mountain View, CA). Anti-human IgD (IADB6) (IgG2a) (Southern Biotechnology Associates, Birmingham, AL) was developed in a second staining step followed, after washing, by incubation with anti-mouse-IgG2a/b. The lectin peanut agglutinin (PNA) was purchased as a FITC conjugate from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-PE was from Becton Dickinson & Co. (Mountain View, CA). Anti-CD3 microbeads (Leu4) (IgG1), anti-CD14 microbeads (LeuM3) (IgG2a), and anti-mouse-IgG2a/b microbeads (rat IgG1) were obtained from Miltenyi Biotec. The lectin peanut agglutinin (PNA) was purchased as a FITC conjugate from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-PE was from Becton Dickinson & Co.

Cell Separation. GCC and IgD+ B cells were enriched by magnetic cell separation using the MACS system (Miltenyi Biotec) (31). To avoid apoptosis of GCC (6), the cells were kept at 4°C throughout the separation. 2 x 10^6 tonsillar mononuclear cells were incubated with anti-CD3 microbeads, anti-CD14 microbeads, and anti-IgD for 20 min. After washing with P/B/A (PBS, 1% BSA, 0.02% sodium azide), the cells were incubated with anti-mouse IgG2a/b microbeads for 20 min. After washing and resuspension in 4 ml P/B/A, the cell suspension was put on top of a separation column (C-column; Miltenyi Biotec). All elutions were performed with P/B/A. The cells that did not bind to the column were eluted with a flow rate of ~6 ml/min ("flow fraction"). The cells that bound to the column were eluted outside the magnetic field with a high flow rate ("microbeads fraction"). The flow fraction was incubated with anti-CD20-PE and PNA-FITC. The B cell-specific anti-CD20 antibody (Leu16) divides tonsillar B cells into bright and dim populations, thereby distinguishing between GCC (CD20+bright [CD20++]) and non-GCC (representing mantle zone and interfollicular B cells, CD20-dim [CD20+]) (32). The lectin PNA stains GCC more brightly than non-GCC (33). Fig. 1 (top left) shows that the CD20++ cells were also stained by PNA, further supporting their GC origin. The microbeads fraction was incubated with anti-IgD and anti-CD20-PE in a first staining step followed, after washing, by incubation with anti-mouse-IgG2a/b. Fig. 1 (bottom left) shows that the majority of non-GCC (CD20+) expressed IgD, whereas GCC (CD20++) were IgD-negative. GCC (PNA+/CD20++) and IgD+ B cells (IgD+/CD20+) were enriched on a FACS® (model 440; Becton Dickinson & Co.) to 99 and 97% purity, respectively (Fig. 1).

PBMC were incubated with anti-CD20-FITC and biotinylated anti-CD5 followed, after washing, by incubation with Streptavidin-PE. All stainings were performed for 15 min on ice. CD5+/CD20+ and CD5-/CD20+ (conventional) B cells were enriched on a FACS® 440 to 73 and 79% purity, respectively (Fig. 2). Approximately 70% of the B cells expressed the CD5 antigen (Fig. 2, left). This is in accordance with the observation that the percentage of CD5-positive B cells among CD20+ B cells decreases from about 70% in the newborn to about 25% in the adult (21).

In all sortings, selective gates were set to exclude propidium iodide-stained dead cells and nonlymphocytes using forward and side...
RNA Isolation, Cloning, and Sequencing of PCR-amplified Material. Total cellular RNA was isolated from 3 × 10⁶ cells of each the PNA+/CD20++ and IgD+/CD20+ fractions and from 5 × 10⁶ cells of each the CD5+/CD20+ and the CD5−/CD20− fractions as described (34). For first strand cDNA synthesis, total cellular RNA of each fraction was hybridized to a primer specific for the first exon of the μ chain constant region (CR) (Cμa: 5′-GAG-GGCACCTGCAATC-3′) and in the case of the PNA+/CD20++ and CD5+/CD20+ fractions, also to a primer specific for a sequence in the first exon of all four γ chain CRs (Cγa: 5′-GACCTTGCACGGGCAGGCCAG-3′) and extended with SuperScript MMLV reverse transcriptase (GIBCO BRL). One twentieth of each the PNA+/CD20++ and IgD+/CD20+ and one fifth of each the CD5+/CD20+ and CD5−/CD20− first strand cDNA mixture was amplified in separate PCR reactions using a Cμ-primer specific for a sequence in the first exon of the μ chain CR (Cμb: 5′-gggaATTCGACAGGAGACGAGGGAA-3′) and a 5′ primer specific for a sequence in framework region I (FRI) of either V,3 or V,4 (9). The V,4FRI-primer exhibits 100% homology to the corresponding sequence in nearly all V,4 germline genes published to date. Only to some it shows a single basepair mismatch in the middle of the sequence which should not prevent successful amplification. One twentieth of the PNA+/CD20++ and one fifth of the CD5+/CD20+ CDNA mixture was amplified in separate PCR reactions using a Cγ-primer specific for a sequence in the first exon of the μ chain CR (Cγb: 5′-ggaTGACCTCGGAAGGAGACGAGGGAA-3′) and a 5′ primer specific for a sequence in framework region I (FRI) of V,4 (9). Genomic DNA from 3 × 10⁶ cells of each the IgD+/CD20+ fraction was isolated according to standard procedures (35) and one tenth of the mixture was PCR amplified with a J, primer (J,9) and V, primers recognizing a sequence in the FRI of either V,3 or V,4 (4). The V,4FRI, Cγb, the J, primers contained SalI, the Cγb, V,4FRI, and V,4FRI primers EcoRI restriction sites for cloning (underlined). PCR amplification was performed in 50-μl reaction mixtures containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTP, 0.125 μM of each primer, and 2.5 U Taq Polymerase (GIBCO BRL), and consisted of 40 cycles of 90 s at 95°C, 30 s at 65°C (65°C for the C, amplification), and 80 s at 72°C. Taq polymerase was added after the first denaturation step. All amplifications were carried out in a Trio Thermoblock (Biometra Biomed. Analytik GmbH, Goettingen, Germany). Amplified cDNA was cloned into pTZ19U by standard methods. After transformation of Escherichia coli DH5α, plasmids were isolated using the Qiaquick Plasmid-Midi-Kit (Qiagen, Hilden, Germany). All inserts were sequenced from both sides by the dideoxynucleotide termination method with T7 DNA polymerase (Pharmacia, Freiburg, Germany) using the PCR primers described above. Sequences were analyzed with DNASIS™ software (Pharmacia).

Results

Cloning and Sequencing of V,4 Transcripts. The characterization and sequencing of the B cell subsets analyzed were described in Materials and Methods and are depicted in Figs. 1 and 2. The insert of 51 individual bacterial colonies were sequenced in the V,4 gene analysis. One insert (IGD7) was identified as a hybrid most likely representing a PCR artifact. The clones were designated according to the B cell subsets from which they were derived, and in the case of the GCC and conventional (CD5−) B cells, also according to the immunoglobulin isotype expressed by the cells. They are: IGD1-10, derived from tonsillar IgD− B cells, μ-expressing; GC1-8 and GCG1-8, derived from tonsillar GCC, μ- and γ-expressing, respectively; CD5/1-8, derived from CD5+ PB B cells, μ-expressing, and CBM1-8 and CBG1-9, derived from conventional (CD5−) PB B cells, μ- and γ-expressing, respectively (Table I).

Assignment of V,4 and J, Sequences to Germline Genes. V,4 sequences of 50 inserts could be assigned to one of eight published V,4 germline genes, namely either V,4.21 (24), V,4.32 (25), V,4.33 (25), V,4.34 (25), 3D216D (30), V,4.21 (24), 3D216D (30), V,4.33 (25), V,4.34 (25), 3D216D (37), or VIV-4 (38) (Fig. 3). Some of the sequences were more homologous to one another than to published germline genes and therefore most likely correspond to novel genes or alleles (pV,4D279D and V,4.335; see below). Also, the V,4 sequence of clone CD5/4 might correspond to a previously unidentified V,4 gene (see below).

The V,4.21, V,71-4, V,2-1, and 3D279D genes or their corresponding alleles to which the majority of V,4 sequences obtained in the present analysis could be assigned have been identified repeatedly in several analyses, showing either no or little sequence divergence (23–30, 36, 38, 39), and also in unmaturated form in a large number of antibodies, as a search in the EMBL (R.35.0) and GenBank (R.77.0) database libraries revealed. These gene segments appear to be highly conserved in the population. Correspondingly, the nucleotide differences of the V,4 sequences which could be assigned to V,4.21, V,71-4, V,2-1, or 3D279D most likely represent somatic mutations. VIV-4, 3D216D, V,4.33, and V,4.34 have so far only rarely been identified in VH4 germ-line gene analyses or in rearranged V,4 genes (25–30, 37, 38, 40, 41). To test whether nucleotide differences in V,4 sequences which were mostly homologous to one of the rarely identified V,4 germline genes affect the analysis for somatic mutation, mutation frequencies were determined in two ways: (a) by considering only V,4 sequences that could be assigned to either V,4.21, V,71-4, V,2-1, or 3D279D; and (b) by con-
Five of the six Jα germline genes (42, 43) were found to be expressed (Table 1). Because of the low level of Jα gene polymorphism (43) it was possible to unambiguously identify somatic mutations in the rearranged Jα gene segments.

**Vα and Jα Gene Usage.** Fig. 3 shows the 50 Vα4 sequences obtained from the tonsillar and PB B cell subsets.

The nucleotide differences to the corresponding germline genes are depicted in Table 1. 15 Vα4 sequences could be assigned to Vα4.21, 11 to V71-4, 10 to 3D279D, 4 to V2-1, 4 to Vα4.33, 2 to Vα4.34, 2 to 3D216D, and 2 to VIV4 (Fig. 3, a–h). The Vα4 sequence of clone CD5/4 which differed by 19 nucleotides from its closest homologue, V71-4, but showed no mutations in the rearranged Jα5 gene, might correspond to a novel Vα4 germline gene on the assumption that a highly mutated V region is likely to harbor point mutations in rearranged Vα as well as Jα gene segments.

Five sequences showed an identical A to G nucleotide exchange at position 3 in codon 69 of 3D279D (Fig. 3 c). This shared nucleotide difference presumably represents a Vα4 gene sequence polymorphism. Accordingly, the Vα4 sequence of CD5/1 which showed no additional nucleotide differences to 3D279D represents a putative novel allele of this gene segment (henceforth designated as [polymorphic] p3D279D; Table 1). The Vα4 sequences of clones CBG2 and GCG6 harbored five identical nucleotide exchanges in codons 53, 67, 73, 74, and 83 of Vα4.33, respectively (Fig. 3 c). Since it is unlikely that these common nucleotide exchanges were introduced by somatic hypermutation, the Vα4 gene segment of CBG2 and GCG6 presumably represents either a novel allele of Vα4.33 or a previously undescribed Vα4 germ-line gene that is closely related to Vα4.33 (henceforth designated as Vα4.33r [related]; Table 1).

Considering p3D279D as an allelic form of 3D279D and the Vα4.33-related sequence as well as the Vα4 sequence of clone CD5/4 as novel genes or duplicates, the number of Vα4 germline genes expressed in peripheral B cells of the present individual comes close to the estimated 10–12 Vα4 germline genes/haploid genome (26, 29, 30).

Of the 50 Vα4 sequences (40%) carried a rearranged Jα4 gene, 13 a Jα3 gene (26%), 9 a Jα6 (18%), 5 a Jα2 (10%), and 3 a Jα5 (6%) (Table 1). Jα gene usage did not differ significantly from that determined by Sanz (2), Huang et al. (14, 18), and Yamada et al. (43) for PB B lymphocytes (Table 1) when all rearrangements are considered. Jα4 was clearly overrepresented among the 50 clones.

Overall, the sequence collection seems to adequately represent the pattern of Vα4 gene expression in the tonsillar and PB B cell subsets.

**CDRIII Regions.** All sequences had unique Vα-Dα/DIR-Jα junctions. Dα and DIR region genes which could be identified between the 3′ end of the Vα4 and the 5′ end of the Jα genes are depicted in Fig. 4. The criteria for the identification of Dα(DIR) elements in the CDRIII region were either (a) 100% homology with a Dα or DIR region element over a stretch of at least 7 bp, or (b) a single basepair difference within a stretch of at least 8 bp that is separated by two or more matching nucleotides from the 5′ or 3′ end of a sequence homologous to a Dα or DIR region element.

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**Table 1. Summary of the Sequence Analysis of Vα4-encoded Transcripts Derived from Isolated Tonsillar and PB B Cell Subpopulations of a 4-yr-old Child**

| Cell population | Clones | Vα4 | nucl. diff. | Jα | nucl. diff. | sum of Vα4/Jα | nucl. diff. |
|-----------------|--------|-----|------------|----|------------|---------------|------------|
| IGD Tonstillar Igα* B cells (IGD) | 3D279 | 0.4% | 0 | 3 | 0 | 0 | 3 |
| | V71-4 | 0.9% | 0 | 1 | 0 | 0 | 1 |
| | Vα4.3 | 9.7% | 1 | 3 | 0 | 0 | 3 |
| | V2-1 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.33 | 3.1% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.34 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | 3D216D | 2.4% | 0 | 1 | 0 | 0 | 1 |
| | VIV-4 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | 3D279D | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | V71-4 | 1.3% | 0 | 1 | 0 | 0 | 1 |
| | Vα4.3 | 13.6% | 0 | 0 | 0 | 0 | 0 |
| | V2-1 | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.33 | 1.7% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.34 | 1.3% | 0 | 0 | 0 | 0 | 0 |
| | 3D216D | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | VIV-4 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | 3D279D | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | V71-4 | 3.5% | 0 | 1 | 0 | 0 | 1 |
| | Vα4.3 | 14.4% | 0 | 0 | 0 | 0 | 0 |
| | V2-1 | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.33 | 2.7% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.34 | 1.3% | 0 | 0 | 0 | 0 | 0 |
| | 3D216D | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | VIV-4 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | 3D279D | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | V71-4 | 6.7% | 0 | 1 | 0 | 0 | 1 |
| | Vα4.3 | 18.9% | 0 | 0 | 0 | 0 | 0 |
| | V2-1 | 0.4% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.33 | 4.2% | 0 | 0 | 0 | 0 | 0 |
| | Vα4.34 | 1.3% | 0 | 0 | 0 | 0 | 0 |
| | 3D216D | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | VIV-4 | 0.7% | 0 | 0 | 0 | 0 | 0 |
| | 3D279D | 0.4% | 0 | 0 | 0 | 0 | 0 |

Clones are designated in Fig. 3. Indicated are the Vα-Dα (Vα4.21, V71-4, 3D279D, V2-1, Vα4.33, Vα4.34, 3D216D and VIV-4; see Fig. 3; Vα4.33r and p3D279D, see text) and Jα germline genes to which the Vα4 and Jα sequences showed highest homology and the nucleotide differences to the corresponding germline genes. The sum of nucleotide differences of the combined Vα4 and Jα sequences is depicted in the right column.

* Nonproductive rearrangement.
Figure 3. Comparison of nucleotide sequences of V{sub}4{sub}>e{sub}>4-encoded transcripts derived from isolated tonsillar and PB B cell subpopulations. V{sub}4{sub}>e{sub}>4 sequences derived from tonsillar IgM{sup}>e<sup>-IgD{sup}>e<sup>- B cells, IgM- and IgG-expressing GCC are abbreviated IGD, GCM, and GCG, respectively. V{sub}4{sub}>e{sub}>4 sequences derived from IgM-expressing CD5{sup}>e<sup>-, IgM- and IgG-expressing conventional (CD5{sup}>e<sup>-) PB cells are abbreviated CD5, CBM, and CBG, respectively. The sequences are compared with the V{sub}4{sub}>e{sub}>4 germline genes (a) V{sub}4{sub}>e{sub}>4.21, (b) V71-4, (c) 3D279D, (d) V2-1, (e) V{sub}4{sub}>e{sub}>4.33, (f) V{sub}4{sub}>e{sub}>4.34, (g) 3D216D, and (h) VIV-4. Only those codons of the respective V{sub}4{sub}>e{sub}>4 germline genes that differ from the sequences below are shown. CDR regions and codons are numbered according to Kabat et al. (44). (Dashes) Nucleotide identity. (Uppercase letters) Replacement mutations. (Lowercase letters) Silent mutations. (Underlined letter) Mutations resulting in the formation of a stop codon. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z30544-563, Z30573-581, Z30590-600, and Z30669-678.
D. or DIR region genes (45-50) could be identified. Most D./DIR region elements were rearranged in the usual presumptive new D. element or PNDE (Hg. 4). CDRIII

Using these criteria, in 47 of 50 clones, one or two published Nucleotide sequences within the joining regions of three clones (IGD6, GCG5, and CBG5) which could not be assigned to regions of 41 clones (82%) harbored one, those of right clones AGCC). This sequence might represent a part of a novel D.

Inverted orientation of the rearranged V.-D. or D.-J. joints (2, 43) were occasionally seen. This might be caused by misincorporation in the PCP.

Somatic Mutation in Tonsillar and PB B Cell Subsets. Table 1 summarizes the nucleotide differences in the rearranged V\(_{\text{d4}}\) and J\(_{\text{h}}\) genes compared to the germline genes to which they showed highest homology. Table 2 shows the V\(_{\text{d4}}\) and J\(_{\text{h}}\) gene segments. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z30544-563, Z30573-581, Z30590-600, and Z30669-678.

Using these criteria, in 47 of 50 clones, one or two published D\(_{\text{n}}\) or DIR region genes (45-50) could be identified. Nucleotide sequences within the joining regions of three clones (IGD6, GCG5, and CBG5) which could not be assigned to any of the published D\(_{\text{n}}\) or DIR region elements showed 100% homology over a stretch of seven nucleotides (CCA-

CBM4 --- GACTTC CAT ATC TGG GGC-3b

CB1 --- GACTTGGGA (D(R)4) ATGTATACATCGGATGATAGTCAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CBB1 --- GACTTGGGA (D(R)4) ATGTATACATCGGATGATAGTCAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/1 --- GAGS (D(R)4) AGTATTACGATGGCTGGTAC AGAGGAA~..rT TTT GAC TAC TGG GGC-6b

CD5/2 --- GAGS (D(R)4) AGTATTACGATGGCTGGTAC AGAGGAA~..rT TTT GAC TAC TGG GGC-6b

CD5/3 --- GA (D(R)4) ATGTATACATCGGATGATAGTCAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/4 --- GATG (D(R)4) ATGTATACATCGGATGATAGTCAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/5 --- GG (D(R)4) ATGTATACATCGGATGATAGTCAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/6 --- GAGS (D(R)4) AGTATTACGATGGCTGGTAC AGAGGAA~..rT TTT GAC TAC TGG GGC-6b

CD5/7 --- CACCTAGTCCA (D(R)1) TGGCTACAGTTATGATAGTAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/8 --- CACCTAGTCCA (D(R)1) TGGCTACAGTTATGATAGTAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/9 --- CACCTAGTCCA (D(R)1) TGGCTACAGTTATGATAGTAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

CD5/10 --- CACCTAGTCCA (D(R)1) TGGCTACAGTTATGATAGTAGTGGTTAT TC TTT GAC TAC TGG GGC-6b

Figure 4. Nucleotide sequences of CDRIII regions of rearranged V\(_{\text{d4}}\) genes derived from isolated tonsillar and PB B cell subpopulations. Clones are designated as in Fig. 3. Indicated are the V end (codon 94) of the respective V\(_{\text{d4}}\) gene, N region nucleotides (N), D\(_{\text{n}}\) and/or DIR region elements (D), and the 5' end of the rearranged J\(_{\text{h}}\) genes (42, 43). D\(_{\text{n}}\) and/or DIR region elements (45-50) which could be identified in between the 3' end of V\(_{\text{d4}}\) and the 5' end of J\(_{\text{h}}\) are indicated. (PNDE) Presumptive new D. element. (Dash) Nucleotide identity. (N) Sequence ambiguities. (I) Inverted orientation of the rearranged D. or DIR region element. (Lowercase letters) Nucleotide differences to the corresponding D\(_{\text{n}}\) or J\(_{\text{h}}\) elements. (Underlined letters) Nucleotides are derived either from the D\(_{\text{n}}\)/DIR element or from the corresponding V\(_{\text{d4}}\) or J\(_{\text{h}}\) gene segments. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z30544-563, Z30573-581, Z30590-600, and Z30669-678.
Table 2. Summary of Mutations in Rearranged V<sub>a</sub> and J<sub>a</sub> Genes Expressed in Tonsillar and PB B Cell Subsets Derived from a 4-year-old Child

| Origin          | Cell population | No. clones | V<sub>a</sub> | J<sub>a</sub> |
|-----------------|-----------------|------------|---------------|---------------|
|                 |                 |            | Range         | % Mutation    | Range         | % Mutation    |
| Tonsil          | IGD             | 9          | 0-3           | 0.4           | 0-1           | 0.0           |
|                 | GCM             | 8          | 1-10          | 2.0           | 0-1           | 0.7           |
|                 | GCG             | 8          | 1-13          | 3.3           | 0-3           | 1.2           |
| Peripheral      | CD5 and CBM<sup>+</sup> | 10         | 0-3           | 0.35          | 0             | 0.0           |
| blood           | CD5<sup>+</sup> and CBM<sup>+</sup> | 5          | 3-11          | 2.6           | 0-1           | 1.7           |
|                 | CBG             | 9          | 1-22          | 3.2           | 0-4           | 3.3           |

* V<sub>a</sub> transcripts derived from IgM-expressing CD5<sup>+</sup> and CD5<sup>-</sup> PB cells were analyzed together. Mutation frequencies were determined for V<sub>a</sub> transcripts that showed (a) three or less, and (b) four or more mutations relative to the corresponding V<sub>a</sub> and J<sub>a</sub> germline genes.

† The V<sub>a</sub> sequence of clone CD5/4 was excluded from the mutation analysis. Cell populations are designated as in Table 1.

Tonsillar IgM- and IgG-expressing GCC showed V<sub>a</sub> gene mutation frequencies of 2.0 and 3.3%, respectively (Table 2). The corresponding frequency (3.2%) determined for IgG-expressing PB B cells was in the same range as that determined for IgG-expressing GCC.

The V<sub>a</sub> gene mutation frequency determined for tonsillar IgM<sup>+</sup>IgD<sup>+</sup> cells was 0.4%. V<sub>a</sub> transcripts derived from CD5<sup>+</sup> and conventional (CD5<sup>-</sup>) IgM-bearing PB B cells were analyzed together (see Discussion). Whereas all V<sub>a</sub> rearrangements derived from tonsillar IgM<sup>+</sup>IgD<sup>+</sup> B cells showed three or less mutations relative to the corresponding V<sub>a</sub> and J<sub>a</sub> germline genes, 5 of 15 V<sub>a</sub> transcripts (excluding CD5/4, see above) derived from CD5<sup>+</sup> and CD5<sup>-</sup> IgM-expressing PB B cells harbored more than three mutations (range of mutations per gene, 4-12; Table 1). Since in the PB μ chain transcripts presumably originate from two distinct B cell subsets (4), mutation frequencies were determined for V<sub>a</sub> transcripts from IgM-bearing PB B cells that showed (a) three or less mutations, which corresponds to the range of mutations observed in tonsillar IgM<sup>+</sup>IgD<sup>+</sup> cells, and (b) four or more mutations in the rearranged V<sub>a</sub> and J<sub>a</sub> genes. The corresponding V<sub>a</sub> gene mutation frequencies were 0.35 and 2.6%, respectively (Table 2).

To confirm the virtual absence of somatic mutation in V<sub>a</sub> transcripts derived from tonsillar IgM<sup>+</sup>IgD<sup>+</sup> B cells, nine V<sub>a</sub> rearrangements were amplified with V<sub>3</sub> and V<sub>4</sub> family specific primers from genomic DNA isolated from this fraction. Recently, all V<sub>a</sub> germline genes have been ordered and sequenced (51). They seem to exhibit little polymorphism, thus allowing the identification of somatic mutations in rearranged V<sub>a</sub> genes. Three of the V<sub>a</sub> rearrangements were nonproductive (data not shown; the sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z30564-572). The V<sub>a</sub> transcripts harbored 0-4-bp differences in the rearranged V<sub>a</sub> and J<sub>a</sub> gene segments (not shown), yielding a mutation frequency of 0.2% (a PCR error of 1/585 was subtracted from the value [4]). This frequency is in a similar range as that obtained for V<sub>a</sub> transcripts derived from tonsillar IgM<sup>+</sup>IgD<sup>+</sup> B cells (0.4%).

Discussion

V<sub>a</sub> Gene Usage. So far, not all potentially functional V<sub>a</sub> germline genes have been found in rearranged antibody V genes. In addition, particular V<sub>a</sub> gene segments appear to be overrepresented over other members of this gene family as judged by their frequent appearance in independent analyses. Among the 50 V<sub>a</sub> sequences obtained in the present work, three gene segments were found to be overrepresented: V<sub>a</sub>4.21 (30%), V<sub>a</sub>71-4 (20%), and 3D279D (20%) (3D279D presumably represents an allelic form of V<sub>a</sub>71-2 [30, 39]). These three genes belong to the group of V<sub>a</sub>4 genes which in the past were repeatedly found to be expressed in peripheral B cells. In particular, the V<sub>a</sub>4.21 gene segment was frequently seen in self-reactive antibodies in various autoimmune diseases (52 and references therein). V<sub>a</sub>4.21-bearing antibodies are known to recognize autologous determinants on red blood cells, the I/i antigens, and such antibodies are found in the serum of healthy individuals (for a review see reference 52). The observation that 30% of the clones in our sequence collection carried V<sub>a</sub>4.21, together with the recurrent finding of V<sub>a</sub> and V<sub>c</sub> gene segments that are associated with autoreactivity in PB B cells of normal individuals (for a review see reference 53), implies that a considerable proportion of the antibody repertoire in normal individuals contains "anti-self" specificities, as previously suggested (54). V<sub>a</sub>71-4 and V<sub>a</sub>71-2-bearing antibodies also appear to be associated with autoreactivity (52, 53). However, we cannot rule out that the overrepresentation of V<sub>a</sub>4.21, V<sub>a</sub>71-4 and 3D279D transcripts in the cDNA libraries is the result of higher Ig-mRNA contents in B cells expressing these gene segments, although we consider this an unlikely possibility. A preferential amplification of these V<sub>a</sub>4 gene segments in the PCR seems also unlikely since the V<sub>a</sub>4FRI-primer used in the present
study exhibits 100% homology to the corresponding sequence in nearly all $V_\mu 4$ germline genes (see also Materials and Methods). That the PCR amplification does not lead to an overrepresentation of particular $V_\mu 4$ genes in the cDNA libraries is further supported by the differing $V_\mu 4$ gene usage between fractions.

10 different $V_\mu 4$ genes were found to be expressed in the present individual. Accordingly, on the assumption that the number of potentially functional $V_\mu 4$ genes in the haploid genome of an individual is 9-11 (26, 29, 30), most of the genes of this family were expressed. This contradicts recent suggestions that at the level of $V_\mu 4$ gene usage the expressed antibody repertoire is considerably smaller than the available gene family.

In summary, it appears that most $V_\mu 4$ germline genes are expressed in peripheral B cells. However, some members of this family are clearly overrepresented over others. It remains to be seen whether this picture can be extrapolated to the other $V$ gene families.

Differences of $V_\mu 4$ gene usage between fractions were particularly evident in the case of the tonsillar B cell subsets: whereas IgM$^+$IgD$^-$ GCC as well as tonsillar IgM$^+$IgD$^+$ cells exclusively expressed $V_\mu 4$ genes which could be assigned to one of the three overrepresented gene segments (8/8), most $V_\mu 4$ transcripts (5/8) derived from IgG$^+$ GCC showed highest homology to one of the genes which were found only rarely in the present analysis (Table 1). This differing $V_\mu 4$ gene usage among the tonsillar B cell subsets is likely to reflect antigenic selection within the GC microenvironment. A striking observation was that of the 15 clones which carried a $V_\mu 4.21$ gene, 13 originated from IgM-expressing cells (13/33, 39%) whereas only two were found among IgG-expressing GCC and PB B cells (2/17, 12%). This gene segment was particularly overrepresented in transcripts derived from tonsillar IgM$^+$IgD$^+$ (naive) B cells (5/9), but was found in only 1/9 transcripts derived from IgG$^+$ (memory) PB B cells. It appears that $V_\mu 4.21$ is only rarely used in T cell–dependent immune responses to exogenous antigens.

In addition to the restricted usage of $V_\mu 4$ gene segments in the tonsillar IgM$^+$IgD$^+$ naive B cells, the $J_\mu 3$ gene segment seems to be overrepresented (4/9) in this population. A high frequency of $J_\mu 3$ utilization is a feature of fetal Ig rearrangements (2, 57, 58). However, other characteristics of the fetal B cell repertoire, namely a high usage of the most proximal $D_\mu$ element, $D_\mu$Q52, short CDRIII lengths and a low frequency of $N$ region addition (2, 57, 58) were not observed in $V$ region genes derived from tonsillar IgM$^+$IgD$^+$ cells.

Naive IgM$^+$IgD$^+$ B Cells Express Virtually Unmutated $V$ Region Genes. Tonsillar IgM$^+$IgD$^+$ B cells, which are localized in the mantle zone surrounding the GC and the interfollicular area, express $V$ region genes with no or little somatic mutation. The low $V_\mu 4$ gene mutation frequency in this population (0.4%) is similar to that determined for $J_\mu$ regions (0.0%) and rearranged $V_\mu 3$ and $V_\mu 4$ genes carried by the same cells (0.2%; the latter genes were amplified from genomic DNA). No or little somatic mutation was also observed in mantle zone B cells (most of which express IgD) derived from human lymph nodes (9). We have previously shown that IgM$^+$IgD$^+$ PB B cells from a 67-yr-old person likewise express virtually unmutated $V_\mu$ genes (4). These cells comprise the majority of PB B cells throughout life and represent a large B cell subpopulation in secondary lymphoid organs.

Thus, as we had already concluded from our earlier data (4), the repertoire of human naive IgM$^+$IgD$^+$ B cells is determined by germline-encoded specificities and by generation of variability in CDRIII through $V_\mu$-D$_\mu$-J$_\mu$ recombination throughout life, like in the mouse (3).

Tonsillar IgM$^+$IgD$^-$ and IgG$^+$ GCC Express Somatically Mutated $V$ Region Genes. Ongoing somatic mutation in human GCC has recently been demonstrated for GCC picked from histological sections of lymph nodes (9). In the present analysis, purified tonsillar GCC were distinguished on the basis of IgM and IgG expression. Both IgM- and IgG-bearing tonsillar GCC express somatically mutated antibody genes (2.0 and 3.3% $V_\mu 4$ gene mutation, respectively). The increased $V_\mu 4$ gene mutation frequency of IgG-expressing GCC compared to IgM-expressing GCC could imply that the former cells represent B cells at a later stage of the GC reaction. This assumption is supported by the differing $V_\mu 4$ gene usage between these subsets.

Somatically Mutated IgM- and IgG-bearing PB B Cells Probably Represent GC-derived B Cells. Since no drastic differences in terms of somatic mutation were observed between the IgM-bearing CD5$^+$ and conventional (CD5$^-$) PB B cell subpopulations—in agreement with results obtained for the corresponding B cell subsets in adults (19)—they are not discussed separately. One third of the $V_\mu 4$ sequences (5/15) derived from these populations harbored more than three mutations (a 0-3-bp difference corresponds to the range of mutations observed in tonsillar IgM$^+$IgD$^+$ cells) relative to the corresponding $V_\mu 4$ and $J_\mu$ germline genes (4-12 mutations; Table 1), yielding a $V_\mu 4$ gene mutation frequency of 2.6% (Table 2). On the other hand, the frequency determined for the $V_\mu 4$ transcripts harboring three or less mutations in the rearranged $V_\mu 4$ and $J_\mu$ genes was in the same range as that determined for tonsillar IgM$^+$IgD$^+$ cells (0.35 and 0.4%, respectively). Somatically mutated $\mu$ chain transcripts have previously been found in PB B lymphocytes from adults (14, 16, 18, 19). Such transcripts are most likely to be derived from IgM$^+$IgD$^+$ cells, which, in contrast to IgM$^+$IgD$^+$ cells, express $V$ region genes diversified by somatic mutation (4).

The mutation frequency determined for $V_\mu 3$ transcripts of IgM$^+$IgD$^-$ PB B cells (4) is similar to that determined for $V_\mu 4$ transcripts of IgM$^+$IgD$^-$ GCC (see Table 2; ~2.0% in both cases). These observations indicate that the IgM$^+$IgD$^-$ PB B cell subpopulation is composed of GC-derived B cells that have left the GC at an early stage of the GC reaction without undergoing isotype switching.

IgG$^+$ PB B cells are thought to be GC-derived memory B cells. The specific analysis of PB memory B cells in individuals of various ages allows one to determine somatic mutation frequencies in these cells regardless of their actual per-
percentage in the PB. 

A comparison between the extent of somatic mutation in IgG+ cells from the child investigated in the present work and IgM-IgD- cells (mostly representing IgG+ and IgA+ B cells) from a 67-yr-old person (4) indicates that the mutation frequency (3.2 versus 3.9%) in PB memory B cells does not increase significantly with age. Together with the fact that the V,4 mutation frequencies determined for IgG-expressing GCC and IgG+ PB B cells were in the same range (3.3 and 3.2%, respectively) this implies that long-lived memory cells do not acquire additional somatic mutations after they have left the GC.

Somatically Mutated IgM+ and IgG+ B Cells Accumulate in the PB during Life. 

Recent experiments by Bridges and Schroeder indicate that the fraction of somatically mutated κ light chain transcripts amplified from PB B lymphocytes increases with age (Bridges, S. L., and H. W. Schroeder, Jr., personal communication, and 58a). These transcripts were most likely derived from both IgG- and IgM-bearing B cells. At the time of birth, IgM-bearing B cells express virtually unmutated V region genes (16, 17), mirroring the situation in the fetal liver (57, 58, 40) (an exceptional case has been reported by Cuisinier et al. [40] who found somatically mutated V,6 transcripts derived from fetal liver B cells). However, the hypermutation mechanism is already active at this time of ontogeny as indicated by the finding of somatically mutated γ and δ chain transcripts derived from cord blood B lymphocytes (22).

These observations, together with our finding of mutated γ and μ chain transcripts in PB B lymphocytes already at young age, suggest that IgG+ as well as IgM+IgD- B cells expressing somatically mutated V region genes accumulate in the PB during ontogeny. Thus, the age-related increase of somatic mutation in PB B cells observed by Bridges and Schroeder probably reflects an increase in the percentage of somatically mutated B cells among PB B lymphocytes since the mutation frequencies in these cells do not appear to vary significantly with age.

Sequence analyses of V gene transcripts derived from PB B lymphocytes of adults showed that the majority of μ chain (14, 16, 18, 19) and κ light chain transcripts (Bridges, S. L., and H. W. Schroeder, Jr., personal communication, and 58a) were somatically mutated. This is surprising since naïve IgM+IgD- B cells represent the major B cell population in the PB throughout life (4), comprising at least 70% of PB B lymphocytes. This discrepancy might be explained by differing Ig-mRNA levels in the various B cell subsets, resulting in an overrepresentation of transcripts derived from IgM+IgD- and class-switched B cells in cDNA libraries. The same might be true for the recent findings that the vast majority of V, transcripts derived from splenic B cells of old individuals were somatically mutated (Bridges, S. L., and H. W. Schroeder, Jr., personal communication, and 58a, 59).

In conclusion, the human PB B cell repertoire is composed of at least three B cell subsets which can be distinguished according to their isotype expression and according to the extent of somatic mutation found in their rearranged V genes. Naïve IgM+IgD- B cells—presumed precursors of GCC—express germline specificities and remain the major PB B cell subset throughout life. Somatically mutated IgM+IgD- cells, as well as class-switched memory B cells, seem to be constantly generated in GC and accumulate in the PB in the course of life.

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