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Research Article

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Molecular Single-cell Analysis Reveals that CD5-positive Peripheral Blood B Cells in Healthy Humans are Characterized by Rearranged V_{κ} Genes Lacking Somatic Mutation

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Abstract

B cells expressing the CD5 cell surface antigen are involved in certain B cell malignancies and autoimmune diseases. From studies in the mouse, it emerged that CD5⁺ B cells represent a separate lineage of B lymphocytes that, in contrast to conventional (CD5⁻) B cells, are not driven into T cell-dependent immune responses in which rearranged variable (V) region genes are diversified by somatic hypermutation. Against this background it came as a surprise that human disease-involved CD5-positive autoreactive B cells as well as B cell chronic lymphocytic leukemias can harbor somatically mutated V region genes. Recent V gene analyses on CD5⁺ B cells in healthy adults did not give rise to a clear picture about the fraction of somatically mutated among all CD5⁺ B cells. In this work we used a molecular single-cell analysis to determine reliably the frequency of mutated CD5⁺ B cells in healthy humans: single, κ light chain-expressing CD5⁺ peripheral blood B cells were isolated by flow cytometry, and rearranged V_{κ} genes were amplified by PCR. From one donor, CD5⁺CD19⁺ B cells were analyzed. Since CD5⁺ B cells were found among IgM⁺IgD⁺ and IgM⁺IgD⁻ cells (but almost not among class-switched cells) from two other donors, individual cells corresponding to these IgM-expressing subsets were investigated separately. The sequence analysis of rearranged V_{κ} genes revealed that most if not all CD5⁺ B cells in healthy humans carry unmutated V region genes. From one of the donors, a novel polymorphic J_x2 gene segment was identified. To explain the discrepancy between the frequent occurrence of disease-associated somatically mutated CD5⁺ B cells and the low incidence or absence of somatic mutation in normal CD5⁺ B cells, we speculate that CD5⁺ B cells usually do not participate in germinal center reactions, but if they occasionally do so, they may be at an increased risk to become involved in autoimmune diseases or B cell malignancies. (J. Clin. Invest. 1997. 100:1667-1676.) Key words: B1 cells • CD5 • single cell PCR • somatic hypermutation • variable region genes

Introduction

B lymphocytes expressing the CD5 antigen are the predominant B cell population early in life (1, 2). Later, their number is reduced, and CD5⁺ B cells account for \sim 10–25% of peripheral blood (PB)¹ B cells in man (1-3). These cells are also found in spleen and tonsil at a comparable frequency (1-3). One characteristic feature of CD5⁺ B cells (also called B1a cells) is that many of them produce autoreactive antibodies, which are usually polyreactive (4). Numbers of $CD5^+$ B cells are elevated in certain autoimmune diseases, and in some of these cases CD5⁺ B cells producing high-affinity autoantibodies have been detected (4-7). A relationship between CD5⁺ B cells and autoimmunity is further suggested by the finding that mouse strains harboring elevated levels of CD5⁺ B cells are prone to autoimmune diseases (1). CD5⁺ B cells are also involved in certain B cell malignancies. More than 95% of cases of B cell chronic lymphocytic leukemia (B-CLL), the most common leukemia of adults in the Western world, express CD5 (1). In addition, CD5 is regularly found on tumor B cells in small lymphocytic lymphoma (1).

The origin of $CD5^+$ B cells in humans and mice is much debated (1, 8). Since CD5 expression can be induced in vitro on CD5-negative B cells, it has been suggested that CD5 represents an activation marker (9). In the induction/selection model, B cells recognizing autoantigens with low affinity are selected to become CD5⁺ B cells (10). On the other hand, transfer experiments in mice support a dual-lineage model that states that CD5-positive and CD5-negative (conventional) B cells represent distinct lineages of B cells (11). Those transfer experiments also revealed that CD5⁺ B cells represent a selfreplenishing population of long-lived lymphocytes (12, 13).

Immunoglobulin V genes expressed by CD5⁺ B cells have been the subject of a large number of studies. A sequence analysis of immunoglobulin V region genes allows one to gain insight into the stage of differentiation of the respective B cell. Immature and naive B cells carry unmutated variable (V) region genes (14-17). Somatic mutations are introduced into rearranged V genes in the course of T cell-dependent immune reactions (14, 18). This process takes place in the microenvironment of the germinal center (GC) (19-21). Consequently, somatically mutated V region genes are found in GC B cells and their descendants, i.e., memory B cells and GC-derived plasma cells (16, 17, 21). Whereas murine CD5⁺ B cells express unmutated V region genes (22-24), the situation appears to be different in the human: somatically mutated V genes have been described in cases of CD5-expressing B-CLL (25-28) and in CD5⁺ B cells producing high-affinity autoantibodies

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^{1.} *Abbreviations used in this paper:* B-CLL, B cell chronic lymphocytic leukemia; CDR, complementarity-determining region; GaH, goat anti-human; GC, germinal center; MC, mononuclear cells; PB, peripheral blood.

(5, 6). Moreover, somatic mutations have been reported to be present in V genes of $CD5^+$ B cells isolated from healthy donors (16, 29, 30). These findings suggest that in terms of somatic mutation, there is no difference between $CD5^+$ B cells and conventional B cells in humans.

Two findings prompted us to reinvestigate the issue of the occurrence and frequency of CD5⁺ B cells carrying somatically mutated V genes in healthy individuals: first, most CD5⁺ B cells are found among IgM^+IgD^+ B lymphocytes (2, 3). The vast majority of these naive B cells, however, carry unmutated V region genes (15–17, 31), which also implies that most $CD5^+ B$ cells express unmutated V genes. Obviously, this result contrasts with the V gene studies on CD5⁺ B cells in healthy individuals (16, 29, 30). A possible explanation for this discrepancy might be that a small subset of cells with mutated V region genes among IgM⁺IgD⁺ B cells could have been missed in the analyses of IgM⁺IgD⁺ B cells, or that the mutated V genes in those studies (16, 29, 30) were derived from IgD^- CD5⁺ B cells. Second, we recently showed that human peripheral blood B lymphocytes carrying somatically mutated V region genes, i.e., IgM⁺IgD⁻ (IgM-only) and class-switched B cells, harbor elevated levels of mRNA coding for immunoglobulin when compared with naive IgM⁺IgD⁺ B cells (31). Since the studies on CD5⁺ B cells of healthy donors were carried out by amplifying V region gene transcripts from cell populations (16, 29, 30), those investigations might be biased towards somatically mutated V genes.

To overcome these problems, we sorted single CD5⁺ B cells by flow cytometry, and amplified rearranged V_{κ} genes from genomic DNA of individual cells. PCR products were sequenced directly. This approach should allow one to determine reliably the frequency of CD5⁺ B cells carrying mutated V region genes.

Methods

Isolation of single cells and flow cytometry. 60-100 ml PB was obtained from three male, healthy donors ages 27-35. PB mononuclear cells (MC) were purified by Ficoll-Isopaque density centrifugation, and were incubated with unconjugated antihuman ĸ light chain (IgG1-isotype, Becton Dickinson, Mountain View, CA) for 15 min on ice. After washing, the cells were incubated with goat anti-mouse-IgG1 microbeads (Miltenvi Biotec Inc., Bergisch Gladbach, Germany) for 20 min at 4°C. Subsequently, ĸ-expressing B cells were enriched to > 90% by magnetic cell separation using the MiniMACSTM system (Miltenyi Biotec Inc.) (15). For isolation of single CD5⁺CD19⁺ B cells from donor HK, the cell suspension was incubated with anti-CD5-PE and anti-CD19-FITC (Becton Dickinson). The cell suspensions from the other two donors were stained with anti-CD5-FITC (Becton Dickinson), goat anti-human (GaH)-IgM-PE (Sigma Chemical Co., München, Germany), and biotinylated GaH-IgD (Southern Biotechnology Associates, Birmingham, AL). After washing, GaH-IgD was developed with Streptavidin-CyChrome (PharMingen, San Diego, CA). All staining procedures were performed for 15 min on ice. Using a FACS 440 (Becton Dickinson), single ĸ-expressing B cells of the following B cell fractions were sorted directly into PCR reaction tubes containing 20 µl PCR buffer and 20 ng 5S rRNA: CD5+CD19+ B cells (donor HK), CD5⁻IgM⁺IgD⁻ B cells, CD5⁺IgM⁺IgD⁻ B cells, and CD5+IgM+IgD+ B cells (donors RK and AB). In addition, a fraction of peripheral blood mononuclear cells (PBMC) was stained with anti-CD3-FITC (Becton Dickinson), and single T cells (CD3⁺) were sorted into reaction tubes as described above.

The size of $CD5^+IgM^+IgD^+$ as well as $CD5^+IgM$ -only PB B cells and their CD5-negative counterparts was determined by forward

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scatter analysis. The populations did not differ from each other in this respect.

For analysis of isotype-switched B cells with regard to CD5-expression, PBMC of seven healthy adult donors were incubated with anti-CD19 microbeads (Miltenyi Biotec Inc.) for 20 min at 4°C, and CD19⁺ B cells were enriched to 99% by MiniMACSTM. The B cellenriched cell suspensions were stained with anti-CD5-PE, GaH-IgD, and with either GaH-IgG-FITC (Southern Biotechnology Associates) or rabbit anti-human-IgA $F(ab')_2$ -FITC (Dako Corp., Hamburg, Germany) as described above. Samples were analyzed on a FACScan (Becton Dickinson).

To analyze tonsillar non-GC B cells consisting of naive as well as memory B cells (17) for expression of CD5, this fraction was enriched by depleting GC B cells (CD38⁺) and T cells using the MACS system (Miltenyi Biotec Inc.). Tonsillar MC were incubated with anti-CD3 microbeads (Miltenyi Biotec Inc.) and unconjugated anti-CD38 (IgG1isotype; Becton Dickinson) for 20 min at 4°C, and after washing with anti-IgG1 microbeads. After magnetic cell separation, tonsillar non-GC B cells were enriched to $\sim 85\%$. This fraction was analyzed for surface expression of CD5 on IgM⁺IgD⁺ and IgM-only cells as well as on isotype-switched memory B cells using the staining procedures described above.

Single cell PCR and sequencing of PCR products. Single cells were incubated in 20 µl PCR buffer containing 0.5 mg/ml proteinase K for 1 h at 50°C. The enzyme was inactivated by denaturation at 95°C (10 min). For the first round of amplification, a primer mix consisting of four V_{κ} family-specific primers, which recognize sequences in the framework region I of the members of the V_k1-4 gene families, and a downstream primer specific for a sequence 3' of J_K5 was used (see Table I). This approach prevents amplification of V_{κ} -J_{κ}1,2,3 & 4 rearrangements that have been located away from the $C_{\!\kappa}$ locus by inversion (32), and therefore presumably do not undergo somatic hypermutation due to their large distance from both the κ intron and the κ 3'-enhancer (33). Owing to the small distance between the five J_{κ} gene segments, however, $V_{\kappa}J_{\kappa}$ rearrangements lying in the proper location in the Igk locus can be efficiently amplified with a primer 3' of the last J_{κ} gene. The first round of amplification was carried out in the same reaction tube in a 50-µl volume containing Expand[™] High Fidelity buffer (Boehringer Mannheim, Mannheim, Germany), 1.75 mM MgCl₂, 100 µM dATP, dGTP, dTTP, and dCTP, respectively, 50 nM of each primer, and 2.5 U Expand[™] High Fidelity polymerase (Boehringer Mannheim). The amplification program consisted of 35 cycles of 60 s at 95°C, 30 s at 61°C, and 80 s at 72°C, followed by a final incubation step at 72°C for 5 min. Enzyme was added after the first denaturation step. For the second round of amplification, the same V_{κ} family-specific primers were used together with three internal J_{κ} specific primers hybridizing to the five human J_{κ} gene segments (see Table I). The second round of amplification was performed in separate reactions for each of the four V_{κ} primers using 1 µl of the firstround reaction mixture in a 50-µl volume containing 10 mM Tris-HCl, pH 8.3 (20°C), 50 mM KCl, 2.0 mM MgCl₂, 100 µM dATP, dGTP, dTTP, and dCTP, respectively, 125 nM of each primer, and 0.7 U Taq DNA polymerase (Boehringer Mannheim). The amplification conditions were as described above, except that the elongation step at 72°C was carried out for 60 s, and 40 cycles were applied. PCR products were purified by gel electrophoresis. An aliquot of the isolated DNA was sequenced directly with ³²P-labeled oligonucleotides of the second round using the dsDNA Cycle Sequencing System (GIBCO BRL, Eggenstein, Germany) as recommended by the supplier. Nucleotide sequences were analyzed using DNASISTM software (Pharmacia, Freiburg, Germany). In some cases, it was not possible to determine by sequence analysis which of the five J_{κ} gene segments had been rearranged. In these cases, the second round of PCR was repeated using the respective V_{κ} family-specific primer together with each of the J_{κ} -specific primers in separate reactions (J_{κ} typing).

Statistical analysis. Differences in the proportions of cells carrying mutated V_{κ} genes, and differences in the proportions of potentially functional V_{κ} genes with complementarity-determining region

Table I. Sequences of Oligonucleotides Used as Primers for the Amplification of Rearranged V_{κ} Genes

| Oligonucleotide | Sequence | | | | | |
|------------------------|---|--|--|--|--|--|
| | 5'-GACATCC <ag>G<at>TGACCCAGTCTCC<at>TC-3'</at></at></ag> | | | | | |
| V _s 2 | 5'-CAG <at>CTCCACTCTCCCTG<ct>CCGTCA-3'</ct></at> | | | | | |
| V _κ 3 | 5'-TTGTG <at>TGAC<ag>CAGTCTCCAG<gc>CACC-3'</gc></ag></at> | | | | | |
| $V_{\kappa}4$ | 5'-AGACTCCCTGGCTGTGTCTCTGGGC-3' | | | | | |
| 3′J ₈ 5 | 5'-CTCTAAA <ag>GTCAATACTGGCCATC-3'</ag> | | | | | |
| 5′J _s 1,2,4 | 5'-ACTCACGTTTGAT <ct>TCCA<gc>CTTGGTCC-3'</gc></ct> | | | | | |
| 5′J _k 3 | 5'-GTACTTACGTTTGATATCCACTTTGGTCC-3' | | | | | |
| 5′J _K 5 | 5'-GCTTACGTTTAATCTCCAGTCGTGTCC-3' | | | | | |

The V_{k} primers hybridize to the framework region I of the respective family. <> denotes a nucleotide mix at this position.

(CDR) III lengths > 9 amino acids between IgM-only CD5⁻, IgM-only CD5⁺, and IgM⁺IgD⁺CD5⁺ B cells were assessed statistically using Fisher's exact test.

Results

 V_{κ} gene sequences of $CD19^+CD5^+$ B cells. After enrichment of κ light chain–expressing PB B cells from donor HK by magnetic cell separation, single cells of the $CD19^+CD5^+$ fraction, which amounted to $\sim 20\%$ of the B cell compartment, were isolated by fluorescence-activated cell sorting (Fig. 1). Rearranged V_{κ} genes of individual cells were amplified from genomic DNA using a seminested PCR strategy (31). The experiment was restricted to analysis of V_{κ} region genes since the human immunoglobulin κ locus is well characterized, and human V_{κ} germline genes seem to exhibit only little polymorphism, which allows unambiguous identification of somatic



Figure 1. Flow cytometric analysis of κ -expressing B cells (after MACS enrichment) derived from PB of a 27-yr-old person (donor HK). Cells were stained with anti-CD19-FITC and anti-CD5-PE. The gate set for sorting of single CD5⁺CD19⁺ B cells is indicated.

mutations in rearranged V_{κ} genes (34, 35). Negative controls consisted of one single T cell, plus one reaction mixture without cells per seven B lymphocytes analyzed. None of these negative controls gave rise to a PCR product.

Of 14 CD5⁺ B cells analyzed by PCR, 11 cells gave rise to a total of 14 amplificates, 12 of which were sequenced (Fig. 2). All sequences represented unique $V_{\nu}J_{\nu}$ joints. Cell X8 carried one in-frame and one out-of-frame rearrangement, whereas cell X9 showed only a single out-of-frame rearrangement. Cell X12 gave rise to two potentially functional V_{κ} region genes. This result may be explained by the fact that two cells had been sorted into the reaction tube at the isolation step. Another possibility is that one of the κ light chains, though the gene segments had been functionally assembled, was unable to pair properly with the H chain expressed by the cell, and therefore an additional rearrangement occurred in the second Igk locus (36, 37). From each of the remaining seven cells, a single in-frame rearrangement was obtained. Whereas V_{κ} gene rearrangements X5K4 and X8K1 showed two basepairs difference each compared to the most homologous V_{κ} germline genes, the other ten V_{κ} sequences turned out to be unmutated (Fig. 2 and Table II). This result indicates that the majority of human CD5⁺ PB B cells carry unmutated V_{κ} region genes. It does not, however, exclude the possibility that a small subset of mutated CD5⁺ B cells might exist in human PB.

Table II. Summary of Mutations in V_{κ} Region Geness Amplified from CD5⁺CD19⁺, CD5⁻IgM-only, CD5⁺IgM-only, and CD5⁺IgM⁺IgD⁺ PB B Cells Derived from Three Donors

| Cell population | Donor | n* | Unmutated [‡] | Range [§] | % Mutation |
|--|-------|----|------------------------|--------------------|------------|
| CD5 ⁺ CD19 ⁺ | НК | 12 | 10 | 0–2 | 0.1 |
| CD5 ⁺ IgM ⁺ IgD ⁻ | RK | 11 | 10 | 0–4 | 0.1 |
| | AB | 10 | 8 | 0–4 | 0.2 |
| CD5 ⁺ IgM ⁺ IgD ⁺ | RK | 11 | 11 | 0 | 0 |
| | AB | 13 | 13 | 0 | 0 |
| CD5 ⁻ IgM ⁺ IgD ⁻ | RK | 11 | 2 | 0-12 | 2.5 |
| | AB | 7 | 1 | 0–16 | 2.0 |

*Number of $V_{\kappa}J_{\kappa}$ rearrangements analyzed; [‡]number of unmutated $V_{\kappa}J_{\kappa}$ rearrangements; [§]range of mutations per gene. ^{||}Mutation frequencies were calculated by considering the nucleotide differences in the V_{κ} region genes relative to the corresponding V_{κ} and J_{κ} germline genes, except for the non-germline-encoded nucleotides at the $V_{\kappa}J_{\kappa}$ junctions.

| | | v _k | 3'V _k | | ۷ | k - J _k jun | ction | 5'J _k | J _k | in frame | bp diff. |
|------------------------------------|-------|----------------|------------------|-------|------|------------------------|-----------|------------------|----------------|----------|----------|
| | | | 93 94 | 95 | 95A | N | 96 97 | 98 99 | | | |
| | X1K4 | B3 | AGT AC | гсс | | | G TGG ACG | TTC GGC | 1 | + | 0 |
| | X2K1 | LFVK431 | AGT TA | C CC1 | • | CTG | ACT | TTC GGC | 4* | + | 0 |
| | X5K4 | B3 | AGT AC | r cci | • | | TGG ACG | TTC GGC | 1 | + | 2 |
| | X6K3 | L6 | AAC TG | G CCT | CC T | | G CTC ACT | TTC | 4 | + | 0 |
| | X8K1 | L5 | AGT TT | C CC1 | C | | AC ACT | TTT GGC | 2 | + | 2 |
| CD5 ⁺ CD19 ⁺ | X8K4 | B3 | AGT AC | r cc | | с | GCT CAC | TTT CGG | 4 | - | 0 |
| | X9K2 | A17 | CAC TG | G CCT | CCC | | TCN CTT | TCG | 4* | - | 0 |
| | X10K2 | A3 | CAA AC | г сст | C C | т | A TTC ACT | TTC GGC | 3 | + | 0 |
| | X11K4 | B3 | AGT AC | г сс | | | G ATC ACC | TTC GGC | 5 | + | 0 |
| | X12K1 | L12A | AGT TA | г тст | ° C | | GG ACG | TTC GGC | 1 | + | 0 |
| | X12K3 | L2 | AAC TG | G CCT | . CC | AG | GG ACG | TTC GGC | 1 | + | 0 |
| | X13K2 | A3 | CAA AC | г сст | C | AG | ACT | TTC GGC | 3* | + | 0 |

Figure 2. Nucleotide sequences of $V_{\kappa}J_{\kappa}$ junctions amplified from CD5⁺CD19⁺ PB B cells by single-cell PCR. Sequences are designated by an X and a sample number of the cell, followed by the corresponding V_{κ} family to which the sequence was assigned. Shown are the 3' end of the respective V_{κ} gene, N-region nucleotides (*N*), and the 5' end of J_{κ} . In addition, the corresponding V_{κ} and J_{κ} germline genes are indicated. J_{κ} genes identified by J_{κ} typing (see Methods) are denoted by *. The sum of nucleotide differences relative to the corresponding germline genes is shown in the right column. Codons are numbered according to Kabat et al. (74). Full-length sequences are available from EMBL/GenBank under accession nos. Z98605–Z98679.

 V_{κ} gene sequences of IgM^+IgD^+ and IgM^+IgD^- CD5expressing B cells. The expression of mutated V genes in human B lymphocytes is mainly restricted to isotype-switched and IgM-only (IgM⁺IgD⁻) B cells (15–17, 31). These two B

cell subsets represent ~ 15% and 10% of human PB B cells, respectively, whereas naive IgM⁺IgD⁺ B cells usually comprise more than 70% of all PB B lymphocytes (15, 31, 38, 39). Consequently, the question arose as to whether CD5⁺ B cells





Figure 3. Peripheral blood. Flow cytometric analysis of MACSenriched CD19⁺ PB B cells derived from a healthy adult person. Cells were stained with anti-CD5-PE, anti-IgD-CyChrome, and either (*left top*) anti-IgA-FITC or (*left bottom*) anti-IgG-FITC. (*Left*) Windows were set around (*a*) the IgA⁺, (*b*) the IgG⁺, and (*c*) the IgD⁺ populations. (*Right*) CD5-expression of (*top*) IgA⁺, (*middle*) IgG⁺, and (*bottom*) IgD⁺ cells.

Figure 4. Peripheral blood. Flow cytometric analysis of κ -expressing B cells (after MACS-enrichment) derived from PB of a 32-yr-old person (donor RK). Cells were stained with anti-IgM-PE, anti-IgD-CyChrome, and anti-CD5-FITC. (*Left*) Windows were set around (*a*) the IgM⁺IgD⁻ (IgM-only), (*b*) the IgM⁺IgD⁺, and (*c*) the IgM⁻IgD⁻ (i.e., predominantly isotype-switched B cells) fractions. (*Right*) CD5-expression of (*top*) IgM⁺IgD⁻, (*middle*) IgM⁺IgD⁺, and (*bottom*) IgM⁻IgD⁻ cells. Indicated are the gates set for sorting of single CD5⁻IgM⁺IgD⁻, CD5⁺IgM⁺IgD⁻, and CD5⁺IgM⁺IgD⁺ cells.

| Α | | v _k | 3'V _k | k - J _k junction 5' | | J _k | in frame | bp diff. | |
|-----------------------------------|---|--|--|--|---|---|---|--|---|
| | | | 93 94 95 95A | N | 96 97 98 99 | | | | |
| CD5 [−] IgM–only | A5K3 A6K1 A9K1 A10K2 A14K1 A30K2 AZ2K2 AZ4K1 AZ5K3 AZ5K3 AZ6K2 AZ7K2 | A27 018 L19A 011 L12A A17 A3 L18A L6 A18§ A3 | AG AGT CTC CC AGT TTC CC AGT TT CCT AGT T CAC TGG CC CAA ACT CC AGT TAC CCT AAC TGG CCT CAC CTT C CAA ACT CC | T GTC G G G G GT GGG G G GAT | TTC ACT TTC G TAC ACT TTT GGC TC ACT TTC GGC C CT TTC GGC C AGT TTC GGC G TAC AGT TTT GGC G TAC AGT TTT GGC CTC ACT TTC GGC CTC ACT TTC GGC C CT C CGC | 3231* 224435 | + + + - + + - | 10 10 7 12 7 10 8 7 0 0 | |
| CD5 ⁺ IgM–on]y | B7K2 B15K3 B16K1 B20K2 B24K1 B25K3 B42K1 B44K3 BZ1K1 BZ2K1 BZ2K1 | A17 A27 018 A3 018 A27 02 L2 L12A L23 A3 | CAC TGG C AGC TCA CC AAT CTC CC CAG ACC CCT AAT CTC CC CC AGC TCA CC AGT CAC CCT C AAC TGG CCT CC AGT TAT T AGT ACC CCC CAA AC | GG GG A GA C CCA TA G GT G A G TTG C TTG C TC | CT TTC GGC G CTC ACT TTC GGC TAC AGT TTT GGC C AGT TTT GGC G ACG TTC GGC TC ACT TTC GGC C AGT TTT GGC G ATC ACC TTC GGC | 4* 4 3* 2 5* 2 1 4 2 4 5 | - + + + + + + + | 0 0 4 0 0 0 0 0 0 | |
| CD5⁺ IgM⁺IgD⁺ | C3K1 C4K1 C4K2 C6K1 C6K2 C8K1 C10K3 C15K1 C31K2 C31K2 C33K2 C21K3 | L5 O2 A3 L23 A17 O2 A27 A30 A17 A3 A27 | AGT TTC CCT C AGT ACC CC CAA ACT CC AAT ACC CCT CAC TGG CC AGT ACC CCT AGT ACC CC AGT TAC CC CAC TGG CAA ACT CC AGC TCA CCT C | C GTA CC TC | AC AGT TTT GGC G TAC ACT GG ACG TTC GGC TTC GGC G TGG ACG TTC G TAC AGT TTT GGC TTTC GGC TGG ACG TTC GGC G TGG ACG TTC GGC TAC AGT TTT GGC | 2 2 1 4 1 2 3 1 2 1 2 1 2 | + - + + + + + + + | | |
| В | | v _k | 3'V _k | V _k - J _k junct | ion ^{5'J} k | J _k | in frame | bp diff. | <i>Figure 5</i> . Nucleotide sequences |
| | | | 93 94 95 95A | N | 96 97 98 99 | | | | CD5 ⁻ IgM-only, CD5 ⁺ IgM-only, and CD5 ⁺ IgM ⁺ IgD ⁺ PB B cells by |
| | D11K3 | L2 | GAC TGG CC | A | ACG TTC GGC | 1 | + | 5 | single-cell PCR. (A) Donor RK; (B) |
| CD5 ⁻ | D14K1 | A20 | AGT GCC CCT C | | A CTT TOG | 4* /* | - | 0 | donor AB. Sequences are desig- |
| TaM-only | D18K3 | 12 | AAC GGG CC | C A HIC CL | TAC ACT TTT GGC | 2 | + | 16 | nated by one or two uppercase let- |
| Igr-only | D20K1 | 1.9 | AGT TAC C | GT | GCT CAC TTT CGG | 4 | - | 2 | ters and a sample number of the |
| | D20K3 | A27 | AGC TCA CC | u. | G ATC ACC TTC GGC | 5 | + | 2 | cell, followed by the corresponding |
| | D24K3 | L6 | AAC TGG CC | | G CTC ACT TTC | 4 | + | 7 | V_{κ} family to which the sequence was |
| | E3K4 | B3 | AGT ACT C | TC | GTA CAC TTT TGG | 2 | - | 0 | assigned. Shown are the 3' end of |
| | E5K3 | A27 | AGC TCA | ACG GA | G CTC ACT TTC | 4 | + | 0 | the respective V_{κ} gene, N-region nu- |
| | E8K1 | 02 | AGT ACC CCT C | GG | CTT TTG GCC | 2 | - | 0 | cleotides (N), and the 5' end of J_{κ} . |
| cost | E8K3 | ATTA | AGG TCA CCT | 0.00 | TGG ACG TTC GGC | 1 | + | 4 | The corresponding V_{κ} and J_{κ} germ- |
| ToM-only | E9K2 F11K3 | 16 | | G GA | G TGG ACG TTC G | 1 | + | 0 | line genes are indicated. The sum of |
| Ign-only | E14K3 | A27 | AGC TCA C | G | G TGG ACG TTC GGC | 1 | + | ů 0 | nucleotide differences relative to |
| | E18K3 | A27 | AGC TCA CC | G AG | C ACT TTC | 4* | + | 1 | the corresponding cormline cones is |
| | E20K1 | 08 | AAT CTC CCT CC | т | TGG ACG TTC GGC | 1 | + | 0 | the corresponding germine genes is |
| | E21K4 | B3 | AGT ACT CCT CC | | G TAC ACT TIT GGC | 2 | + | 0 | shown in the right column. The C to G nucleotide exchange at position |
| | F16K1 | L12A | AGT TAT | CCC AC | G TGG ACG TTC GGC | 1 | + | 0 | two of codon 97 of the novel poly- |
| | F17K3 | A27 | AGC TCA | TCT | TGG ACG TTC GGC | 1 | + | 0 | morphic L2 gape segment (donor |
| | F18K3 | A27 | AGC TCA CCT CC | 0.70 | G ATC ACC TTC GGC | 5 | + | 0 | $\mathbf{P}_{\mathbf{K}}$ is underlined. Full length so |
| | F19K4 | B3 | AGI ACI CC | C IC | C ACC TTC GGC | 5 2* | + | U | KK) is under mied. Full-length se- |
| CD5 ⁺ | F20K3 | 12 | AGE TAGE OCT OC | G CT | G ACG TTC GG | 3* 1 | - | 0 | quences are available from EMBL/ |
| IgH ⁺ IgD ⁺ | F21K1 | 08 | AAT CTC CC | C CCT | TTT CGG | 3* | - | õ | GenBank under accession nos. |
| | F22K1 | L12A | AGT TAT | с | GC TCA CTT TCG | 4 | - | 0 | Z98605–Z98679. *J _k genes identi- |
| | F22K3 [‡] | A27 | | TT TTG A | GG ACG TTC GGC | 1 | + | 0 | fied by J_{κ} typing (see Methods); |
| | F23K1 | L8 | AGT TAC CCT C | GG | ACT TTC GG | 4* | + | 0 | [§] pseudogene; [‡] homology to germ- |
| | F23K3 | L16A | AAC TGG CCT CC | | G CTC ACT TTC GG | 4 | + | 0 | line gene A27 of sequence F22K3 |
| | F25K3 | U2 L6 | AGT ACC CCT AAC TGG CCT CC | G GA | G CTC ACT TTC GG | 5 4 | ++ | 0 | ends at position one of codon 90. |

occur among class-switched and/or IgM-only B cells, and, if this was the case, whether those CD5-positive B lymphocytes harbor mutated V genes. Although class-switched cells are thought to be negative for CD5 expression, in the literature one finds surprisingly little data on this subject (40). To clarify this matter, MACS-enriched CD19⁺ PB B cells derived from seven healthy adult donors were stained with anti-IgD and anti-CD5, and with either anti-IgA or anti-IgG. FACScan analysis revealed in all cases that CD5-expressing cells were barely detectable (i.e., < 1%) among isotype-switched B cells (Fig. 3). On the other hand, we reported in a previous study on a small fraction of CD5⁺ B lymphocytes within the PB IgMonly compartment (31). This result was confirmed by flow-cytometric analysis of PB B cells (from donors RK and AB) that were enriched by magnetic cell separation and stained with anti-IgM, anti-IgD, and anti-CD5 (Fig. 4). The fraction of $CD5^+$ B cells among the IgM-only subset accounted for $\sim 10\%$ each, whereas the fraction of $CD5^+$ B cells among the IgM⁺IgD⁺ compartment amounted to $\sim 20\%$ in both cases.

To determine the level of somatic mutation within rearranged V_{κ} genes, single κ -positive B cells of both donors were isolated from the CD5⁺IgM⁺IgD⁺, the CD5⁺IgM⁺IgD⁻, and, as a control, from the CD5⁻IgM⁺IgD⁻ subset. From donor RK, single cells of all three fractions were isolated twice in two separate experiments. Single-cell PCR and sequencing were performed as described above. Taking both donors together, a total of 106 cells were analyzed by PCR. 52 cells gave rise to a single, and 10 cells to two distinct PCR products. 63 amplificates from 55 cells were sequenced, all of which represented unique $V_{\kappa}J_{\kappa}$ rearrangements (Fig. 5). Eleven cells showed a single out-of-frame rearrangement, whereas six cells carried both a productive and a nonproductive $V_{\kappa}J_{\kappa}$ joint. Again, two cells (C6 and F23) gave rise to two distinct in-frame rearrangements each. The remaining 36 cells revealed a single potentially functional V_{μ} region gene each.

15 out of 18 rearrangements of the CD5-IgM-only compartment turned out to be mutated (range: 2-16 mutations, Figs. 5 and 6 and Table II). Interestingly, the three unmutated sequences represented out-of-frame $V_{\kappa}J_{\kappa}$ joints (Fig. 5). These three genes may be derived from cells that carry somatic mutations in the heavy chain and functional light chain genes: in some κ light chain–expressing B cells, the second (presumably nonfunctional) rearranged k allele is inactivated by deletion of the C_k gene and the κ enhancers, leaving the V_kJ_k joint untouched (41). This deletion most likely abolishes somatic mutation in the respective $V_{\kappa}J_{\kappa}$ joint (33, 42). The frequency of somatic mutation of CD5-IgM-only PB B lymphocytes was calculated to be 2.5% for donor RK, and 2.0% for donor AB (Table II). Both values are in a similar range to mutation frequencies observed for IgM-only PB B cells in previous studies (15, 31). In contrast, only 3 out of 21 rearrangements of the CD5⁺IgM-only subset harbored somatic mutations (1, 4, and 4 nucleotide exchanges), whereas the other 18 sequences were identical to the corresponding V_{κ} and J_{κ} germline genes (Figs. 5 and 6 and Table II). In addition, among 24 rearrangements obtained from CD5⁺IgM⁺IgD⁺ PB B cells, not a single point mutation was found (Figs. 5 and 6 and Table II).

Evidence for a novel polymorphic $J_{\kappa}2$ gene segment. The $J_{\kappa}2$ gene segment was found to be rearranged in eleven clonally independent V_{κ} region genes obtained from cells of donor RK



Figure 6. Distribution of V_{κ} region genes with respect to the number of mutations they carry. Each part depicts the proportion of sequences with mutations as specified. Sequences of donors RK and AB are summarized. (*n*) Total number of sequences of each population.

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(Fig. 5 *A*). Position two of codon 97 of the respective rearranged $J_{\kappa}2$ genes differed from the published $J_{\kappa}2$ germline gene (43) by a C to G nucleotide exchange in nine of these rearrangements. Since six of those V region genes carried no mutation in the rest of their sequence, it is unlikely that the C to G exchanges represent somatic mutations. The result rather suggests that donor RK is heterozygous for a previously unidentified polymorphic $J_{\kappa}2$ gene which replaces a threonine (encoded by ACT) by a serine (AGT) at the last position of the CDRIII. A search in the European Molecular Biology Laboratories database (release 95) revealed that a $J_{\kappa}2$ gene with AGT at codon 97 was previously sequenced from three other individuals, although this was not identified a novel, albeit rare $J_{\kappa}2$ gene polymorphism.

 V_{κ} gene usage of CD5⁺ B cells. We obtained 43 potentially functional $V_{\kappa}J_{\kappa}$ joints from CD5⁺ B cells. The sequences could be assigned to a total of 16 different V_{κ} germline genes of the V_{k} gene families 1, 2, 3, and 4. Nine sequences showed highest homology to the $V_{\mu}3$ gene A27, five sequences each to the single V_{κ}4 (B3) gene and the A3/A19 (V_{κ}2) gene, and four sequences to O2/O12 (V_{κ} 1). Three sequences each could be assigned to L12A (V_{κ}1), L2 (V_{κ}3), and L6 (V_{κ}3). Nine V_{κ} germline genes were found to be rearranged either once or twice (Figs. 2 and 5). The V_{κ} genes that have been used frequently by CD5⁺ B cells in this study, in particular A27, were also found to be overrepresented in compilations of rearranged V_{κ} sequences, the majority of which were derived from conventional B cells (35, 47). Thus, it appears that human CD5⁺ PB B cells do not differ considerably from conventional B cells with regard to V_{μ} gene usage.

N-region additions at $V_{\kappa}J_{\kappa}$ *junctions of* $CD5^+$ *B cells.* A hallmark of murine B cells developing early in ontogeny, a substantial fraction of which belongs to the CD5-expressing subset (1, 48), is the rare occurrence of non-germline-encoded nucleotides at the V_HD_HJ_H junctions (49, 50). On the other hand, N-region additions have frequently been detected in CD5⁺ B cells obtained from adult mice, even though less frequently than in conventional B cells (62% versus 93%) (49–51).

In contrast, N sequences are a common feature of human B lymphocytes already in the fetus, and they appear to be consistently present at $V_H D_H J_H$ junctions of B cells in the adult (52, 53). Moreover, IgH CDRIII sequences derived from CD5⁺ and CD5⁻ human cord blood B cells revealed no significant difference with respect to the frequency of N-region additions (54). In our collection of 57 V_k gene rearrangements amplified from CD5⁺ B cells of three adult donors, 34 contained non-germline-encoded nucleotides at their V_kJ_k junctions (60%), whereas N sequences were found at 10 out of 18 V_kJ_k junctions derived from CD5⁻ B cells (56%) (Figs. 2 and 5). These data, therefore, support the notion that N-region additions occur frequently in human CD5⁺ B cells, and that the frequency, unlike in the mouse, does not differ from that of conventional B cells.

CDRIII lengths of potentially functional V_{κ} region genes. Comparison of the CDRIII lengths of potentially functional V_{κ} region genes demonstrated distinct patterns between CD5⁻ and CD5⁺ B cells: whereas none of the 13 rearrangements obtained from CD5⁻IgM-only B cells revealed CDRIIIs exceeding 9 amino acids, those CDRIIIs were found in 7 of 15 rearrangements (10 codons each) of the CD5⁺IgM-only and in 7 of 18 rearrangements (6 with 10 and one with 11 codons) of the CD5⁺IgM⁺IgD⁺ subpopulation (Fig. 5). The difference between the CD5⁻ subset on the one hand, and the CD5⁺IgMonly as well as the CD5⁺IgM⁺IgD⁺ subset on the other, was statistically significant (P = 0.005 and P = 0.012, respectively). In contrast, the two CD5⁺ B cell fractions did not differ significantly from each other (P = 0.461).

Since it has been suggested that CD5-expressing B cells are involved in autoimmune diseases, particularly in rheumatoid arthritis (1, 5–7), it is interesting to note that Bridges et al. recently reported on increased proportions of V_{κ} transcripts with unusually long CDRIIIs amplified from synovia and PB of patients with rheumatoid arthritis as compared to healthy individuals (55). This overrepresentation, however, was rather due to an increase in $V_{\kappa}J_{\kappa}$ joints containing CDRIIIs of 11 amino acids, than to an increase in those with CDRIIIs of 10 amino acids, the latter being overrepresented in the CD5+ subsets of this study. Thus, the relationship between CD5-positive B cells carrying V_{κ} region genes with extended CDRIII lengths and B cells expressing V_{κ} region genes with long CDRIIIs in patients suffering from rheumatoid arthritis remains to be clarified.

Analysis of tonsillar B cell subsets with regard to CD5 expression. To determine whether the distribution of CD5 on B cell subsets derived from secondary lymphoid organs resembles that seen in the PB, we analyzed tonsillar B cells for expression of CD5 on IgM-expressing, as well as on isotypeswitched B lymphocytes. Besides naive and memory B cells, the tonsil contains a large fraction of GC B cells (17). These cells are known to be CD5-negative (56). To avoid masking in the flow cytometric analysis of tonsillar memory and naive B cells for CD5 expression by the numerous GC B cells, we depleted the latter, and, in addition, T cells, by magnetic cell separation. Concerning the pattern of IgM and IgD expression on the enriched cells, we were surprised to find only two of seven tonsils showing a clearly discernible IgM-only subpopulation. Whereas most tonsils were derived from children or young adults (ages 5–19), these two tonsils were from donors over 30 years old, suggesting that IgM-expressing memory cells in this tissue accumulate with age. In one of these cases, expression of CD5 on IgM^+IgD^+ on the one hand, and IgM-only cells on the other, resembled that seen on their counterparts in the PB (data not shown). In the other case, the sample size was too small to determine the fraction of CD5-positive IgM-only B cells. The IgM⁺IgD⁺ cells of the remaining tonsils, like IgM⁺IgD⁺ PB B cells, comprised $\sim 20\%$ CD5⁺ cells (data not shown). Additionally, fractions enriched for non-GC B cells from three tonsils were analyzed for the CD5 expression on class-switched B cells. Fig. 7 shows that, whereas a sizeable proportion of IgD-positive B cells expressed CD5, few if any CD5-positive cells could be detected among IgG⁺ and IgA⁺ B cells. Thus, at least in the case of the tonsil, the expression pattern of CD5 on B cell subsets of secondary lymphoid organs mirrors that seen on the corresponding PB B lymphocyte subsets (Figs. 3, 4, and 7).

Discussion

Previous investigations suggested that $CD5^+$ B cells in healthy individuals regularly acquire somatic mutations. In three RNA-based studies, somatic mutations were found in 12/ 16 (29), 16/23 (30), and 5/8 (16) V gene transcripts amplified from populations of CD5⁺ B cells isolated by flow cytometry. In yet another RNA-based study, the ratio of mutated to un-



Figure 7. Tonsil. Flow cytometric analysis of MACS-enriched tonsillar non-GC B cells (naive and memory B cells) derived from a 32-yr-old adult. Cells were stained with anti-CD5-PE, anti-IgD-CyChrome and either (*left top*) anti-IgA-FITC or (*left bottom*) anti-IgG-FITC. Windows were set around (*a*) the IgA⁺, (*b*) the IgG⁺, and (*c*) the IgD⁺ populations. (*Right*) CD5 expression of (*top*) IgA⁺, (*middle*) IgG⁺, and (*bottom*) IgD⁺ cells.

mutated V genes (3/9) was lower (46). Moreover, 16 cell lines generated by EBV transformation of $CD5^+$ B cells from healthy adult donors have been described in the literature (25, 57–59). Among those lines, 12 have been reported to be mutated. Collectively, these studies suggested that the majority of $CD5^+$ B cells in healthy humans express somatically mutated V genes.

Despite the conformity of these data, the following observations prompted us to reinvestigate V genes of human CD5⁺ B cells for somatic mutation: first, the finding that most CD5⁺ B cells are found among IgM⁺IgD⁺ B lymphocytes, the vast majority of which carry unmutated V region genes (15-17, 31), implies that most CD5⁺ B cells express unmutated V region genes. Second, we recently observed that IgM-only and classswitched B cells harbor elevated levels of mRNA for k light chains when compared with IgM^+IgD^+ naive B cells (31). Since IgM-only as well as class-switched B cells carry somatically mutated V genes (15, 16, 31), the presence of such cells in a given sample will bias population-based studies of V gene transcripts towards mutated sequences. This consideration is particularly important in analyses of CD5⁺ B cells: since CD5 is expressed on B cells only weakly, the populations of conventional and CD5⁺ B cells overlap in flow cytometric analyses (see Figs. 1, 3, 4, and 7) (2, 3, 16, 29, 30). Thus, sorted CD5+ populations are likely to be contaminated by conventional B cells, \sim 20–30% of which are IgM-only or class-switched cells. We therefore conclude that RNA-based population studies do

not represent a suitable approach to determine the frequency of $CD5^+$ B cells harboring mutated V genes.

Furthermore, the collection of EBV lines derived from CD5⁺ B cells is clearly not representative of the CD5⁺ B cell population; two of the lines harboring mutated V genes express isotypes other than IgM (57), which is atypical for $CD5^+$ B cells, as we and others have shown (Figs. 3 and 7) (40, 60). In addition, in the work of Schutte et al. (59), the two lines analyzed were the only ones that expressed CD5 among 500 lines established from adult PB. This underrepresentation of CD5expressing EBV lines is not surprising, since sorted CD5⁺ B cells usually lose CD5 expression upon EBV immortalization (59, 61). Under these circumstances, the two CD5-positive lines can hardly be regarded as being representative of CD5⁺ B cells. Nevertheless, analyses of the EBV lines indicate that some CD5⁺ B cells carrying somatic mutations can be found in healthy adults, although their frequency cannot be reliably determined from those investigations (25, 57–59).

The vast majority of CD5⁺ PB B cells in healthy humans carry unmutated V region genes. In this study, CD5-expressing PB B cells derived from healthy adults were analyzed for the level of somatic mutation in rearranged V_{κ} genes by single-cell PCR. This approach has several advantages when compared with the analysis of cell lines or cDNA-libraries generated from cell populations (62); a single-cell experiment is obviously not biased by varying transcript levels or by preferential EBV transformation of particular subsets of cells. By analyzing single cells, the frequency of B cells harboring somatically mutated V region genes within a B cell subset can be reliably determined. Furthermore, single-cell PCR allows unambiguous identification of somatic mutations in contrast to cell population analyses that involve cloning of PCR products. Amplificates obtained from single cells can be sequenced directly without being cloned, and therefore sequence errors due to misincorporation by Taq DNA polymerase are negligible.

In a first experiment, V_{κ} region genes of single CD5⁺ CD19⁺ PB B cells were investigated. Among 10 cells analyzed, 2 $V_{\kappa}J_{\kappa}$ joints showed 2 mutations each, whereas the remaining rearranged V_{κ} genes were identical to their corresponding germline genes (Fig. 2 and Table II). This result indicates that the majority of CD5⁺ B cells in healthy humans carry unmutated V genes.

Since the finding of two slightly mutated V genes in the analysis of CD5⁺CD19⁺ PB B cells might imply that a subset of CD5⁺ B cells carries mutated V genes, we sought a way to identify such a putative subpopulation. In man, CD5⁺ B cells are found among IgM-expressing, but not among isotype-switched PB B lymphocytes (Figs. 3 and 4) (40). The IgM⁺ PB B cell compartment consists of $\sim 90-95\%$ IgM⁺IgD⁺ B cells that harbor predominantly unmutated V region genes (15, 17), and of about 5–10% IgM-only B cells, the majority of which were shown to carry mutated V gene rearrangements (31). Both of these subsets contain a fraction of CD5-expressing B lymphocytes (~ 20 and 10%, respectively) (Fig. 4). Thus, if CD5⁺ B cells harboring mutated V genes exist, one might expect to find them within the IgM-only population.

Single IgM-only and single IgM⁺IgD⁺ B cells of the CD5positive fraction were isolated from PB of two donors, and rearranged V_{κ} genes were amplified and sequenced. Whereas all 19 cells of the IgM⁺IgD⁺CD5⁺ B cell subset showed unmutated V_{κ} rearrangements, 3 of the 19 IgM-only CD5⁺ B cells analyzed carried slightly mutated $V_{\kappa}J_{\kappa}$ joints (Figs. 5 and 6 and Table II). In contrast, mutated V_{κ} region genes were obtained from 14 out of 17 cells of the control population, i.e., IgM-only CD5⁻ B cells. The fraction of B cells harboring mutated V_{κ} genes of the latter subset was significantly larger than were the respective fractions of the IgM-only CD5⁺ and IgM⁺IgD⁺ CD5⁺ subsets (P < 0.001 and P < 0.001, respectively). On the other hand, the difference in the proportion of mutated among all cells between the IgM-only CD5⁺ and the IgM⁺IgD⁺CD5⁺ subset (3/19 versus 0/19) did not reach statistical significance (P = 0.115). Owing to the overlap of CD5⁺ and CD5⁻ B cell subsets, we consider it likely that the mutated cells of the IgMonly CD5⁺ fraction are due to cellular contamination of IgMonly CD5⁻ memory B cells.

Nevertheless, we cannot exclude the possibility that mutated CD5⁺ B cells exist infrequently in humans. On the assumption that the mutated CD5⁺ B cells in this analysis do not represent contaminating conventional IgM-only memory B cells, the frequency of mutated CD5⁺ B cells in human PB, based on the results of this study, can be estimated as follows: 90-95% of CD5⁺ B cells express both IgM and IgD; all of those cells analyzed carried unmutated V_{κ} region genes (Figs. 5 and 6 and Table II); and 5-10% of CD5+ B lymphocytes represent IgMonly B cells, 16% of which were mutated (Figs. 5 and 6 and Table II). As a result, the frequency of mutated cells would be < 2% of all CD5⁺ PB B cells. We conclude, therefore, that the vast majority of PB CD5+ B cells express unmutated V region genes. The finding that tonsillar CD5⁺ B cells show the same isotype distribution as do PB CD5⁺ B cells (see Figs. 3, 4, and 7) implies that this conclusion also holds true for CD5⁺ B cells of secondary lymphoid organs.

This sequence analysis shows that IgM-only PB B cells in humans are composed of two separate subsets; $CD5^-$ (conventional) memory B cells harboring mutated V genes, and a small fraction of $CD5^+$ B cells carrying unmutated V genes. Whether the two subsets of $CD5^+$ B cells analyzed in this study, i.e., IgM-only and IgM⁺IgD⁺CD5⁺ B cells, differ in other aspects than the level of IgD expression, remains unclear.

 $CD5^+$ *B* cells involved in diseases can be somatically mutated. B-CLL, small lymphocytic lymphoma, and some cases of prolymphocytic leukemia are considered to represent malignancies of CD5⁺ B cells. Although early studies of antibody genes expressed in B-CLL and small lymphocytic leukemia suggested that the tumor B cells carry unmutated V genes (63– 67), it has become clear that a considerable fraction of cases of CD5-positive B-CLL and prolymphocytic leukemia harbor somatically mutated V region genes (25–28, 68–70). In addition, mutated V genes were also found in each of six EBV-immortalized, flow-cytometrically isolated CD5⁺ B cells producing high-affinity, monoreactive autoantibodies derived from patients suffering from autoimmune diseases (5, 6). Thus, CD5⁺ B cells involved in autoimmunity and/or B cell malignancy frequently harbor somatically mutated V genes.

 $CD5^+$ B cells drawn into a GC reaction in rare instances may be at an increased risk to become involved in autoimmunity and/or B cell malignancy. The frequent occurrence of mutated CD5⁺ B cells in disease contrasts with the finding of this analysis, that the CD5⁺ B cell population of healthy individuals is characterized by unmutated V region genes. How can this apparent discrepancy be explained?

One possibility would be that the disease-involved CD5expressing B cells are not derived from CD5⁺ B cells, but represent conventional B cells that began to express CD5 on the cell surface after they left the GC. This hypothesis finds support in the observation that CD5 expression can be induced on conventional B cells in vitro (9).

One can also, however, envision an alternative scenario (see reference 8). $CD5^+$ B cells represent a population of cells, many of which produce low-affinity, poly- and autoreactive antibodies (4). These cells usually do not participate in T celldependent immune reactions, and hence do not establish GCs. In rare cases, however, CD5⁺ B cells may be drawn into a GC reaction, and within this microenvironment, undergo somatic hypermutation. Through this process, low-affinity autoantibodies may give rise to high-affinity, pathogenic autoantibodies, as they have been detected in patients suffering from autoimmune diseases (5, 6). The vigorous clonal expansion of GC B cells and the processes of somatic hypermutation and class switch recombination may put the long-lived CD5⁺ B cells (71) at an increased risk for malignant transformation, because these activities may raise the chance to acquire transforming events. As a consequence, cases with mutated V genes would be overrepresented among B-CLLs.

The assumption that $CD5^+$ B cells normally do not participate in GC reactions is supported by transfer experiments in the mouse that showed that $CD5^+$ B cells only poorly reconstitute GCs (8, 72). In addition, these results argue against the possibility that $CD5^+$ B cells may establish GCs, and upon doing so, lose CD5 expression, thereby generating $CD5^-$ GC descendants. The scenario discussed in the previous paragraph finds further support by many similarities between the tumor cells in $CD5^+$ B-CLL and $CD5^+$ B cells, indicating their derivation from $CD5^+$ and not from conventional B cells (73). Collectively, the aspects mentioned here support the view that $CD5^+$ B cells, only in rare instances proliferate and mutate within a GC, but if they do so, they are at an increased risk to become involved in immune dysfunctions.

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