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 $J_\kappa 2$ 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 ~ 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 repesent a self-replenishing 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 κ 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 (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) for 20 min at 4°C. Subsequently, κ-expressing B cells were enriched to > 90% by magnetic cell separation using the MiniMACS™ 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 κ-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 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 MiniMACS™. The B cell–enriched 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 (IgG1-isotype; 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 ~ 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_κ1–4 gene families, and a downstream primer specific for a sequence 3′ of J_κ5 was used (see Table I). This approach prevents amplification of V_κ-J_κ1,2,3 & 4 rearrangements that have been located away from the C_κ 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 Igκ 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 $MgCl_2$, 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 first-round reaction mixture in a 50-μl volume containing 10 mM Tris-HCl, pH 8.3 (20°C), 50 mM KCl, 2.0 mM $MgCl_2$, 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 ^{32}P-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 DNASIS™ 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
$V_\kappa 1$	5′-GACATCC<AG>G<AT>TGACCCAGTCTCC<AT>TC-3′
$V_\kappa 2$	5′-CAG<AT>CTCCACTCTCCCTG<CT>CCGTCA-3′
$V_\kappa 3$	5′-TTGTG<AT>TGAC<AG>CAGTCTCCAG<GC>CACC-3′
$V_\kappa 4$	5′-AGACTCCCTGGCTGTGTCTCTGGGC-3′
$3'J_\kappa 5$	5′-CTCTAAA<AG>GTCAATACTGGCCATC-3′
$5'J_\kappa 1,2,4$	5′-ACTCACGTTTGAT<CT>TCCA<GC>CTTGGTCC-3′
$5'J_\kappa 3$	5′-GTACTTACGTTTGATATCCACTTTGGTCC-3′
$5'J_\kappa 5$	5′-GCTTACGTTTAATCTCCAGTCGTGTCC-3′

The V_κ 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 ~ 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 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 $V_\kappa J_\kappa$ 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 Igκ 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.

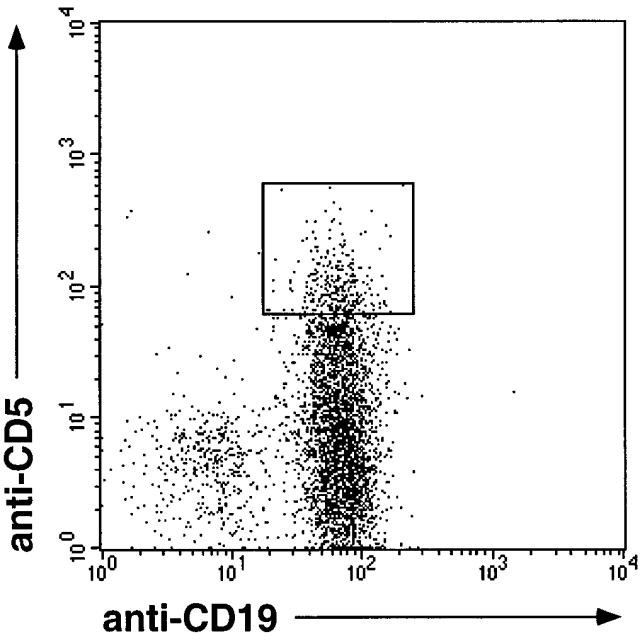

Figure 1. Flow cytometric analysis of κ-expressing B cells (after MACS enrichment) derived from PB of a 27-yr-old person (donor HK). Cells were stained with anti-CD19-FITC and anti-CD5-PE. The gate set for sorting of single $CD5^+CD19^+$ B cells is indicated.

Table II. Summary of Mutations in V_κ Region Genes 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‖
$CD5^+$ $CD19^+$	HK	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	$3'V_k$	$V_k - J_k$ junction	$5'J_k$	J_k	in frame	bp diff.
			93 94 95 95A	N	96 97 98 99			
	X1K4	B3	AGT ACT CC		G TGG ACG TTC GGC	1	+	0
	X2K1	LFVK431	AGT TAC CCT	CTG	ACT TTC GGC	4*	+	0
	X5K4	B3	AGT ACT CCT		TGG ACG TTC GGC	1	+	2
	X6K3	L6	AAC TGG CCT CC		G CTC ACT TTC	4	+	0
	X8K1	L5	AGT TTC CCT C		AC ACT TTT GGC	2	+	2
$CD5^+CD19^+$	X8K4	B3	AGT ACT CC	C	GCT CAC TTT CGG	4	-	0
	X9K2	A17	CAC TGG CCT CCC		TCN CTT TCG	4*	-	0
	X10K2	A3	CAA ACT CCT C	T	A TTC ACT TTC GGC	3	+	0
	X11K4	B3	AGT ACT CC		G ATC ACC TTC GGC	5	+	0
	X12K1	L12A	AGT TAT TCT C		GG ACG TTC GGC	1	+	0
	X12K3	L2	AAC TGG CCT CC	A G	GG ACG TTC GGC	1	+	0
	X13K2	A3	CAA ACT CCT 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^- CD5-expressing 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

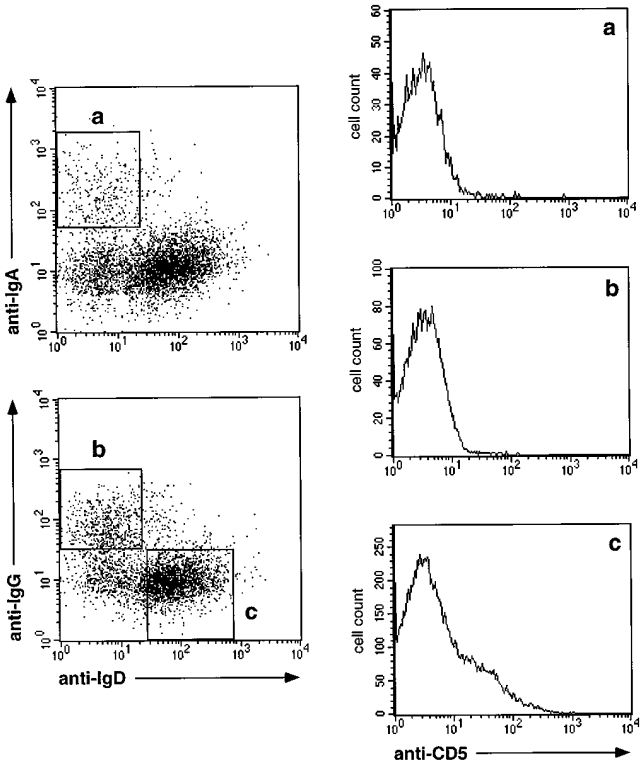

Figure 3. Peripheral blood. Flow cytometric analysis of MACS-enriched $CD19^+$ PB B cells derived from a healthy adult person. Cells were stained with anti-CD5-PE, anti-IgD-CyChrome, and either (*left top*) anti-IgA-FITC or (*left bottom*) anti-IgG-FITC. (*Left*) Windows were set around (*a*) the IgA^+,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

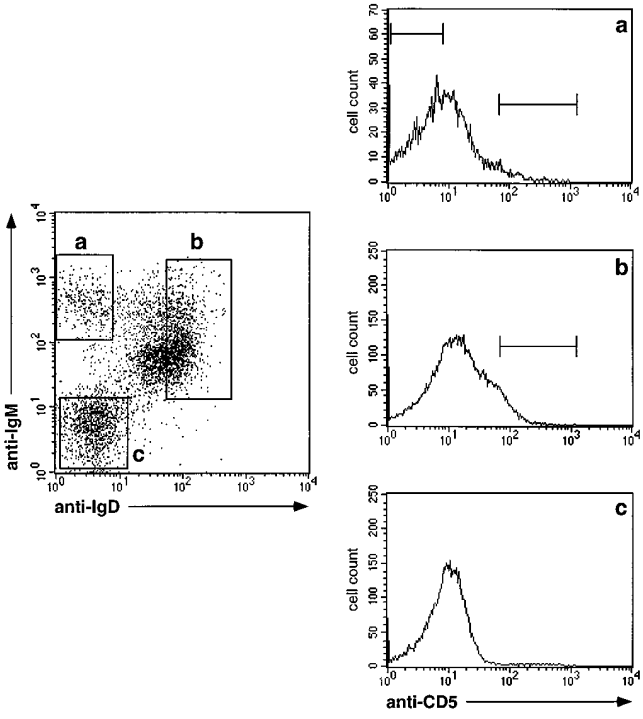

Figure 4. Peripheral blood. Flow cytometric analysis of κ-expressing B cells (after MACS-enrichment) derived from PB of a 32-yr-old person (donor RK). Cells were stained with anti-IgM-PE, anti-IgD-CyChrome, and anti-CD5-FITC. (*Left*) Windows were set around (*a*) the IgM^+IgD^- (IgM-only), (*b*) the 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.

A

		V_k	3′V_k	V_k – J_k junction	5′J_k	J_k	in frame	bp diff.
			93 94 95 95A	N	96 97 98 99			
	A5K3	A27	AG	T GTC	TTC ACT TTC	3	+	10
	A6K1	O18	AGT CTC CC		G TAC ACT TTT GGC	2	+	10
	A9K1	L19A	AGT TTC CC	G G	TC ACT TTC GGC	3	+	7
	A10K2	O11	GAG TTT CCT		TGG ACG TTC GGC	1	+	2
CD5⁻	A14K1	L12A	AGT T		CT TTC GGC	3*	+	12
IgM-only	A30K2	A17	CAC TGG CC	G	C AGT TTT GGC	2	–	7
	AZ2K2	A3	CAA ACT CC		G TAC AGT TTT GGC	2	+	10
	AZ4K1	L18A	AGT TAC CCT		CTC ACT TTC GGC	4	+	8
	AZ5K3	L6	AAC TGG CCT		CTC ACT TTC G	4	+	7
	AZ6K2	A18§	CAC CTT C	GT GGG G	A TTC ACT TTC GGC	3	–	0
	AZ7K2	A3	CAA ACT CC	G GAT	CC TTC GGC	5	–	0
	B7K2	A17	CAC TGG C	GG GG	CT TTC GGC	4*	–	0
	B15K3	A27	AGC TCA CC	A GA	G CTC ACT TTC	4	+	0
	B16K1	O18	AAT CTC CC	C CCA TA	GGC	3*	–	0
	B20K2	A3	CAG ACC CCT		TAC AGT TTT GGC	2	+	4
CD5⁺	B24K1	O18	AAT CTC CCT CC	G A	TTC GGC	5*	–	0
IgM-only	B25K3	A27	AGC TCA CC	G GT	C AGT TTT GGC	2	+	0
	B42K1	O2	AGT ACC CCT C		GG ACG TTC GGC	1	+	0
	B44K3	L2	AAC TGG CCT CC	G A	TC ACT TTC GGC	4	+	0
	BZ1K1	L12A	AGT TAT T	G	C AGT TTT GGC	2	+	0
	BZ2K1	L23	AGT ACC CC	C TTG	TC GGC	4	–	0
	BZ2K2	A3	CAA AC	C TC	G ATC ACC TTC GGC	5	+	0
	C3K1	L5	AGT TTC CCT C		AC AGT TTT GGC	2	+	0
	C4K1	O2	AGT ACC CC		G TAC ACT	2	+	0
	C4K2	A3	CAA ACT CC		GG ACG TTC GGC	1	–	0
	C6K1	L23	AGT ACC CCT		TTC GGC	4	+	0
CD5⁺	C6K2	A17	CAC TGG CC		G TGG ACG TTC	1	+	0
IgM⁺IgD⁺	C8K1	O2	AGT ACC CC		G TAC AGT TTT GGC	2	+	0
	C10K3	A27	AGC TCA CCT C		T TTC GGC	3	–	0
	C15K1	A30	AGT TAC CC	C	TGG ACG TTC GGC	1	+	0
	C31K2	A17	CAC TGG	GTA CC	GT TTT GGC	2	–	0
	C33K2	A3	CAA ACT CC		G TGG ACG TTC GGC	1	+	0
	CZ1K3	A27	AGC TCA CCT C	TC	TAC AGT TTT GGC	2	+	0

B

		V_k	3′V_k	V_k – J_k junction	5′J_k	J_k	in frame	bp diff.
			93 94 95 95A	N	96 97 98 99			
	D11K3	L2	GAC TGG CC	A	ACG TTC GGC	1	+	5
	D14K1	A20	AGT GCC CCT C	AA AT	A CTT TCG	4*	–	0
CD5⁻	D17K2	A2	CA	A TTC CC	C ACT TTC	4*	+	3
IgM-only	D18K3	L2	AAC GGG CC	C	TAC ACT TTT GGC	2	+	16
	D20K1	L9	AGT TAC C	GT	GCT CAC TTT CGG	4	–	2
	D20K3	A27	AGC TCA CC		G ATC ACC TTC GGC	5	+	2
	D24K3	L6	AAC TGG CC		G CTC ACT TTC	4	+	7
	E3K4	B3	AGT ACT C	TC	GTA CAC TTT TGG	2	–	0
	E5K3	A27	AGC TCA	ACG GA	G CTC ACT TTC	4	+	0
	E8K1	O2	AGT ACC CCT C	GG	CTT TTG GCC	2	–	0
	E8K3	A11A	AGG TCA CCT		TGG ACG TTC GGC	1	+	4
CD5⁺	E9K2	A17	CAC TGG CCT CC	G GA	G ACG TTC	1	+	0
IgM-only	E11K3	L6	AAC TGG CCT CC		G TGG ACG TTC G	1	+	0
	E14K3	A27	AGC TCA C	G	G TGG ACG TTC GGC	1	+	0
	E18K3	A27	AGC TCA CC	G AG	C ACT TTC	4*	+	1
	E20K1	O8	AAT CTC CCT CC	T	TGG ACG TTC GGC	1	+	0
	E21K4	B3	AGT ACT CCT CC		G TAC ACT TTT GGC	2	+	0
	F16K1	L12A	AGT TAT	CCC AC	G TGG ACG TTC GGC	1	+	0
	F17K3	A27	AGC TCA	TCT	TGG ACG TTC GGC	1	+	0
	F18K3	A27	AGC TCA CCT CC		G ATC ACC TTC GGC	5	+	0
	F19K4	B3	AGT ACT CC	C TC	C ACC TTC GGC	5	+	0
	F20K1	L12A	AGT TAT TCT		TCA CTT TCG GC	3*	–	0
CD5⁺	F20K3	L2	AAC TGG CCT CC	G CT	G ACG TTC GG	1	+	0
IgM⁺IgD⁺	F21K1	O8	AAT CTC CC	C CCT	TTT CGG	3*	–	0
	F22K1	L12A	AGT TAT	C	GC TCA CTT TCG	4	–	0
	F22K3‡	A27		TT TTG A	GG ACG TTC GGC	1	+	0
	F23K1	L8	AGT TAC CCT C	GG	ACT TTC GG	4*	+	0
	F23K3	L16A	AAC TGG CCT CC		G CTC ACT TTC GG	4	+	0
	F24K1	O2	AGT ACC CCT	TC	G ATC ACC TTC GGC	5	+	0
	F25K3	L6	AAC TGG CCT CC	G GA	G CTC ACT TTC GG	4	+	0

Figure 5. Nucleotide sequences of $V_\kappa J_\kappa$ junctions amplified from $CD5^-IgM$-only, $CD5^+IgM$-only, and $CD5^+IgM^+IgD^+$ PB B cells by single-cell PCR. (*A*) Donor RK; (*B*) donor AB. Sequences are designated by one or two uppercase letters 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. The corresponding V_κ and J_κ germline genes are indicated. The sum of nucleotide differences relative to the corresponding germline genes is shown in the right column. The C to G nucleotide exchange at position two of codon 97 of the novel polymorphic $J_\kappa 2$ gene segment (donor RK) is underlined. Full-length sequences are available from EMBL/GenBank under accession nos. Z98605–Z98679. *J_κ genes identified by J_κ typing (see Methods); §pseudogene; ‡homology to germline gene A27 of sequence F22K3 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 IgM-only 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_κ 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 κ allele is inactivated by deletion of the C_κ gene and the κ enhancers, leaving the $V_\kappa J_\kappa$ 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 (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 as a polymorphism in those studies (44–46). Thus, we identified a novel, albeit rare $J_\kappa 2$ gene polymorphism.

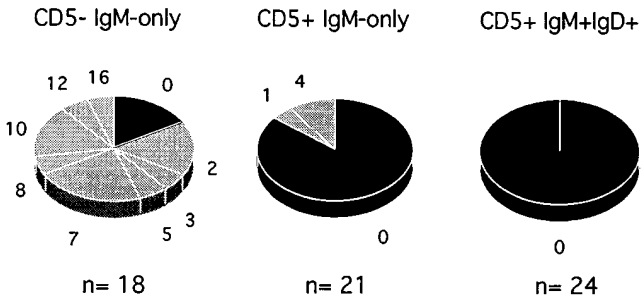

Figure 6. Distribution of V_κ region genes with respect to the number of mutations they carry. Each part depicts the proportion of sequences with mutations as specified. Sequences of donors RK and AB are summarized. (*n*) Total number of sequences of each population.

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_κ gene families 1, 2, 3, and 4. Nine sequences showed highest homology to the $V_\kappa 3$ gene A27, five sequences each to the single $V_\kappa 4$ (B3) gene and the A3/A19 ($V_\kappa 2$) gene, and four sequences to O2/O12 ($V_\kappa 1$). Three sequences each could be assigned to L12A ($V_\kappa 1$), L2 ($V_\kappa 3$), and L6 ($V_\kappa 3$). 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_κ 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 $V_H D_H J_H$ 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_κ gene rearrangements amplified from $CD5^+$ B cells of three adult donors, 34 contained non-germline-encoded nucleotides at their $V_\kappa J_\kappa$ junctions (60%), whereas N sequences were found at 10 out of 18 $V_\kappa J_\kappa$ 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 $CD5^+IgM^-$ only 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 isotype-switched 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).

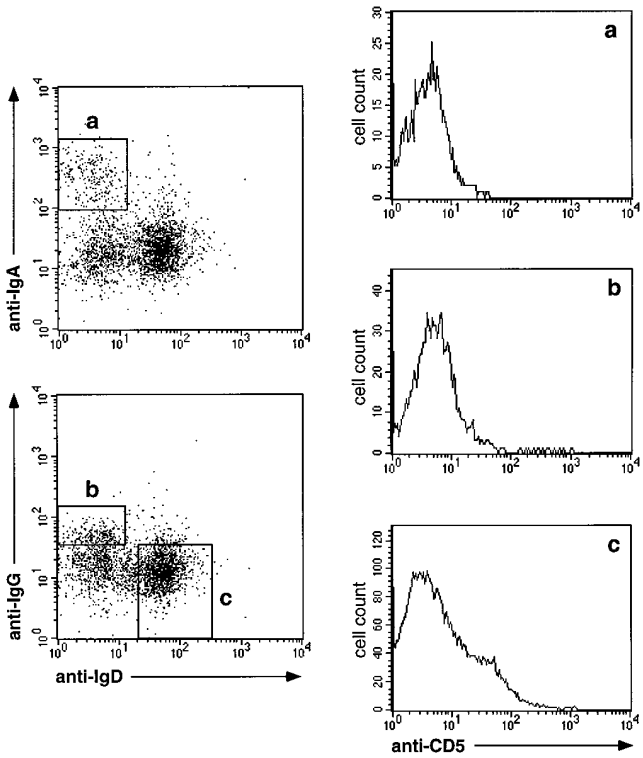

Figure 7. Tonsil. Flow cytometric analysis of MACS-enriched tonsillar non-GC B cells (naive and memory B cells) derived from a 32-yr-old adult. Cells were stained with anti-CD5-PE, anti-IgD-CyChrome and either (*left top*) anti-IgA-FITC or (*left bottom*) anti-IgG-FITC. Windows were set around (*a*)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

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 unmutated 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 class-switched B cells harbor elevated levels of mRNA for κ 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 CD5-expressing 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 ($\sim$ 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 CD5-positive 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 IgM-only $CD5^+$ fraction are due to cellular contamination of IgM-only $CD5^-$ 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 IgM-only 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 CD5-expressing B cells are not derived from $CD5^+$ B cells, but rep-

resent 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 cell–dependent 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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