Human Immunoglobulin (Ig)M$^+$IgD$^+$ Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells

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Summary

Immunoglobulin (Ig)M$^+$IgD$^+$ B cells are generally assumed to represent antigen-inexperienced, naive B cells expressing variable (V) region genes without somatic mutations. We report here that human IgM$^+$IgD$^+$ peripheral blood (PB) B cells expressing the CD27 cell surface antigen carry mutated V genes, in contrast to CD27-negative IgM$^+$IgD$^+$ B cells. IgM$^+$IgD$^+$CD27$^+$ B cells resemble class-switched and IgM-only memory cells in terms of cell phenotype, and comprise ~15% of PB B lymphocytes in healthy adults. Moreover, a very small population (<1% of PB B cells) of highly mutated IgD-only B cells was detected, which likely represent the PB counterpart of IgD-only tonsillar germinal center and plasma cells. Overall, the B cell pool in the PB of adults consists of ~40% mutated memory B cells and 60% unmutated, naive IgD$^+$CD27$^-$ B cells (including CD5$^+$ B cells). In the somatically mutated B cells, V$_H$ region genes carry a two- to threefold higher load of somatic mutation than rearranged V$_\kappa$ genes. This might be due to an intrinsically lower mutation rate in κ light chain genes compared with heavy chain genes and/or result from κ light chain gene rearrangements in GC B cells. A common feature of the somatically mutated B cell subsets is the expression of the CD27 cell surface antigen which therefore may represent a general marker for memory B cells in humans.

Key words: B cell • CD27 • immunoglobulin D • memory B cell • somatic hypermutation

In T cell–dependent immune responses, naive B cells are recruited into the germinal centers (GC)[1] of peripheral lymphoid organs after antigen activation. Within these structures, antibody mutants are generated through the process of somatic hypermutation. Eventually, high affinity B cells are selected either into the plasma cell or the memory B cell pool (1). In the mouse, memory B cells have mostly switched from the initial expression of IgM to that of other Ig classes. Therefore, it came as a surprise that in humans, substantial numbers of IgM-expressing memory B cells seem to occur along with the "classical" class-switched memory B cells (2, 3). We identified a population of somatically mutated IgM-bearing B cells in the peripheral blood (PB), namely IgM$^+$IgD$^-$ (IgM-only) cells (4), that phenotypically, and presumably also functionally (5), resemble class-switched cells. In the PB, IgM-only and class-switched cells each comprise 10–15% of all B lymphocytes. IgM-only cells also occur at high numbers in the various

lymphoid organs (see reference 4), notably in the splenic marginal zone (6, 7).

Despite the extensive characterization of human B cell subsets at the level of rearranged V genes in recent years, no concordant picture arose as to whether somatically mutated B cells also accumulate in the IgD-expressing compartment. Several studies on tonsillar IgM$^+$IgD$^+$ B cells (8–10) as well as IgD-expressing PB B cells (11, 12) have indicated that somatically mutated IgD memory B cells occur, if at all, at a low frequency. This implies that in humans, as in the mouse, IgD represents a marker for naive B cells. However, the recent work of Paramithiotis and Cooper (13) challenges this view: based on the findings of (*a*) mutated μ-transcripts in cDNA libraries generated from mature bone marrow B cells and (*b*) a high number of mature IgM$^+$IgD$^+$ cells in the marrow, they concluded that IgD-bearing memory B cells home to this primary lymphoid organ. In the study of Paramithiotis and Cooper, IgD-expressing B cells were not selectively analyzed, and because somatically mutated B cells express higher levels of Ig mRNA than naive B cells (4), it remains uncertain which fraction of the mutated μ-transcripts was indeed de-

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[1]*Abbreviations used in this paper:* FR, framework region; GC, germinal center(s); PB, peripheral blood; R/S, ratio of replacement to silent mutations.

rived from the presumed IgM⁺IgD⁺ memory B cells in the bone marrow, and not from IgM-only memory cells or (contaminating) IgM plasma cells.

A special case represents tonsillar IgD⁺IgM⁻ GC B cells that have deleted the cμ gene and harbor an exceptionally high load of somatic mutations (12). These cells probably differentiate into somatically mutated IgD-only plasma cells homing to the tonsillar subepithelium (14), but descendents of those cells have not been observed in the PB (12).

Maurer et al. (15) and Agematsu et al. (16) recently described two subsets of IgD⁺ B cells in human tonsils and PB, respectively, that can be distinguished by the expression of the CD27 cell surface antigen, a member of the TNF receptor family. This antigen is expressed on essentially all IgD⁻, i.e., class-switched and IgM-only, PB B cells. In vitro, IgD⁺CD27⁺ but not IgD⁺CD27⁻ cells respond to activation stimuli in the same way as IgD⁻CD27⁺ B cells. Here we characterize this newly described IgD⁺ CD27⁺ B cell subset with respect to both the level of V gene mutation and cell phenotype, and compare those cells with class-switched and IgM-only memory B cells as well as with IgD⁺CD27⁻ cells.

Materials and Methods

Cell Separation and Flow Cytometry. Buffy coats of healthy adult donors were obtained from the blood bank of the Institut für Transfusionsmedizin of the Cologne University Hospital. PBMC were isolated by Ficoll-Isopaque density centrifugation, and CD19⁺ B cells were enriched to >98% by magnetic cell separation using the MiniMACS® system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described (11). For the analysis of cell surface antigens, the B cell–enriched cell suspension was incubated with biotinylated goat anti–human IgD (GaH-IgD; Southern Biotechnology Associates, Inc., Birmingham, AL), with FITC- or PE-conjugated anti-CD27 (PharMingen, San Diego, CA) and with either anti-CD23–FITC, anti-CD5–FITC (both from Becton Dickinson, Mountain View, CA), or GaH-IgM–PE (Sigma, München, Germany) for 10 min on ice. After washing with PBS/0.5% BSA, GaH-IgD was developed with Streptavidin-CyChrome (PharMingen). The cell suspensions were analyzed on a FACScan® (Becton Dickinson).

For the isolation of single IgD⁺CD27⁺ and IgD⁺CD27⁻ B cells from the PB of three healthy donors, the B cell–enriched cell suspensions were preincubated for 5 min with 1 mg/ml Beriglobin (Behringwerke AG, Marburg, Germany), a human Ig fraction, followed by an incubation with FITC (isomer 1)-conjugated anti-CD27 and biotinylated GaH-IgD for 15 min. After washing and a further 5-min incubation with Beriglobin, the cell suspension was stained with digoxygenated anti–FITC-isomer 1 (a gift of A. Thiel, Deutsches Rheuma-Forschungszentrum, Berlin, Germany). After two washing steps, the cells were incubated on ice with FITC-containing antidigoxigenin liposomes for 30 min under constant agitation (for the manufacturing of liposomes, see reference 17). After three washing steps, single IgD⁺CD27⁺ and IgD⁺CD27⁻ B cells were sorted on a FACS® 440 (Becton Dickinson) directly into PCR tubes containing 20 μl Expand High Fidelity PCR buffer (Boehringer Mannheim, Mannheim, Germany) and 20 ng 5S rRNA. Likewise, single IgM-only as well as IgD-only PB B cells were isolated from donor 3 after staining the CD19⁺-enriched cell suspension against IgD and IgM as described above.

For the isolation of single κ-expressing IgG⁺&IgA⁺ PB B lymphocytes, PBMC were isolated from 50 ml PB of a healthy volunteer (donor 4). PBMC were incubated with anti-κ (Becton Dickinson), and, after washing, goat anti–mouse (GaM)-κ microbeads (Miltenyi Biotec GmbH) as described (18). The cell suspension was stained with biotinylated GaH-IgD and both GaH-IgG–FITC (Southern Biotechnology Associates, Inc.) and GaH-IgA–FITC (Dako, Hamburg, Germany), followed, after washing, by an incubation with Streptavidin-CyChrome. Individual IgG⁺&IgA⁺ B cells were isolated as described above.

Single-cell PCR. Single cells in PCR buffer (see above) were incubated with 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 six V_H and four V_κ family–specific primers, which recognize sequences in framework region (FR) I of the members of the $V_H1–6$ and the $V_\kappa1–4$ gene families, and both a $3'J_H$ and a $3'J_\kappa$ primer mix were used (18-20). 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), 2.5 mM MgCl₂, 100 μM dATP, dGTP, dTTP, and dCTP, 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 59°C, and 60 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 and the V_H primers for the three largest V_H families (V_H 1, 3, and 4) were used together with a $5'J_H$ or $5'J_\kappa$ primer mix (18, 20). The second round of amplification was performed in separate reactions for each of the three V_H and 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, 50 mM KCl, 1.5 mM MgCl₂, 100 μM dATP, dGTP, dTTP, and dCTP, 125 nM of each primer, and 0.7 U Taq DNA polymerase. The amplification conditions were as described above, except that 45 cycles were applied and the annealing temperature raised to 61°C. For the amplification of rearranged V_H genes from IgD-only cells, V_H family–specific primers were used that hybridize to sequences in the leader-peptide region of the members of the V_H1, 3, and 4 gene families (21). The respective PCR reaction mixtures and cycling conditions were as described above. PCR products were purified by gel electrophoresis. An aliquot of the isolated DNA was sequenced directly using the Ready Reaction DyeDeoxy-Terminator or BigDye cycle sequencing kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) and an ABI377 sequencer (Perkin-Elmer Applied Biosystems). Nucleotide sequences were analyzed using DNASIS software (Pharmacia GmbH, Freiburg, Germany) and the IMGT database (http://www.genetik.uni-koeln.de/dnaplot/). Some of the sequenced PCR products turned out to represent either non-Ig sequences or double sequences of two rearrangements using the same V gene family. These sequences are not further considered. Some PCR products were not sequenced.

Results

IgD⁺CD27⁺ PB B Lymphocytes Phenotypically Resemble Class-switched and IgM-only Memory Cells. Functional studies performed on isolated IgD⁻CD27⁺, IgD⁺CD27⁺, and IgD⁺CD27⁻ PB B cells revealed that IgD⁺CD27⁺ B cells respond in a similar way to activating stimuli as IgD⁻ B cells (16). To examine whether IgD⁺CD27⁺ cells share

phenotypic similarities with "classical" class-switched and IgM-only memory cells, we stained this subset for cell surface markers which are differentially expressed on memory B cells on the one hand and naive B cells on the other, namely CD23 and CD5 (see reference 4). B cell–enriched fractions from several donors were stained for IgD, CD27, and the respective antibodies against CD23 and CD5 and analyzed on a FACScan® (Fig. 1). Like class-switched and IgM-only cells, IgD^+CD27^+ B cells were predominantly $CD23^-$ and $CD5^-$. The IgD^+CD27^- fraction, on the other hand, contained both $CD23^+$ and $CD5^+$ cells (Fig. 1). Staining for IgM expression revealed that the majority of IgD^+CD27^+ cells—in contrast to IgD^+CD27^- cells—express high levels of surface IgM (Fig. 1). In this respect, IgD^+CD27^+ cells resemble IgM-only B cells (4). To confirm the observation of Agematsu et al. that IgD^- B lymphocytes express CD27 (16), IgG^+, IgA^+, and IgM-only B cells were selectively analyzed for CD27 expression. As expected, cells of those subsets were $CD27^+$ (data not shown).

To determine the percentages of the IgD^-CD27^+, IgD^+CD27^+, and IgD^+CD27^- subsets among PB B lymphocytes, B cell–enriched fractions from the PB of eight healthy adults were stained for IgD and CD27 and analyzed flow-cytometrically (not shown). The PB of the donors showed considerable variation in the frequencies of the
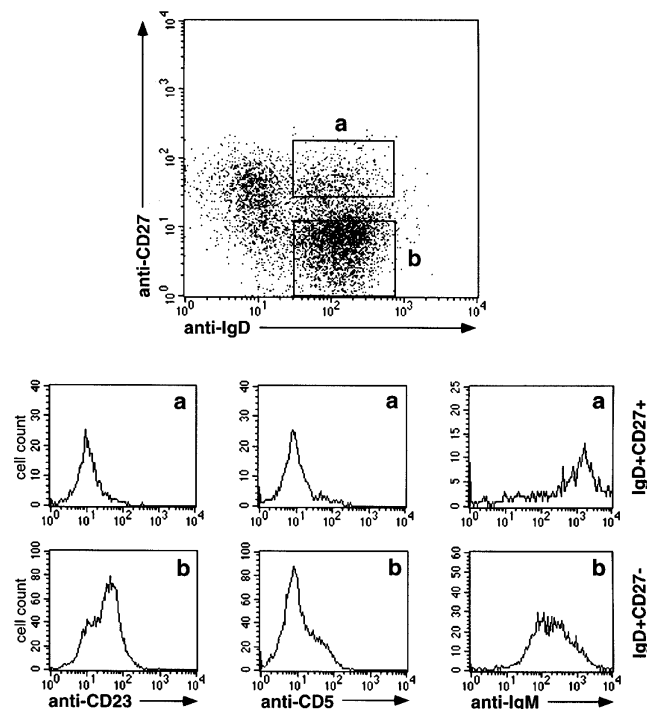
PB B cell subsets, with 29–65% IgD^+CD27^-, 6.5–22% IgD^+CD27^+, and 13–43% IgD^-CD27^+ B cells. In accord with previous results (16), the B cell pool in the PB comprises on average 60% IgD^+CD27^-, 15% IgD^+CD27^+, and 25% IgD^-CD27^+ B cells. The latter population can be further subdivided in on average 15% IgG^+&IgA^+ and 10% IgM-only cells.

IgD-expressing PB B Cells Can Be Subdivided into Somatically Mutated $IgM^+IgD^+CD27^+$ B Cells and Unmutated $IgM^+IgD^+CD27^-$ B Cells. IgD^+CD27^+ and IgD^+CD27^- PB B cells were analyzed for the level of somatic mutation in their rearranged V_H genes. After enrichment of $CD19^+$ B cells derived from the PB of three healthy adults by magnetic cell separation, the corresponding cell suspensions were stained for IgD and CD27 (using FITC-conjugated liposomes; see Materials and Methods), and single IgD^+CD27^+ and IgD^+CD27^- cells were isolated flow-cytometrically. The use of FITC-conjugated liposomes, which stain 100–1,000 times brighter than FITC or PE, was necessary to achieve a good separation of the $CD27^+$ and $CD27^-$ populations on the FACS® 440. Sorting gates set for the isolation of single cells of the respective subsets are indicated in Fig. 2. While IgD^+CD27^+ cells were isolated from all three donors, IgD^+CD27^- cells were sorted only from donors 1 and 2. Since the vast majority of both IgD^+CD27^+ and IgD^+CD27^- B cells coexpress IgM (see Fig. 1), these populations are designated below as $IgM^+IgD^+CD27^+$ and $IgM^+IgD^+CD27^-$ B cells, respectively.

Rearranged V_H genes were amplified by PCR from the genomic DNA of the cells using a seminested approach. Negative controls consisted of reaction mixtures without cells and were always negative. From the 3 donors, a total of 156 cells were analyzed. From 69 cells a potentially functional rearrangement, and from 6 cells only a nonfunctional rearrangement, were obtained. 12 cells gave rise to 2 V gene rearrangements; in 9 cases a nonfunctional $V_HD_HJ_H$ joint was amplified in addition to a potentially functional rearrangement; and in the remaining cases, 2 potentially functional joints were obtained. The amplification of two potentially functional rearrangements from one sample could either be due to sorting of two cells into one reaction tube or could indeed reflect the presence of two functional V_H gene rearrangements in a B cell, as has been described for cases of B cell chronic lymphocytic leukemia (22). However,

Figure 1. Fluorescence analysis of IgD^+ PB B cell subsets with regard to the expression of CD23, CD5, and IgM. $CD19^+$ PB B cells enriched by magnetic cell separation (99% purity) were stained with FITC- or PE-conjugated anti-CD27, anti-IgD–CyChrome, and anti-CD23–FITC, anti-CD5–FITC, or anti-IgM–PE. (*Top*) Windows were set around the IgD^+CD27^+ and the IgD^+CD27^- populations and analyzed for CD23, CD5, and IgM staining within the respective fractions (*histograms*).
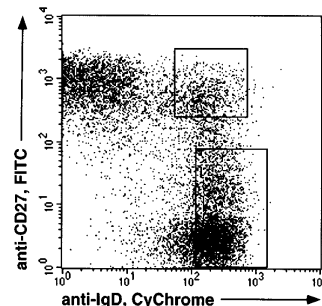

Figure 2. Fluorescence analysis of B cells derived from the PB of a healthy adult. Anti-IgD/anti-CD27 two-color staining of $CD19^+$ PB B cells enriched by magnetic cell separation (>98% purity). Indicated are the gates set for sorting of single IgD^+CD27^+ and IgD^+CD27^- cells.

Table 1. *V_H Gene Sequences of IgD⁺CD27⁺ and IgD⁺CD27⁻ B Cells*

	CD27⁺					CD27⁻			
Cell	V_H gene	Pot. funct.	Mutations		Cell	V_H gene	Pot. funct.	Mutations	
Donor 1			*no.*	*%*				*no.*	*%*
D1/7	DP46 (3)	+	3	1.5	D1/2	(Z80675) (3)	+	1	0.5
D1/8	V3-23	+	6	3.0	D1/3	V3-30	+	0	0
D1/9	V3-48	+	2	1.0	D1/4	V3-9	+	0	0
D1/10	3d279d (4)	+	6	3.0	D1/5	V4-34	+	0	0
D1/11	V3-23	+	10	5.0	D1/7	V3-53	−	0	0
	DP70 (4)	+	5	2.6		3d279d (4)	+	0	0
D1/12	V3-7	+	4	2.0	D1/8	DP48 (3)	+	0	0
D1/14	V3-23	+	0	0	D1/10	V3-30	+	0	0
D1/15	DP50 (3)	+	6	3.0	D1/11	V3-30	+	0	0
D1/16	V3-9	+	0	0	D1/13	V3-15	−	0	0
D1/17	DP46 (3)	+	12	6.0	D1/14	V3-11	+	0	0
D1/19	V3-21	+	3	1.5	D1/16	V3-11	+	0	0
D1/20	V3-53	−	13	5.9	D1/19	DP64 (4)	+	0	0
D1/22	V3-53	+	12	6.7	D1/20	V3-23	+	0	0
D1/23	DP7 (1)	+	5	2.2	D1/21	LSG12.1 (3)	+	0	0
D1/25	V3-11	−	4	2.3	D1/22	DP46 (3)	+	0	0
D1/26	V3-48	+	24	12.4	D1/23	V3-23	+	0	0
D1/27	V3-23	+	5	2.5	D1/26	DP10 (1)	−	0	0
D1/28	DP29 (3)	+	13	6.5		V3-23	+	0	0
D1/29	V3-53	−	6	3.4	D1/27	V3-15	+	0	0
	4.30 (4)	+	12	6.1	D1/29	V3-30	+	0	0
D1/30	cos8 (3)	+	9	4.5					
D1/31	DP71 (4)	+	5	2.6					
D1/32	V3-23	+	11	5.6					
Means				**3.7**					**0**
Donor 2									
D2/1	DP50 (3)	+	13	6.5	D2/1	DP78 (4)	+	0	0
	V4-4b	−	11	5.6	D2/2	V3-7	+	0	0
D2/2	DP29 (3)	+	2	1.0	D2/8	DP88 (1)	+	0	0
D2/3	DP58 (3)	+	9	4.5	D2/13	V1-18	+	0	0
D2/5	V3-9	+	9	4.5	D2/16	V3-21	+	0	0
D2/6	V4-31	−	9	3.3	D2/18	V3-15	+	0	0
D2/7	V3-30	+	11	5.5	D2/23	V4-34	+	0	0
D2/8	V3-53	+	9	4.7	D2/25	V3-30	+	0	0
D2/9	DP67 (4)	+	6	3.1	D2/26	V4-31	+	0	0
	V3-23	−	20	9.0	D2/28	V3-9P	+	0	0
D2/11	DP67 (4)	+	30	13.5	D2/30	V3-30	+	0	0
	DP46 (3)	−	24	13.9					
D2/15	DP58 (3)	+	9	4.5					
D2/17	V3-9	+	1	0.5					
D2/18	V1-18	+	9	4.5					
	DP46 (3)	−	13	6.6	Means				**0**

(continued)

Table 1. *(Continued)*

	CD27+			
Cell	VH gene	Pot. funct.	Mutations	
D2/19	V3-15	+	1	0.5
D2/20	V3-15	+	11	5.6
	VH4.16	+	0	0
D2/23	V4-34	+	10	5.2
D2/25	V3-30	+	3	1.5
D2/26	V3-23	+	18	10.0
D2/27	V4-31	+	0	0
Means				**5.0**
Donor 3				
D3/1	DP79 (4)	+	4	2.0
D3/3	V4-34	+	9	4.5
D3/4	V3-23	+	26	12.9
D3/5	V3-23	+	23	11.0
D3/7	V3-23	+	24	12.7
	DP79 (4)	+	14	7.1
D3/8	DP50 (3)	−	15	7.9
	3d279d (4)	+	11	5.5
D3/9	DP50 (3)	−	14	7.1
D3/10	V4-4	+	1	0.5
D3/12	Yac-5 (3)	+	0	0
D3/15	V3-23	+	8	4.9
D3/17	V1-18	+	12	6.2
D3/18	V3-9	−	7	4.3
D3/24	DP75 (1)	+	7	4.0
D3/27	V3-22P	−	13	8.0
	DP75 (1)	+	5	3.0
D3/28	V3-13	+	15	8.9
D3/32	GLSJ2a (3)	+	6	3.6
D3/33	V3-7	+	8	4.8
Means				**5.9**

On average, 195 bp of the V_H gene segment were sequenced. If possible, the V_H gene nomenclature by Matsuda et al. was used (reference 51). For other genes, the V_H family is indicated in parentheses after the gene name. Several sequences from CD27+ B cells harbored deletions and/or insertions in addition to point mutations: D1/20, 1-bp deletion in FRI and 1-bp deletion in CDRII; D1/25, 28-bp deletion in CDRII; D1/29 (V3-53), 11-bp deletion in CDRII; D1/32, 3-bp deletion in CDRII; D2/6 bp, two overlapping duplications (28 and 21 bp) in FRIII; D2/9 (V3-23), 1-bp deletion in CDRII; D2/11 (DP46), 17-bp duplication in FRI and 47-bp deletion in CDRII; D2/11 (DP67), 6-bp duplication in CDRII; D3/7 (V3-23), 3-bp deletion in CDRII; D3/8 (DP50), 6-bp deletion in CDRII; D3/27 (V3-22P), 34-bp duplication in J_H. The sequences reported in this paper are available from EMBL/GenBank/DDBJ under accession no. AJ231545–AJ231685. *Pot. funct.*, Potentially functional.

it is also possible that in those cells one of the potentially functional rearrangements was in reality not functional. All sequences represented unique $V_HD_HJ_H$ joints (not shown). The results of the sequence analysis are listed in Table 1.

Whereas all but 1 of the 32 rearranged V_H genes amplified from the IgM+IgD+CD27− cells were unmutated, 63 of 67 V_H genes analyzed from the IgM+IgD+CD27+ population showed somatic mutations (1–30-bp differences compared with the most homologous germ-line genes; Table 1). In 11 of the rearrangements of the IgM+IgD+CD27+ fraction, deletions/insertions of variable sizes were identified in addition to point mutations (Table 1). This is in accord with recent findings of a considerable frequency of deletions and/or insertions in B cells undergoing somatic mutation (20, 23). The average somatic mutation frequencies of the IgM+IgD+CD27+ B cells (considering only nucleotide exchange mutations) were determined to be 3.7% for donor 1, 5.0% for donor 2, and 5.9% for donor 3 (see Table 3). These results demonstrate that in addition to IgM-only B cells, IgM+IgD+CD27+ cells represent a further population of IgM-bearing B cells in the PB that express somatically mutated V genes. Furthermore, this analysis shows that IgM+IgD+ B cells which carry unmutated V genes are CD27−.

Since replacement mutations are usually counterselected within the FRs of antibody V region genes to preserve the structure of the V domain, antigen-selected B cells show on average a replacement/silent (R/S) mutation ratio between 1.0 and 1.5, i.e., considerably smaller than the value expected assuming random mutagenesis (∼3.0; reference 24). For the IgM+IgD+CD27+ B cells, an R/S value of the mutations within the FRs of 1.5 was determined (not shown), which is in the same range as that typical for class-switched and IgM-only memory B cells (24).

A Minute Fraction of PB B Cells Consists of IgM− IgD+CD27+ Cells Expressing Highly Mutated V_H Region Genes. In most samples of B cell–enriched cell suspensions stained for IgM and IgD, an IgD+IgM− population could be recognized that comprised usually <1% of PB B lymphocytes. These cells were found to be CD27+ (Fig. 3). To determine whether such cells harbor somatically mutated V genes, single IgM−IgD+ cells were flow-cytometrically isolated from the B cell–enriched cell suspension of donor 3 stained against IgD and IgM (not shown). IgD-only cells comprised <0.5% of PB B lymphocytes in this case. Rearranged V genes were amplified from the genomic DNA using V_H leader primers in a seminested PCR strategy as described above. 34 cells were analyzed. From 13 cells, 1 V_H gene rearrangement each was amplified. The sequences of all amplicates were unique (not shown). Four rearrangements were unmutated. The remaining nine $V_HD_HJ_H$ joints carried a high load of somatic mutations ranging from 15 to 59 bp differences to their respective V_H germline genes (Table 2). The high load of somatic mutation within the rearranged V region genes of IgD-only B cells might also explain why the PCR efficiency for these cells was relatively low (13 of 34 cells positive): mutations at the primer binding sites may have often resulted in fail-
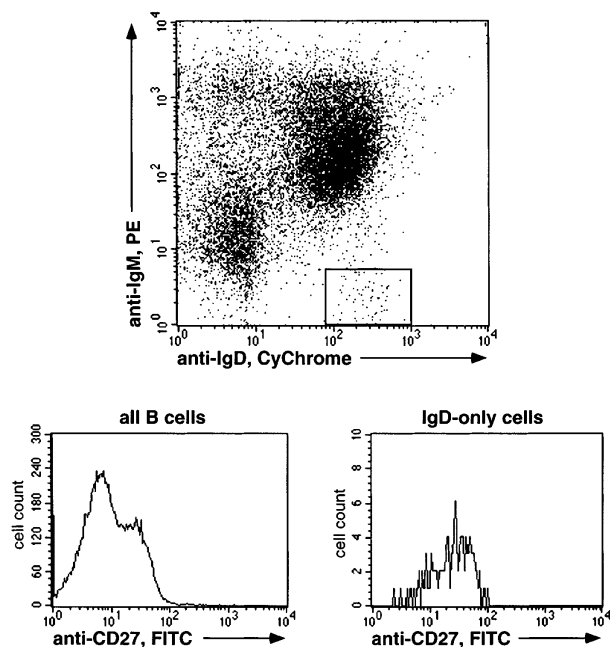
Figure 3. Fluorescence analysis of PB B cells with regard to the expression of CD27. Anti-IgD/anti-IgM double-fluorescence analysis of CD19^{+} PB B cells enriched by magnetic cell separation (99% purity). (*Top*) A window is set around the IgD-only population analyzed for CD27 staining compared with all B cells (*histograms*). The sample depicted here showed an exceptionally high percentage of IgD-only cells, which is usually in the range of 0.5% of all PB B lymphocytes. In total, 10^5 events were collected on a FACScan®.

ure of successful amplification. Six of these sequences were potentially functional, one represented an out-of-frame rearrangement, and in two instances the functionality of the original rearrangement before the accumulation of mutations was uncertain (Table 2). Three of the mutated rearrangements showed deletions and/or insertions.

The unmutated sequences likely stemmed from contaminating IgM^{+}IgD^{+}CD27^{−} B cells, which represent a much larger cellular compartment than IgD-only cells. The mutated sequences show an exceptionally high mutational load (average 15.2%) which has previously been described only for V regions expressed by IgD-only GC B cells (~12% mutation; reference 12) and IgD-only tonsillar plasma cells (14). Therefore, it appears that the IgM^{−}IgD^{+} B cells analyzed here represent the PB descendents of IgD-only GC B cells. Since IgM^{−}IgD^{+}CD27^{+} cells were included in the sorter gate set for the isolation of IgD^{+}CD27^{+} cells, it is possible that some of the (highly mutated) sequences in the analysis of those cells (Table 1) were indeed derived from IgD-only B cells. The low average R/S value for mutations within the FRs of the six potentially functional V_H region genes of the IgD-only cells (1.4) indicates selection of these cells for antigen receptor expression.

The Level of Somatic Mutation in V_H Regions Expressed by IgM^{+}IgD^{+}CD27^{+} B Cells Is in the Same Range as That of IgM-only B Cells. The average V_H gene mutation frequency of IgM^{+}IgD^{+}CD27^{+} B cells was determined to be ~5%. In our previous work, we focused on rearranged V_κ

Table 2. *V_H Gene Sequences of IgD-only B Cells from Donor 3*

Cell	V_H gene	Pot. funct.	Mutations		Deletions/insertions
			no.	*%*	
D3/1	V1-18	+	0	0	
D3/4	I.9III (3)	?*	27	9.6	2-bp del., 9-bp del.
D3/5	DP38 (3)	+	23	7.7	
D3/10	V3-23	?	59	22.1	1-bp ins. in FRI, 13-bp del. in CDRI/FRII
D3/11	V1-18	+	48	16.5	
D3/12	DP38 (3)	?‡	0	0	
D3/15	DP75	+	15	5.1	
D3/16	DP75	+	49	16.7	
D3/18	V1-18	+	44	15.0	
D3/21	V3-23	−	57	33.5	12-bp del. in CDRI, 23-bp del. in CDRII
D3/22	V3-9P	+	24	10.8	
D3/26	VIV-4	+	0	0	
D3/27	V3-30	+	0	0	
Mean			**15.2**§		

On average, 256 bp of the V_H gene segment were included in the mutation analysis. If possible, the V_H gene nomenclature by Matsuda et al. was used (reference 51). For other genes, the V_H family is indicated in brackets after the gene name. *del.*, Deletion. *ins.*, Insertion. *Pot. funct.*, Potentially functional.
*In-frame rearrangement rendered nonfunctional by a 2-bp deletion and a mutation in codon 91 resulting in a stop codon.
‡Reading-frame unclear.
§Only mutated rearrangements considered.

genes to determine mutation frequencies of PB B cell subsets (4, 11, 18). To reliably compare the average V gene mutation frequency of the $IgM^+IgD^+CD27^+$ fraction with that of the other somatically mutated, IgM-expressing subset (IgM-only cells), we isolated single IgM-only B cells from donor 3 and determined the level of somatic mutation in V_H regions. 32 IgM-only cells were analyzed. From 16 cells, 1 potentially functional rearrangement per cell was amplified, and from 1 cell, both a nonproductive and a productive $V_HD_HJ_H$ joint (not shown). One potentially functional V_H gene rearrangement showed a 3-bp deletion in CDRII. All sequences showed unique $V_HD_HJ_H$ joints (not shown). All but one of the rearrangements were somatically mutated (5–21-nucleotide differences), yielding an average mutation frequency of 5.8% (Table 3). Thus, the V_H gene mutation frequency determined for IgM-only cells is in the same range as that of the $IgM^+IgD^+CD27^+$ cells analyzed from the same donor (5.9%; Table 3).

In Humans, V_κ Genes Are Considerably Less Mutated than V_H Region Genes. To determine whether the V_κ genes of $IgM^+IgD^+CD27^+$ and IgM-only B cells also harbor a similar load of somatic mutations, rearranged V_κ genes were amplified from the genomic DNA of IgD^+CD27^+ B cells in which mutated V_H regions had already been identified. From the 3 donors, a total of 44 IgD^+CD27^+ B cells, from which V_H gene rearrangements had been obtained, were analyzed for V_κ rearrangements. From 20 cells, 1 rearrangement was obtained per cell; 3 cells gave rise to 2, 1 cell to 3 $V_\kappa J_\kappa$ joints (Table 4). From 12 cells only a potentially functional, from 9 cells 1 or 2 nonfunctional, and from 3 cells both a productive and a nonproductive rearrangement were amplified (Table 4).

All nonproductive V_κ rearrangements were found to be unmutated. This is most likely due to the fact that in λ- (and some κ-) expressing B cells, κ loci harboring nonproductive V_κ rearrangements are usually inactivated by a de-

Table 3. *Somatic Mutation in V_H Region Genes Amplified from Single PB B Cells*

Population	Donor	No. of sequences		Mutations	
		Total	Mutated	Range	Average
					%
$IgM^+IgD^+CD27^-$	1	21	1	0–1	0
	2	11	0	0	0
$IgM^+IgD^+CD27^+$	1	24	22	0–24	3.7
	2	23	21	0–30	5.0
	3	20	19	0–26	5.9
IgM-only	3	18	17	0–21	5.8
IgD-only	3	13	9	15–59*	15.2*

*Only mutated sequences are considered.

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Table 4. *V_H and V_κ Region Genes from IgD^+CD27^+ B Cells*

Cell	V_κ gene	Pot. funct.	Mut.	% mutations	
				V_κ gene	V_H gene
Donor 1					
D1/8	A19 (2)	−	0		
	DPK8 (1)	+	0		
D1/9	Vg (3)	+	4	1.9	1.0
D1/11	A20 (3)	+	3	1.5	5.0, 2.6
D1/12	L12a (1)	+	1	0.5	2.0
D1/15	Ve (1)	−	0		
D1/17	LFVK431 (1)	−	0		
D1/19	O12 (1)	−	0		
	Vg (3)	+	2	1.0	1.5
D1/22	A27 (3)	−	0		
D1/26	DPK21 (3)	+	7	3.5	12.4
D1/28	A17 (2)	−	0		
	DPK21 (3)	−	0		
	DPK8 (1)	+	3	1.4	6.5
Donor 2					
D2/1	A19 (2)	+	8	3.5	6.5, 5.6
D2/2	LFVK432 (1)	+	2	1.0	1.0
D2/3	A20 (1)	−	0		
D2/5	O12 (1)	+	1	0.5	4.5
D2/6	A17 (2)	−	0		
D2/8	Vg (3)	−	0		
	B3 (4)	−	0		
D2/11	DPK21 (3)	+	11	5.6	13.5, 13.9
D2/20	A19 (2)	+	3	1.2	5.6, 0.0
D2/23	A27 (3)	−	0		
Donor 3					
D3/2	B3 (4)	−	0		
D3/7	A17 (2)	+	7	3.2	12.7, 7.1
D3/8	A19 (2)	+	4	1.6	7.9, 5.5
D3/9	DPK21 (3)	+	4	1.9	7.1
D3/16	B3 (4)	−	0		
Mean				**2.0**	**6.1**

Details of the V_H gene sequences are listed in Table 1. V_κ family is indicated in parentheses.

letion of both the C_κ gene and the κ-enhancers (25). $V_\kappa J_\kappa$ joints are often retained on the chromosome, and such $V_\kappa J_\kappa$ joints appear not to be subject to somatic mutation (26, 27). Therefore, it seems reasonable to consider only potentially functional V_κ regions for the determination of the average V_κ gene mutation frequency in $IgM^+IgD^+CD27^+$ cells.

14 of the 15 cells that carried a potentially functional V_κ rearrangement showed somatic mutations (1–11-bp differences; Table 4) in the respective $V_\kappa J_\kappa$ joints. The average

mutation frequency was 2.0% (Table 4). The corresponding V_H gene mutation frequency of the cells in which a mutated V_κ region could be identified amounts to 6.1% (see Table 4). Thus, $IgM^+IgD^+CD27^+$ cells show a level of somatic mutation in their V_κ regions that is in the same range as that seen in IgM-only cells (2%; reference 4), and in both populations of B cells, the V_κ regions are considerably less mutated than the V_H region genes.

To further confirm that the lower level of somatic mutations in κ versus heavy chain genes is a general feature of human B cells, we also amplified V_H and V_κ region genes from single class-switched, κ-expressing B cells of a fourth adult donor. Indeed, the average mutation frequency of 18 V_H region genes amounted to 6.1%, whereas the average mutation frequency of 8 V_κ region genes was twofold lower, namely 3.0% (not shown).

Discussion

$IgM^+IgD^+CD27^+$ B Cells Presumably Represent a Third Phenotypically Defined Memory B Cell Subset in Humans. Almost all $IgM^+IgD^+CD27^+$ B cells analyzed carried somatically mutated V region genes, in contrast to $IgM^+IgD^+CD27^-$ B lymphocytes (Table 1). $IgM^+IgD^+CD27^+$ cells phenotypically resemble both class-switched and IgM-only cells in that they are $CD27^+$, $CD23^-$, and $CD5^-$ (Fig. 1; reference 4), and generally appear to be larger than $CD27^-$ B cells (15, 16; our unpublished observations). Unlike $IgM^+IgD^+CD27^-$ cells, they express high levels of membrane IgM (Fig. 1), similar to IgM-only cells (4). That $IgM^+IgD^+CD27^+$ cells may represent memory B cells is further supported by the following observations. (*a*) Upon stimulation in in vitro assays, both IgD^-CD27^+ and IgD^+CD27^+ cells—in contrast to IgD^+CD27^- cells—are quickly activated and secrete large amounts of Ig (15, 16). This is consistent with one of the key features of memory B cells, as reported for splenic marginal zone and tonsillar subepithelial memory B cells (28, 29). (*b*) A small fraction of tonsillar GC B cells expresses IgD, and half of those cells carry somatically mutated V region genes (30). These GC B cells might be the precursors of the $IgM^+IgD^+CD27^+$ PB B cells. A second potential precursor population for the $IgM^+IgD^+CD27^+$ B cells is represented by a rare type of tonsillar GC which are populated by somatically mutated IgD^+CD70^+ GC B cells (31; Küppers, R., and C. van Noesel, unpublished observations). $IgM^+IgD^+CD27^+$ PB B cells are also $CD70^+$ (32; our unpublished observations). (*c*) CD27-expressing B cells are almost absent from cord blood (16, 33), which is devoid of memory B cells. Although it presently cannot be ruled out that $IgM^+IgD^+CD27^+$ B lymphocytes are generated in a GC-independent pathway (34), these observations collectively suggest that these cells, besides class-switched and IgM-only cells, represent a third phenotypically defined memory B cell subset in human PB. Regarding the mutated μ-transcripts identified in cDNA libraries generated from mature bone marrow B cells (13), it seems likely that those transcripts were derived from both IgM-only and $IgM^+IgD^+CD27^+$ memory cells homing to or circulating through the bone marrow.

IgD-only B Cells in the PB Carry Highly Mutated V Region Genes. We observed a minute fraction of IgD-only B cells in the PB, usually representing <1% of PB B cells (our unpublished observations). They are $CD27^+$ (Fig. 3) and express V region genes with an exceptionally high load of somatic mutation (Table 2), as has been described for IgD-only GC B cells (12) and tonsillar IgD-only plasma cells (14). Based on these similarities, it seems likely that the IgD-only cells in the PB represent the descendents of IgD-only GC B cells. Thus, it appears that IgD-only GC cells can differentiate not only into tonsillar plasma cells, but also into recirculating $sIgD^+$ cells. The fact that these highly mutated cells express surface Ig and that the average R/S mutation value for the FRs of the potentially functional V_H region genes amplified from IgD-only cells (1.4) is in the range typical for antigen-selected memory B cells indicates that these cells have been selected within the GC for antigen receptor expression.

In Human Memory B Cells, V_H Region Genes Harbor on Average a Two- to Threefold Higher Load of Somatic Mutations than V_κ Region Genes. In our previous V gene analyses on B cell subsets derived from healthy adults, we repeatedly determined somatic mutation frequencies of ~2% for IgM-only and 4% for class-switched cells (4, 11, 18). Those values were derived from analyzing rearranged V_κ genes. The average V_H gene mutation frequency of ~5% in IgD^+CD27^+ B cells (Tables 1 and 3) made us wonder whether V_H regions may in general carry a higher load of somatic mutations than V_κ regions. To clarify this, we sequenced rearranged V_H genes from IgM-only cells as well as V_κ regions from IgD^+CD27^+ cells. Furthermore, from one donor V_H and V_κ region genes were amplified from single class-switched B cells. Indeed, within a given cell population, V_H regions show a two- to threefold higher average mutation frequency than V_κ regions (Tables 3 and 4). The same conclusion can be drawn from the results of both a V gene analysis of sporadic Burkitt's lymphomas ($n = 9$; V_κ: 1.8% average mutation frequency, V_H: 3.4% [35]) and a single cell study of rearranged V_H as well as V_κ genes expressed by IgM-bearing PB B lymphocytes (36). The present data also demonstrate that IgD^+CD27^+ B cells show a level of somatic mutation in the same range as that of IgM-only cells (2% for V_κ [Table 4, and reference 4]; ~5% for V_H [Table 3]).

The simplest interpretation of the lower mutation frequency of V_κ than V_H genes is that the intrinsic mutation rate is higher in the latter. However, the lower mutation load of V_κ genes could also be due to novel V_κ gene rearrangements in GC B cells (37, 38), which would have gone through fewer rounds of somatic mutation than the corresponding V_H gene rearrangements.

The V_H gene mutation values discussed above hold true for adults. For μ- and γ-transcripts derived from tonsillar and PB memory B lymphocytes of children, we and others previously reported mutation frequencies of 2 and 4%, respectively (9, 10). What could be the explanation for the

discrepancy between the mutation frequencies of memory cells in children and in adults? Perhaps the hypermutation mechanism is not yet fully active in GC B lymphocytes of children. Alternatively, memory B cells may be driven repeatedly into GC reactions where they acquire additional somatic mutations. However, the mutation load does not appear to increase considerably in adults with advancing age (4, 11).

The Peripheral B Cell Pool in Humans. On the basis of the present data, B cells that express unmutated V genes are IgM$^+$IgD$^+$CD27$^-$ and comprise ~60% of PB B cells. Lipsky and colleagues find a similar fraction (55%) of unmutated or slightly mutated V genes amplified from individual CD19$^+$ PB B lymphocytes (39). IgM$^+$IgD$^+$CD27$^-$ B cells can be further distinguished into a large population of CD5$^-$ cells and a smaller subset of CD5$^+$ B cells (18). The latter are thought to belong to a separate B cell lineage (for a review, see reference 40) and presumably do not regularly participate in T cell–dependent immune responses (41, 42). They comprise 10–20% of B cells in the adult PB. CD5-negative IgM$^+$IgD$^+$CD27$^-$ cells are termed naive B cells as they represent the presumed precursors of GC B cells in T cell–dependent immune responses. These cells make up between 40 and 50% of PB B lymphocytes (Fig. 4).
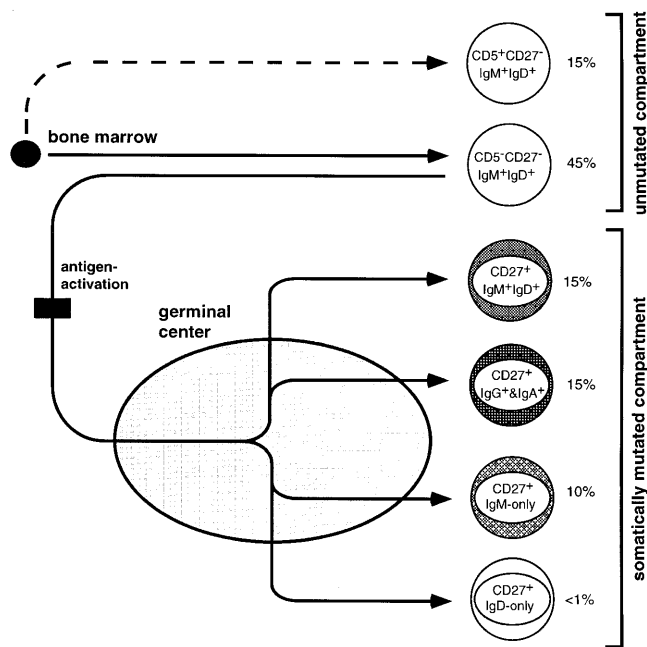
Figure 4. The PB B cell pool and its presumptive generation. The unmutated B cell compartment (CD27$^-$) is distinguished into CD5-positive IgM$^+$IgD$^+$ cells, whose derivation is debated, and CD5-negative IgM$^+$IgD$^+$, bone marrow–derived, naive B cells. Whereas the CD5$^+$ B cells usually do not participate in T cell–dependent immune responses, naive B cells upon antigen encounter are activated and establish GC. In the course of the GC reaction, somatically mutated class-switched, IgM-only, and IgM$^+$IgD$^+$ cells, all of which express the CD27 membrane antigen, are generated. After selection, they leave the GC as memory B cells. In some GC, IgD-only B cells are generated, which carry an exceptionally high load of somatic mutation. Indicated are the percentages of the respective populations among all PB B lymphocytes.

About 40% of PB B cells represent memory B cells (Fig. 4). There is evidence that such cells occur at similar numbers also in secondary lymphoid organs (summarized in reference 4). The large fraction of memory B cells in humans contrasts with the situation in the mouse, where the frequency of memory B cells (in old, nonintentionally immunized mice) is in the range of 5% of all peripheral B lymphocytes (43). This difference might largely be explained by the longer life span of humans, leading to the accumulation of a much larger fraction of memory B cells. IgD and/or IgM-expressing memory B cells were described in rodents and chickens already 20 years ago (24, 44–46). However, these cells were not phenotypically or molecularly characterized in detail, and there is no information on the size of this memory compartment in those species. In models of T cell–dependent immune responses in the mouse, memory B cells almost exclusively express isotypes other than IgM and IgD, indicating that the memory B cell pool shows a different composition in mice and in humans. In humans, IgM-bearing memory cells seem to predominate over class-switched memory cells (4; Fig. 4): human memory B cells can be distinguished into (on average) 40% class-switched, 20% IgM-only, and 40% IgM$^+$IgD$^+$CD27$^+$ cells (and probably a small population of IgD-only cells). A common characteristic of these subsets is the expression of the CD27 cell surface antigen, which thus may represent a general marker for memory B cells in humans. CD27-expressing B cells can be activated through interaction with the CD27 ligand, CD70 (47), a molecule belonging to the TNF receptor family which is found on peripheral T cells, and recently it was shown that B cells stimulated via CD27–CD70 interaction acquire a plasma cell phenotype in vitro (48). This finding suggests that memory B cells, activated by antigen in the context of T–B cell interaction, may quickly, as a result of an additional CD27 stimulation, differentiate into Ig-secreting cells. Although in vitro the differentiation of memory B cells into plasma cells can be achieved without CD27 stimulation (29), it is tempting to speculate that in vivo CD27–CD70 interaction represents the key signal to bias memory B cells to the plasma cell differentiation pathway.

Kindler and Zubler note that in vitro–activated IgM-only PB B cells differentiate into plasma cells, but do not change isotype even under conditions that promote switching in a large fraction of IgM$^+$IgD$^+$ B cells (5). This indicates that IgM-only memory B cells are committed to secrete IgM. Such behavior of IgM-only cells may be explained by an internal rearrangement between the 5′ and 3′ ends of the sμ regions, resulting in deletion of this region (49, 50). Thus, further switching to downstream isotypes in IgM-only (and potentially also IgM$^+$IgD$^+$CD27$^+$) B cells may be abolished.

Despite the prevalence of IgM-expressing over "classical" class-switched memory B cells, the role of IgM-expressing memory cells in T cell–dependent immune responses remains elusive. Are IgM-only and IgM$^+$IgD$^+$ CD27$^+$ cells as a rule generated in the course of a T cell–dependent immune response from a fraction of GC B cells

that do not undergo class switching? Or are they generated specifically against particular pathogens? Do IgM⁺IgD⁺ CD27⁺ memory cells upon antigen stimulation secrete IgD in addition to IgM? Pentameric IgM enables efficient cross-linking of antigen and permits a strong activation of the complement system. These features are advantageous in the defense against bacteria. In this regard, it is interesting to note that the splenic marginal zone, the entry port of blood-borne antigens, is mainly populated by IgM-only memory B cells (6, 7). Perhaps these cells have been generated to quickly respond to bacteria that invade the blood-stream.

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