

Analysis of Somatic Mutation in Five B Cell Subsets of Human Tonsil

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Summary

Using a series of phenotypic markers that include immunoglobulin (Ig)D, IgM, IgG, CD23, CD44, Bcl-2, CD38, CD10, CD77, and Ki67, human tonsillar B cells were separated into five fractions representing different stages of B cell differentiation that included sIgD⁺ (Bm1 and Bm2), germinal center (Bm3 and Bm4), and memory (Bm5) B cells. To establish whether the initiation of somatic mutation correlated with this phenotypic characterization, we performed polymerase chain reaction and subsequent sequence analysis of the Ig heavy chain variable region genes from each of the B cell subsets. We studied the genes from the smallest V_H families (V_H4, V_H5, and V_H6) in order to facilitate the mutational analysis. In agreement with previous reports, we found that the somatic mutation machinery is activated only after B cells reach the germinal center and become centroblasts (Bm3). Whereas 47 independently rearranged IgM transcripts from the Bm1 and Bm2 subsets were nearly germline encoded, 57 Bm3-, and Bm4-, and Bm5-derived IgM transcripts had accumulated an average of 5.7 point mutations within the V_H gene segment. γ transcripts corresponding to the same V_H gene families were isolated from subsets Bm3, Bm4, and Bm5, and had accumulated an average of 9.5 somatic mutations. We conclude that the molecular events underlying the process of somatic mutation takes place during the transition from IgD⁺, CD23⁺ B cells (Bm2) to the IgD⁻, CD23⁻, germinal center centroblast (Bm3). Furthermore, the analysis of Ig variable region transcripts from the different subpopulations confirms that the pathway of B cell differentiation from virgin B cell throughout the germinal center up to the memory compartment can be traced with phenotypic markers. The availability of these subpopulations should permit the identification of the functional molecules relevant to each stage of B cell differentiation.

The variable regions of the two critical antigen receptors of the immune system, the T cell receptor and the immunoglobulin molecule, are encoded by five different genetic elements that, in the germline, are separated by thousands of base pairs (1, 2). A recombination machinery shared by T and B cells brings these elements together into functional TCR V _{β} /V _{α} and Ig V_H/V_L chains (3). Availability of a broad array of germline genes, generation of random amino acids during the process of rearrangement, and combinatorial association of V _{β} /V _{α} and V_H/V_L chains are essential steps in the generation of diversity within the T and B cell repertoires. B cells display the unique property of accumulating somatic mutations in their Ig variable region genes, further contributing to increase the almost limitless number of antigenic specificities (1).

Although a large body of information has accumulated in recent years concerning the repertoire of human Ig variable region genes, both at the level of genomic organization and

expression, our current knowledge about the mechanism of somatic mutation remains elementary. Mutations are introduced only into rearranged Ig genes of transcriptionally active heavy and light chains, at a rate of $\sim 10^{-3}$ /bp/generation (4). Although both productively and nonproductively rearranged V genes are targeted, mutations predominantly occur within the region which surrounds the rearranged variable region gene, spanning ~ 2 kb of DNA (5). Even though somatic mutation is thought to be near random, strand polarity (6–9) and mutational hot spots have been reported (reviewed in 10). There is considerable evidence that the peripheral lymphoid organs provide the microenvironment for the activation of virgin and memory B cells and the accumulation of somatic mutation during the humoral immune response. Early B cell activation during antigen-specific antibody responses occurs in the T cell and interdigitating cell areas of the lymph nodes, tonsils, Peyer's patches, and the periarteriolar lymphocytic sheaths (PALS) of the spleen. This early B cell activa-

tion gives rise to short lived plasma cells, IgM-positive splenic marginal zone B cells, and primary B cell blasts that colonize the primary follicles (11–14). The subsequent germinal center (GC)¹ reaction is initiated by the rapid proliferation of three to five primary blasts in association with follicular dendritic cells (11, 12, 15). The primary B blasts follow a differentiation pathway from centroblasts to centrocytes, and then to either plasma cells or memory B cells (16–19). During these processes, somatic hypermutation (20–23), positive selection (24–26) and differentiation of high affinity GC B cells occurs (27–33). To date, progress in understanding the molecular mechanisms underlying somatic mutation has been hampered by the lack of an experimental in vitro system.

Kinetic analyses of V region mutation and selection of microdissected murine GC B cells have been recently reported (34). These studies indicate that mutant B cells are not detected in early GC, and that the estimated three to six B cells that give rise to each GC undergo substantial proliferation before the initiation of Ig hypermutation. Using a similar approach, Kuppers et al. (35) have recently reported that the human GC is initially populated by a polyclonal set of antigen-activated B cells that proliferate in the dark zone and largely express unmutated V region genes. The same group has been able to establish that human peripheral blood B cells can be phenotypically separated into three subsets (IgM⁺IgD⁺, IgM⁺IgD⁻, and IgM⁻IgD⁻), only one of which (IgM⁺IgD⁺) expresses unmutated genes (36).

In this report, we describe the phenotypic characterization of five B cell subsets (Bm1 to Bm5) representing different stages of B cell maturation, from the naive IgD⁺ state (Bm1 and Bm2), through the GC CD38⁺ stage (Bm3 and Bm4) to the IgD⁻CD38⁻ memory B cell (Bm5). The analysis of the Ig heavy chain variable region gene transcripts from these B cell populations indicates that the initiation of somatic mutation correlates with the phenotypic characteristics of the GC centroblast (sIgD⁻, CD38⁺, CD77⁺).

Materials and Methods

Isolation of Tonsil B Cells

Tonsil B cells were taken from patients during routine tonsillectomy, minced, and the resulting cell suspensions were subjected to two rounds of T cell depletion using 2-aminoethyl-isothiocyanide bromide-modified sheep red blood cells. The resulting cells were >97% CD19⁺, and <1% CD14⁺ and CD3⁺, and were further separated into high density and low density B cells by centrifugation through 15, 60, and 65% Percoll gradients (Pharmacia LKB, Uppsala, Sweden). The resulting total tonsil B cells and the high and low density B cells were used for phenotypic analysis, immunomagnetic bead sorting, and FACS[®] sorting into five B cell subsets.

Labeling of Cell Surface Antigens

Direct Immunofluorescence Staining. Labeling was performed using

the following mAbs directly conjugated with PE or FITC: anti-CD23-PE (Serotec, Ltd., Oxford, UK), anti-CD23-FITC (ImmunoTch, Marseille, France), anti-CD38 ascitic fluid (Ortho Diagnostic Systems, Roissy, France), anti-CD38-PE (Becton Dickinson & Co., Mountain View, CA), anti-CD77 supernatant (Immunotech), anti-human IgD Biot. (Amersham Corp., Arlington Heights, IL), anti-CD39-Biot. (The Binding Site, Ltd., Birmingham, UK).

Indirect Immunofluorescence Staining. Labeling was performed with a panel of uncoupled or biotinylated murine mAbs that were detected by FITC-conjugated sheep anti-mouse Ig F(ab')₂ or PE-conjugated streptavidin.

Double Immunofluorescence Staining. Cells were sequentially incubated with two mAbs using two protocols: (a) mAbs conjugated to FITC and PE; and (b) one antibody conjugated to FITC and another biotinylated, which was detected by PE-labeled streptavidin.

Cell Sorting

Cells were sorted with a FACStar[®] (Becton Dickinson & Co.) equipped with a 2-W argon laser.

Sequencing the Ig V_H Transcripts from the Five B Cell Subsets

Total RNA was extracted from 1–5 × 10⁵ cells using guanidinium thiocyanate-phenol-chloroform in a single step (37). The total RNA yield was reverse transcribed using oligo d(T) as primer and avian myeloblastosis virus reverse transcriptase in 100 μl final volume. First strand cDNA (1–5 μl) was directly used for second strand synthesis and amplification via the PCR (38) in a final volume of 100 μl containing 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, at 37°C, 1.5 mM MgCl₂, 2.5 U Taq polymerase, and 50 pmol of primers that consisted of oligonucleotides corresponding to the C_μ and C_γ constant regions (μ1: 5'CGG GTG CTG CTG ATG TCA GAC3'; μ2: 5'TGG GGC GGA TGC ACT CCC3'; γ1: 5' CAC CGT CAC CGG TTC GG3'; γ2: 5'GTA GTC CTT GAC CAG GCA GC3') the V_H family-specific leaders (V_H4, V_H5, and V_H6) (39), and the FW1 sequences of the V_H4-21 (5'CTA CAG CAG TGG GGC GCA3') (40). PCR was carried out for 40 cycles under standard conditions (denaturation 1 min at 94°C, annealing 2 min at 54–58°C, extension 1 min at 72°C). The PCR products were purified using microconcentrators (Microcon 100; Amicon, Beverly, MA), phosphorylated, and blunt-end ligated into an EcoRV-digested, dephosphorylated plasmid (pBluescript; Stratagene, La Jolla, CA). The ligation mixtures were used to transform BSJ-72 competent cells, and two replicas of the colonies were screened with internal end-labeled oligonucleotides. Positive colonies were sequenced in both directions by the dideoxy chain-termination method (41) using either γ-³⁵S-ATP and Sequenase (42), or fluorescent labeled ddNTP and Taq-Polymerase (auto-mated sequencer protocol; ABI Advanced Biotechnologies Inc., Columbia, MD).

Analysis of DNA Sequences

A total of 146 IgM and IgG transcripts were analyzed using DNASTar (DNASTar Inc., Madison, WI). Clonal relatedness was established by analyzing the CDR3 regions. Sequences displaying 100% identity throughout the VDJ region were considered as a single transcript in the mutational analysis. Sequences with similar CDR3 length and sequence but with scattered nucleotide differences were considered the result of in vivo clonal expansion. Parallel mutations in these types of related clones were counted only once in the analysis.

¹ Abbreviations used in this paper: GC, germinal center; R/S, replacement vs. silent.

Results

Isolation of the B Cell Subpopulations. In recent years, the use of mAbs has allowed the identification of a large number of surface B cell markers. Immunohistochemical analysis using these mAbs has facilitated the tentative assignment of B cell subsets (43–45). In our study, tonsil B cells were double stained with anti-CD38 and anti-IgD, since these two markers have been shown to differentiate follicular mantle (IgD⁺) from GC (CD38⁺) B cells. Accordingly, three major B cell subpopulations could be identified (Fig. 1).

CD38⁻, IgD⁺ B cells were purified from high density B cells by depletion of CD38⁺, IgG⁺, and IgA⁺ B cells. The resulting cells are small resting B cells that express high levels of IgD, IgM, CD44, and cytoplasmic Bcl-2 protein. They are negative for CD38, CD10, CD77, and IgG, and display low levels of CD20 (data not shown). Since ~30% of these cells express CD23, a marker associated with B cell activation (46), we separated via FACS[®] CD23⁻ from CD23⁺ cells and defined them as Bm1 and Bm2 cells, respectively.

CD38⁺, IgD⁻ B cells were purified from low density B cells by depletion of IgD⁻ and CD39⁺ B cells. They express high levels of CD38, CD10, CD20, IgG, and the nuclear antigen Ki67 but are negative for CD39, IgD, CD23, and the cytoplasmic protein Bcl-2 (data not shown). About 40% of these cells express CD77, and only 10% express CD44. As Fig. 1 shows, CD77 was used to separate via FACS[®] two cell subpopulations, Bm3 (CD77⁺) and Bm4 (CD77⁻), since CD77 has been shown to differentiate dark zone cen-

troblasts from light zone centrocytes (43–45). These cells are large to medium size with characteristic nuclear clefts.

CD38⁻ IgD⁻ B cells were isolated from total B cells by depletion of CD38⁺ and IgD⁺ B cells. These cells express high levels of IgG, CD39, CD44, and cytoplasmic Bcl-2 protein, are negative for IgD, CD23, CD38, CD10, CD77, and nuclear antigen Ki67, and display low levels of CD20 (data not shown). They are small- to medium-sized lymphocytes with cytoplasmic processes.

PCR Analysis of IgM and IgG Transcripts from the Bm1-Bm5 Subsets. Oligonucleotide primers specific for the human V_H4, V_H5, and V_H6 gene families were used in combination with IgM and IgG constant region primers to amplify via PCR the heavy chain variable region mRNAs from each of the B cell subpopulations isolated from two different tonsils. After a single round of PCR (40 cycles), we successfully amplified V_H4 and V_H5 IgM transcripts from each of the Bm subsets. However, a second round of seminested PCR with internal constant region primers was required to obtain Bm1, Bm2, and Bm5 IgM V_H6 products. A second round of PCR was also undertaken to selectively amplify the V_H4-21 gene segment (40) from the initial V_H4 PCR products. IgG transcripts from the same V_H4, V_H5, and V_H6 families could only be amplified from subpopulations Bm3, Bm4, and Bm5. All the PCR amplifications were performed in duplicate with identical results. Although our PCR conditions were not designed to be quantitative, the comparable amplification of

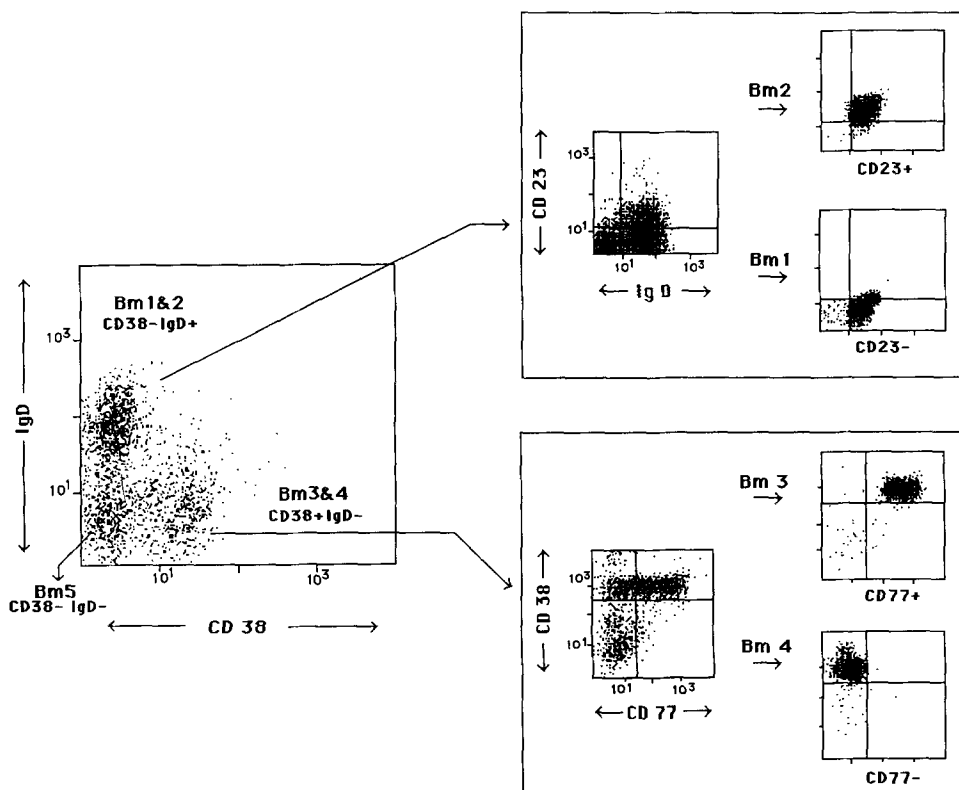


Figure 1. Immunofluorescence FACS[®] analysis of tonsil B cells to identify IgD⁺CD38⁻ FM B cells, IgD⁻CD38⁺ GC B cells, and IgD⁻CD38⁻ memory B cells. IgD⁺ B cells were further sorted into CD23⁻ (Bm1) and CD23⁺ Bm2 cells. CD38⁺ B cells were sorted into CD77⁺ (Bm3) and CD77⁻ (Bm4) cells.

IgM V_H4 and V_H5 messages from the five subpopulations provides an internal control that rules out major biases introduced by differences in the starting amounts of template.

Sequence Analysis Reveals That Somatic Mutation Involves the GC (Bm3 and Bm4) and Memory B Cell (Bm5) Subpopulations. We analyzed 146 V_H sequences derived from the different B cell subpopulations and compared the regions encoded by the V_H gene segment with their corresponding V_H germline counterparts. D segments were not included in the mutational analysis due to the usual difficulty in accurately establishing their germline origin. As Table 1 shows, 28/47 Bm1 and Bm2 IgM transcripts were 100% identical to their germline counterparts, 12/47 displayed a single nucleotide difference, 6/47 had two differences, and only one sequence contained four nucleotide substitutions in the region encoded by the V_H gene segment. Strikingly, analysis of IgM Bm3 transcripts disclosed that only 4/25 were 100% identical to the germline, whereas 19/25 had accumulated more than three nucleotide substitutions. Finally, none of the 17 Bm4 IgM and only 1/15 Bm5 transcripts displayed complete identity with the germline. Accordingly, all the Bm3, Bm4, and Bm5 IgG transcripts (15, 6, and 8, respectively) were mutated (Table 1 and Fig. 2, A and B).

Fig. 3 A shows the average number of substitutions among individual nonclonally related IgM rearrangements. Fig. 3 B depicts the number of substitutions within IgM and IgG transcripts from the GC and memory B cell compartments. Interestingly, IgG transcripts had accumulated almost twice as many substitutions as IgM transcripts.

Fig. 4 shows the percentage of silent versus replacement mutations among IgM (Fig. 4 A) and IgG (Fig. 4 B) transcripts from the B cell subpopulations. Two thirds of the nucleotide changes encoded amino acid replacements, suggesting that the nucleotide substitutions within a codon were random. Analysis of the number of substitutions in each of the intervals encoded by the V_H gene segment indicated that, regardless of the isotype, the two hypervariable regions (CDR1 and CDR2) accumulated the highest number of substitutions when normalized for length (Fig. 5). Also as expected, the percentage of amino acid replacements within the CDRs was slightly higher than within the FWs (72.1 vs. 61%, respectively). Table 2 depicts the ratio of replacement vs. silent substitutions (R/S) among the B cell subpopulations. Interestingly, in the frameworks this ratio is maintained close to 1.5, the expected value predicted by Shlomchik et al. (47) after taking into account the possibility of deleterious framework replacement mutations leading to negative B cell selection. The high R/S ratio observed within Bm1 transcripts should be cautiously analyzed, since most of the mutated V_H sequences derived from this B cell subpopulation display only one to two nucleotide differences compared with the germline. Because this low mutation frequency falls into the average Taq polymerase error (see below), the Bm1 R/S ratio could at least partially be explained by an in vitro bias. Thus, analysis of the R/S ratio within the CDRs seem to reflect the process of progressive antigenic selection, as it increases along the proposed model of differentiation from Bm2 to

TABLE I

CLONE	# MUTATIONS (R/S)					JH
	FW1	CDR1	FW2	CDR2	FW3	
1-6MG	-	-	-	-	-	JH4
1-6MH	-	0/1	-	-	-	JH4
1-6MV	-	-	-	-	-	JH4/5
1-6MA	-	-	-	-	-	JH3
1-6MGG	-	-	-	-	-	JH6
1-6MC	-	0/1	-	-	-	JH4
1-6MJ	1/0	-	-	-	-	JH4
1-6ME	-	-	-	-	-	JH6
1-6ML	1/0	-	-	-	1/0	JH4 #
1-6MM	-	-	-	-	-	JH4
1-4M11	-	-	-	-	1/1	JH4 @
1-4M1	-	-	-	-	1/0	JH5
1-4M2	-	-	-	-	-	JH6
1-4M6	-	-	-	-	-	JH5
1-4M12	-	-	-	-	-	JH3
1-4M3	-	-	-	-	-	JH6
1-4M7	-	-	-	-	-	JH5
1-4M5	-	1/0	1/0	-	-	JH2
1-4M9	-	-	1/0	-	-	JH3
1-4M10	-	-	-	1/0	-	JH6
1-4M8	-	-	-	0/1	1/0	JH4
1-4M4	-	-	-	-	1/0	JH3
2-6MAA	-	-	-	-	-	JH5
2-6MAA'	-	-	-	-	-	JH5
2-6ML	-	-	-	-	0/1	JH5 @
2-6MY	-	-	-	-	-	JH6
2-6MB	-	-	-	-	1/0	JH4
2-6MB'	-	-	-	-	1/0	JH4
2-6M11	-	-	-	-	-	JH5
2-6MCC	-	-	-	-	-	JH6
2-6MA	-	-	-	-	-	JH3
2-6MBB	-	-	-	-	-	JH4
2-6MC	-	-	-	-	-	JH4
2-6MH	-	-	-	-	-	JH4
2-6MD	-	-	0/1	-	-	JH6
2-6MF	-	-	-	-	1/0	JH4
2-6MG	1/0	-	2/0	-	0/1	JH6
2-6ME	-	-	-	-	-	JH4
2-6MK	-	-	-	-	-	JH3
2-6MM	-	-	-	-	1/0	JH1/4
2-5M3.1	-	0/1	-	1/0	-	JH6
2-5M3.2	-	-	-	-	-	JH6
2-5M3.3	-	-	-	-	-	JH5
2-5M3.4	-	-	-	-	-	JH6
2-5M3.6	-	-	-	-	-	JH4
2-5M7.1	-	-	-	-	-	JH4
2-5M7.3	-	-	-	-	-	JH6
2-5M7.4	-	-	-	1/0	0/1	JH4
2-5M7.5	-	-	1/0	-	-	JH1

Analysis of the nucleotide substitution ratio (R/S) among the five Bm subpopulations. Clone numbers denote the Bm subset followed by the V_H family. Capital letters (M or G) correspond to the isotype, and are followed by the individual clone designation.

Nucleotide difference due to base pair deletion.

@ Nucleotide difference(s) in FW3 can be explained by recombination.

* 5-bp nucleotide insertion in CDR2.

*, **, *** Clonally related sequences.

+ Possible PCR hybrid sequence.

3-6MB	0/1	1/0	-	-	-	JH5
3-6MC	0/1	1/0	-	1/0	4/0	JH4
3-6MF	1/0	1/0	-	3/1	2/0	JH3
3-6MI	1/3	1/1	-	5/4	>2/1	JH4 #
3-6MV	-	1/0	-	2/0	2/1	JH4
3-6MBB	-	-	1/0	2/1	0/2	JH4
3-6MAA	1/1	1/0	0/1	-	1/2	JH6
3-6MII	-	-	1/0	2/0	5/0	JH4
3-6MDD	-	-	-	3/0	6/0	JH3
3-6MEE	1/2	-	0/1	1/3	1/1	- #
3-6MK	-	-	-	**	5/1	JH4 ~
3-6ML	1/1	2/0	1/0	6/2	1/2	JH5
3-6MM	1/2	-	0/2	3/1	2/2	JH3
3-6M2	-	-	-	-	-	JH4
3-6M3	-	0/1	-	1/0	2/0	JH6
3-6M4	-	-	-	1/1	1/0	JH4
3-6M6	-	1/0	-	1/2	3/1	JH5
3-5M4.1	-	4/2	1/0	4/0	1/1	JH3
3-5M4.2	-	-	-	-	-	JH4
3-5M4.3	-	-	-	-	-	JH4
3-5M4.4	1/0	0/1	-	0/1	-	JH5
3-5M4.5	-	-	-	-	-	JH4/5
3-5M8.1	-	1/0	-	1/0	1/3	JH4
3-5M8.5	-	-	-	0/1	-	JH4
3-5M8.6	-	-	-	1/0	1/0	JH6
4-6MC	-	-	2/0	4/0	1/0	JH3
4-6MF	0/1	-	-	-	2/1	JH5
4-6MI	1/0	1/0	-	-	1/0	JH4
4-6MB	-	-	-	1/0	-	JH5
4-6MY	1/0	3/0	-	2/1	2/1	JH4
4-5MA	-	1/0	-	-	5/1	JH4 @
4-5MB	1/1	-	0/1	1/0	2/1	JH5
4-5MD	2/0	1/0	-	1/1	1/0	JH5
4-5MG	2/1	1/0	-	-	-	JH4
4-5MH	1/1	1/0	1/0	1/1	2/0	JH6 @
4-5MP	-	-	-	1/0	1/0	JH4 @
4-4M21	1/1	1/0	3/1	6/1	4/4	JH4
4-4M22	1/1	-	-	-	1/1	JH2
4-4M26	-	-	-	-	2/0	JH4 @
4-4M27	1/3	0/1	1/0	1/1	2/3	JH6
4-4M28	2/1	0/1	-	1/1	3/0	JH5 @
4-4M29	-	0/1	-	1/0	0/2	JH6
5-6MB	-	-	-	1/0	-	JH4
5-6MD	-	1/1	-	2/0	-	JH4
5-6MG	-	1/1	-	1/0	4/2	JH1
5-6MH	-	-	-	2/0	1/0	JH3
5-6MM	-	-	-	2/0	2/2	JH3
5-5ME	0/1	-	0/1	-	1/1	JH4 @
5-5MH	1/2	-	-	-	1/1	JH4 *
5-5MK	-	-	-	-	-	JH4
5-5MR	1/0	2/0	-	-	-	JH4
5-5M0	3/1	0/1	1/1	3/0	2/3	JH5 @
5-5MS	1/1	-	-	-	1/0	JH3
5-5MU	-	-	-	-	1/1	JH4 *
5-4M44	-	-	-	2/0	-	JH5
5-4M45	-	-	-	0/1	1/0	JH4
5-4M47	-	-	2/0	1/0	2/1	JH4

3-6G1	3/0	1/0	0/1	4/1	1/1	JH6
3-6G3	3/1	2/1	-	2/1	0/3	JH6 **
3-6G5	-	-	-	3/4	2/1	JH4
3-6G5'	0/1	-	-	3/4	2/1	JH4
3-6G10	2/1	-	-	1/0	1/0	JH4
3-6G10'	3/1	1/0	-	9/0	1/0	JH4 +
3-6G11	-	2/0	0/1	2/1	6/0	JH3
3-6G12	2/1	2/1	-	2/1	1/0	JH6 **
3-6G12''	-	1/0	-	3/1	2/1	JH6 **
3-6G12'''	-	-	1/1	4/0	4/1	JH6 **
3-6G12''''	2/0	2/1	-	2/2	1/1	JH6 **
3-6G13	1/0	-	-	2/1	1/0	JH4 ***
3-6G13'	3/2	2/0	-	2/1	3/2	JH4 ***
3-6G16	1/0	1/0	-	-	0/1	JH4
3-6G20	2/0	2/0	-	2/4	2/1	JH6

4-6G1	1/1	1/0	-	1/1	2/2	JH3
4-6G3	1/2	1/0	-	1/0	3/3	JH6
4-6G8	-	2/1	0/1	3/0	1/0	JH6
4-6G10	1/1	1/0	-	1/1	2/1	JH4
4-6G12	1/0	-	-	1/0	0/2	JH4 ***

5-6G2	1/2	3/0	0/1	1/2	4/4	JH6
5-6G3	0/2	1/0	-	5/2	5/3	JH4
5-6G4	1/1	1/0	-	-	1/2	JH5
5-6G5	-	-	0/1	1/0	5/1	JH6
5-6G8	0/2	1/2	-	0/4	5/1	JH4
5-6G11	-	1/0	-	1/0	1/0	JH6

Bm5 cells. However, Bm5 IgG transcripts only reach a R/S ratio of 1.4 (Table 2).

The Pattern of Nucleotide Substitutions Supports the Model of Mutational Preference/Strand Polarity. The analysis of 484 independent substitutions is depicted in Table 3. In agreement with previous reports (6-10, 34), transitions predominate over transversions (226 vs. 218, respectively), even though there can be potentially twice as many possible transversion events. Our data also favor the notion of a bias against mutations at T nucleotides (15% of the substitutions), and a preferential targeting at A:N over N:A changes (39% vs. 16.7%), supporting the view that hypermutation is preferentially targeted to one strand of the DNA molecule (6-9, 34). In the interpretation of these results it is important to consider however that A:G substitutions (22% of our sample) have been reported to be the most frequent Taq polymerase misincorporation events (48).

The Small V_H Families Contribute Considerable Diversity within the Human Tonsil B Cell Repertoire. To determine the extent of diversity contributed by B cells expressing members of the smallest V_H families, we had to discriminate between those PCR artifacts mimicking in vivo restriction and/or clonal expansion. We defined clonal expansion as the presence of scattered nucleotide differences throughout the regions encoded by the V_H, D, and J_H gene segments between two or more clones sharing the same V_H-D-J_H rearrangements. Transcripts sharing identical CDR3/FW4 regions but displaying extensive differences in the region encoded by the V_H gene segment were considered PCR hybrid artifacts, and only distinct mutations were included in the study.

Our analysis of 146 transcripts from the five B cell subpopulations (Bm1-Bm5) disclosed 131 nonidentical and 125 nonrelated V_H-D-J_H rearrangements, 74 of which were encoded by the single member of the V_H6 gene family. Dupli-

A
CDR II

VH6	AGGACATACTACAGGTCCAAGTGGTATAATGATTATGCAGTATCTGTGAAAAGT
1MG-VHT.....C.....C.....T.G.....
1MH-VHT.....C.....C.....T.G.....
1MV-VHG.....CT.C..C..A..T.....
1MA-VHG.....CT.C..C..A..T.....
1MGG-VHT.....
1MC-VHC..A..T.....C.....C.....CC..A..G..
1MJ-VHG.....G.....A.....G..
1ME-VHT.....G.....G.....
1ML-VHG.....C..C.....
1MM-VHC.....G.....G.T.....
2MAA-VHT.....G.....C.....G.....
2ML-VHG.....C.....G.T.....G..
2MY-VHG.....C.....T.....
2MB-VHG.....T.C.....T.....G..
2MI-VHG.....T.C.....T.....G..
2MCC-VHG.....T.C.....T.....G..
2MA-VHG.....T.C.....T.....G..
2MBB-VHG.....T.C.....T.....G..
2MC-VHG.....T.C.....T.....G..
2MH-VHG.....T.C.....T.....G..
2MD-VHG.....T.C.....T.....G..
2MF-VHG.....T.C.....T.....G..
2MG-VHG.....T.C.....T.....G..
2ME-VHG.....T.C.....T.....G..
2MK-VHG.....T.C.....T.....G..
2MM-VHG.....T.C.....T.....G..
3MB-VHG.....T.C.....T.....G..
3MC-VHG.....T.C.....T.....G..
3MF-VHC.....G.....G.....G.....G..
3MI-VH	*****GG.....G..TC.G.GA.....G..
3MV-VHC.....
3MBB-HC.....G.....C.....
3MAA-VHC.....
3MI-VHC.....
3MDD-VHT.....C..G.....
3MEE-VHC.G.....C..C.....
3MK-VH	*****
3ML-VHA.....CT.A...C...A...A..G..
3MM-VHT..T.....A.....G.....
3M2-VHG.....
3M3-VHG.....
3M4-VHT.....C.....
3M6-VHG..T.C.....
4MC-VHT..C.....G.....G..
4MF-VHG.....
4MI-VHC.....
4MB-VHC.....
4MY-VHG.....A.....G..
5MB-VHC.....
5MD-VHGT.....
5MG-VHG.....
5MH-VHAT.....
5MM-VHT.....G.....

B
CDR II

VH6	AGGACATACTACAGGTCCAAGTGGTATAATGATTATGCAGTATCTGTGAAAAGT
3G1-VHT.....C.....C.....T.G.....
3G3-VHT.....C.....C.....T.G.....
3G5-VHG.....CT.C..C..A..T.....
3G5'-VHG.....CT.C..C..A..T.....
3G10-VHT.....
3G10'-VHC..A..T.....C.....C.....CC..A..G..
3G11-VHG.....G.....A.....G..
3G12-VHT.....G.....G.....
3G12'-VHG.....C..C.....
3G12''-VHC.....G.....G.T.....
3G12'''-VHT.....G.....C.....G.....
3G13-VHG.....C.....G.T.....G..
3G13'-VHG.....C.....T.....
3G16-VHG.....T.C.....T.....G..
3G20-VHG..T.C.....T.....G..
4G1-VHG.....A.....
4G3-VHT.....
4G3'-VHT.....
4G8-VHCGG.....
4G10-VHT..T.....
4G12-VHG.....
5G2-VHG...C.A.....
5G3-VHGT.....T.....G..G..C
5G4-VHG.....
5G5-VHT.....
5G5'-VHT.....
5G8-VHC.....G..T..A.....
5G8'-VHC.....G..T..A.....
5G11-VHT.....

Figure 2. Nucleotide sequence comparison of the CDR2 of V_H6 containing transcripts from each of the subsets. (A) IgM transcripts. (B) IgG transcripts (see Table 1 for clone designations). Asterisk, base pair deletion.

cated and triplicated transcripts were preferentially found in those subgroups requiring reamplification (Bm1, Bm2, and Bm5), suggesting that they resulted from in vitro rather than in vivo expansion. Clonally related transcripts could only be identified within the IgG-expressing Bm3 and Bm4 subsets (Table 1 and Fig. 6), very likely as result of the selection process with progressive recruitment of single clones. In some instances however, the possibility of PCR hybrid artifacts was difficult to rule out (see below). The fact that we did not find clonal expansion within the Bm5 memory cell pool might be due to the smaller Bm5 sample size.

Overall, most of the analyzed sequences represented independent rearrangements, implying that B cells expressing V_H gene segments from the smallest families fully participate in GC reactions.

Characteristics of PCR Errors in the Analysis of Polyclonal Populations of B Cells Expressing a Single V_H Gene Segment. We

expected the most common form of PCR error in our study to be nucleotide misincorporation. To calculate the internal Taq polymerase error rate, we used the IgM expressing Bm1 and Bm2 subsets as controls. Out of the 47 IgM transcripts independently sequenced from these two populations, 28 were completely germline encoded and the remaining 19 contained a total of 28 nucleotide differences. Assuming that these differences were all due to Taq polymerase, the error rate would be 0.2% or 1/500 bp. Considering that the Bm1 and Bm2 IgM transcripts were the result of two rounds of amplification (total = 80 cycles), we calculate a misincorporation rate of <1/1000 bp. Deletions involving large areas within V_H-D_H-J_H transcript and hybrid sequences were most commonly found among the IgM expressing Bm3 and Bm4 subsets (sequences not included in the analysis). Since artifacts of this kind arise when nicked DNA is used as a PCR template, it is not surprising to find them preferentially among the B

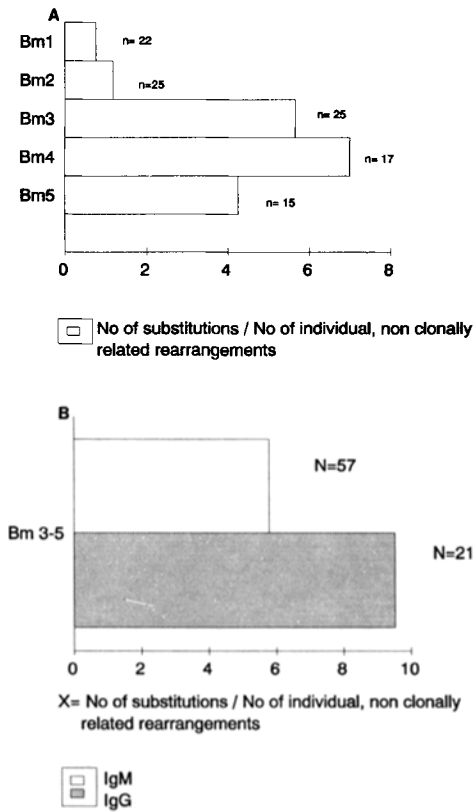


Figure 3. (A) Average number of nucleotide substitutions within the IgM transcripts from each of the Bm subpopulations. (B) Average number of nucleotide substitutions within the IgG transcripts from the Bm3, Bm4, and Bm5 subpopulations.

cell populations undergoing rapid expansion and apoptosis within the GC.

Discussion

One of the hallmarks of secondary immune responses is the recruitment of long-lived memory B cells secreting high

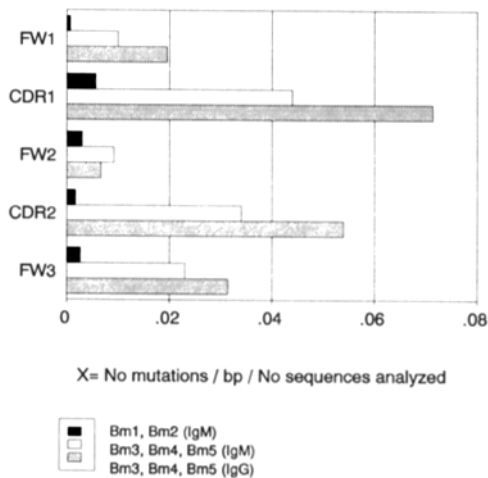


Figure 5. Average number of mutations within each of the regions encoded by the V_n gene segment.

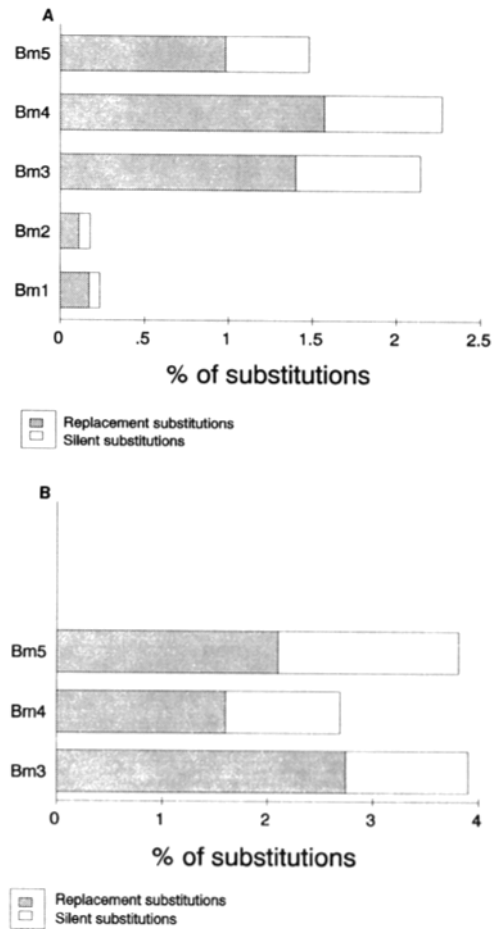


Figure 4. Percentage of replacement (hatched bars) and silent (light bars) substitutions within IgM (A) and IgG (B) transcripts from the different Bm subpopulations.

Table 2. Replacement vs. Silent Substitutions in IgM and IgG Transcripts from Tonsil B Cell Subsets

	FWs		CDRs	
	R/S	R/S	No. of substitutions	No. of sequences
IgM				
Bm1	9	0.6	15	22
Bm2	1.7	2	14	25
Bm3	1.6	2.3	156	25
Bm4	1.8	3.2	113	17
Bm5	1.6	4.5	55	15
IgG				
Bm3	1.7	2.9	114	10
Bm4	0.9	4	40	5
Bm5	1	1.4	68	6

R/S, Replacement/Silent substitutions.

Table 3. Transitions vs. Transversions in IgM and IgG Transcripts from Tonsil B Cell Subsets

	Total substitutions					Proportion of the total number of substitutions for each base			
	T	C	A	G		T	C	A	G
T	—	0.09	0.03	0.02	T	—	0.65	0.22	0.13
C	0.12	—	0.02	0.08	C	0.52	—	0.12	0.36
A	0.08	0.08	—	0.22	A	0.22	0.21	—	0.57
G	0.03	0.07	0.10	—	G	0.16	0.36	0.48	—

affinity antibodies specific for the triggering antigen. Memory B cells derive from precursor virgin B cells that undergo proliferation and differentiation in GC. With the aid of phenotypic markers we have isolated five subpopulations corresponding to the progression of B cells from the virgin to the memory compartment. To aid in this classification, we have sequenced a total of 146 rearranged heavy chain variable region transcripts from the different subpopulations. Our results show that the B cell subpopulations derived from the GC (Bm3 and Bm4) have accumulated a large number of somatic mutations, whereas two of the mantle zone subpopulations (Bm1 and Bm2) display only IgM transcripts with virtually no evidence of having been subjected to somatic diversification. Interestingly, the remaining subset (Bm5) thought to represent the memory compartment displayed the same level of somatic mutation seen in the GC subsets, further supporting its GC origin.

The existence of a natural IgM⁻, IgD⁻ memory compartment has been described in mice (49). This compartment represents up to 2–6% of the total splenic B cell population of unimmunized animals and, although it is basically com-

posed of B cells that have undergone class switching to IgG, a fraction of the IgG transcripts have been shown to contain V_H genes identical to the germline. It has been suggested that memory B cells expressing unmutated V_H genes are selected based on their display of high affinity immunoglobulin receptors for antigen (49). A similar phenomenon has been described for memory B cells arising during intentionally induced murine immune responses (50). In the present study we identified both IgM and IgG transcripts within the population of Bm5 cells characterized as the human memory compartment (Liu, Y.-J., and J. Banachereau, manuscript in preparation). However, while all the IgG transcripts contained at least three nucleotide substitutions within the region encoded by the V_H gene segment, 26% of the IgM transcripts (4/15) displayed only zero to two nucleotide differences from the corresponding germline counterpart. Whether these transcripts represent examples of germline encoded high affinity antibodies as opposed to contaminants carried over through the cell sorting and/or PCR manipulations cannot be addressed at this point.

Even though we have analyzed the V_H sequences of a

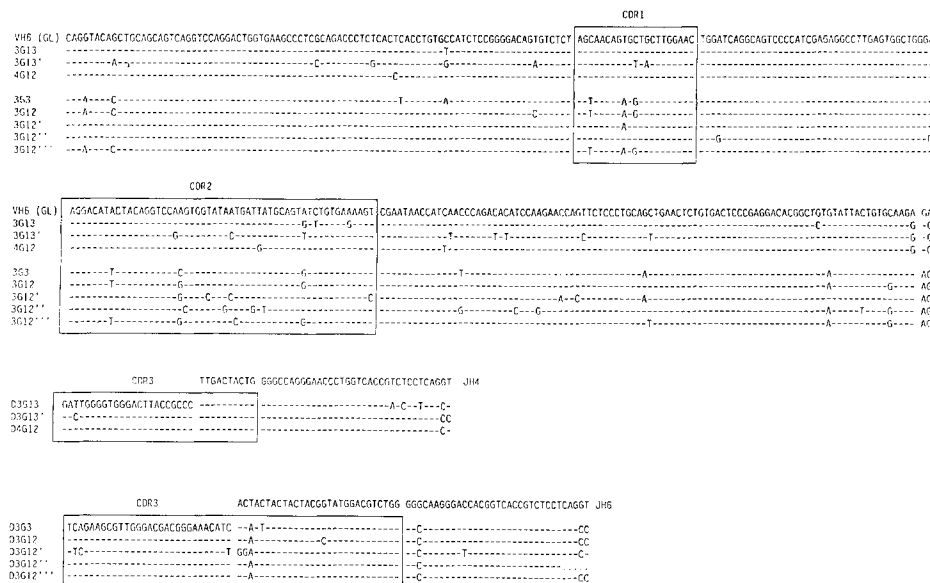


Figure 6. Nucleotide sequences of the two sets of clonally related sequences expressing V_H6 gene segments from subgroups Bm3 and Bm4 (see Table 1 for clone designations).

polyclonal population of B cells that very likely arose in response to a myriad of antigens, several interesting conclusions can be drawn from these studies. We found that within any given subpopulation, IgG transcripts had accumulated twice as many mutations as their IgM counterparts, suggesting either that the somatic mutation machinery is more active in IgG-expressing B cells, or, as suggested by Kepler et al. (51), reentry of positively selected cells into the GC takes place as a way of generating even higher affinity mutants. If this is the case, whether recirculation involves both IgM and IgG Bm5 cells, and the efficiency with which IgM-expressing cells undergo further class switches upon consecutive GC reactions are interesting questions that remain to be addressed.

Analysis of the distribution of nucleotide substitutions disclosed that CDR1 was the most mutated among the different regions encoded by the V_H gene segment. Since the vast majority of sequences in our study represent independent rearrangements which very likely encode unrelated specificities, it is possible that an intrinsic mutational bias exists around CDR1. Betz et al. (52) have reported a similar observation in an elegant study compiling data from the mutations generated in murine Ig genes during the response to phenyl-oxazolone as well as in unselected passenger transgenes.

The analysis of the ratio of R/S substitutions within the five B cell subsets included in this study suggests that IgM-bearing B cells are progressively selected from the virgin (Bm1), to the memory compartments (Bm5). The same observation, however, does not apply to Bm5 IgG transcripts that display the lowest R/S ratio among the GC-derived subpopulations.

It is important to recall that only a small proportion of GC B cells survive the selection process involved in the generation of the memory pool, since many of the substitutions found among Bm3 and Bm4 cells may not confer an advantage for antigen binding.

In situ studies of the kinetics of the murine GC reaction to the hapten 4-hydroxy-3-nitrophenyl acetyl have been recently reported. These studies disclosed the progressive reduction in clonal diversity, increased restriction in the usage of canonical gene segments, and lack of somatic mutation within the noncanonical Ig genes expressed within the GC (34). We found reduction in clonal diversity (i.e., clonal expansion) only in GC B cells (Bm3 and Bm4) containing IgG transcripts, even though our sample includes more than twice as many IgM transcripts. Although this observation can be explained based on the large sample size of the B cell pool contained within a human tonsil, it suggests that antigenic selection may operate preferentially on IgG expressing cells. The pattern of somatic mutation found within the IgM transcripts (high R/S ratios) rules out the possibility that they are the product of nonselected "passenger" B cells. The fact that they are transcribed in frame also argues against the possibility that they represent nonproductive rearrangements cotranscribed within IgG expressing B cells.

The possibility of tracing B cells through their various stages of differentiation in peripheral lymphoid organs provides the first substrate to address some of the fundamental questions that remain unresolved regarding the generation of T cell-dependent antibody responses in humans.

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