The humoral immune response relies on mature B cells, each producing a unique Ig. After a primary antigenic (Ag) challenge, triggered naive B cells can differentiate directly into plasma cells producing a first wave of specific, low-affinity IgM antibodies. In parallel, germinal center (GC) reactions are initiated that are critically dependent on T helper cells and are essential to generate B cells with high-affinity antibodies of different classes and to produce memory cells. During the GC reaction, B cells undergo a phase of brisk cell division thereby creating the GC dark zone (1–3). These rapidly dividing cells, centroblasts, accumulate nucleotide substitutions in their Ig variable region (IgV) genes, a process designated as somatic hypermutation (SHM) (4–6). Based on competition for survival signals elicited by native Ag presented at the surface of follicular dendritic cells, B cells with high-affinity antibodies of different classes and to produce memory cells. During the GC reaction, B cells undergo a phase of brisk cell division thereby creating the GC dark zone (1–3). These rapidly dividing cells, centroblasts, accumulate nucleotide substitutions in their Ig variable region (IgV) genes, a process designated as somatic hypermutation (SHM) (4–6). Based on competition for survival signals elicited by native Ag presented at the surface of follicular mantle zone; GC, germinal center; IgV, Ig variable region; SHM, somatic hypermutation; TZ, T cell zone.

The kinetics of the GC reaction have been extensively studied in rodents after immunization with sheep red blood cells or with haptens coupled to carrier proteins. Immunization experiments with T cell–dependent Ags revealed that recognizable GCs are formed within 4–5 d and are maintained for 21 d (1–3, 9, 10). In spleens from mice immunized with 4-hydroxy-3-nitrophenylacetyl coupled to chicken gamma globulin, SHM in the GCs was detectable starting from day 8 to reach approximately three mutations on average per IgV gene by day 14. Based on stringent selection, GCs finally become oligoclonal and are reported to contain three to six Ag-specific B cell clones on average (11). In man, in situ analyses on LNs (12, 13) and Peyer’s patches (14) showed that the GCs contained 4–13 B cell clones with functional IgV. As yet, it is uncertain whether B cells can engage in a GC reaction more than once. In secondary anti-arsonate responses in mice, GC B cells were found to carry higher numbers of somatic mutations in their IgV genes as compared with the primary response, whereas affinity-enhancing mutations seemed to appear more rapidly. It remained unclear, however, whether this was due to accelerated SHM rates or recruitment of memory B cells into these responses (15). At least two groups have reported that in man the IgV gene mutation frequencies in both peripheral B cells and intestinal plasma cells increase with age, suggesting repeated rounds of Ag-driven hypermutation (16, 17).

To reveal migration trails of antigen-responsive B cells in lymphoid tissue, we analyzed immunoglobulin (Ig)M–VH and IgG–VH transcripts of germinal center (GC) samples micro-dissected from three reactive human lymph nodes. Single B cell clones were found in multiple GCs, one clone even in as many as 19 GCs. In several GCs, IgM and IgG variants of the same clonal origin were identified. The offspring of individual hypermutated IgG memory clones were traced in multiple GCs, indicating repeated engagement of memory B cells in GC reactions. These findings imply that recurring somatic hypermutation progressively drives the Ig repertoire of memory B cells to higher affinities and infer that transforming genetic hits in non-Ig genes during lymphomagenesis do not have to arise during a single GC passage, but can be collected during successive recall responses.

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To gain insight into the expansion and dissemination of Ag-responsive B cells in man, we analyzed the clonal B cell composition of 48 GCs of reactive LNs originating from three donors. We observed that single B cell clones seed into multiple GCs, often located at considerable distances, and evidence was obtained for active class-switch recombination (CSR) of B cell clones within individual GCs. Importantly, in the LNs of three donors we encountered the offspring of single, hypermutated IgG clones in multiple GCs, indicative of repeated involvement of Ag-experienced B cells in this unique microenvironment.

RESULTS

Laser-aided microdissection and IgVH amplification of GC B cells

Small tissue samples of 40–80 cells were isolated out of hematoxylin-stained, frozen sections of three reactive LNs from different donors. To distinguish GCs with cycling B cells, adjacent sections were immunohistochemically stained for the proliferation marker Ki-67. Thus, we microdissected tissue samples of 30, 11, and 16 GCs out of sections of LN1, LN2, and LN3, respectively. As controls, samples from follicular mantle zones (FMs) surrounding the GCs and samples from T cell zones (TZs) were collected. IgVH transcripts were amplified by RT-PCR using VH1, VH3, and VH4 family-specific leader primers in combination with a fluorochrome-labeled Cμ primer, allowing analysis by “genescanning” on automated capillary sequencing equipment (18). Based on length variability of the complementarity determining regions 3 (CDR3s), the samples generally yielded multiple peaks, representing different B cell clones (not depicted). In LN1, we observed in 19 GCs a recurrent 481-bp peak in the VH4-Cμ PCR (Fig. 1). In LN3, products of the same lengths were obtained out of GC1 and GC2 in the VH1-Cμ PCR (not depicted). We thus decided to extensively clone and sequence VH4-Cμ PCR products of LN1 and LN2 and VH1-Cμ PCR products of LN3. RT-PCR products that were used for cloning were generated in parallel using an unlabeled Cμ primer. Depending on the availability of material, IgG transcripts were also amplified, cloned, and sequenced.

The offspring of single B cell clones inhabits multiple follicles

Out of most samples, IgVH products of different lengths were amplified representing B cell clones with unique IgVH compositions. Interestingly, by PCR and cloning we now identified in LN1 and LN3 as many as seven distinct IgM clones of which the offspring was detected in more than one GC. In LN3, four IgM-VH1 clones were identified, termed B-μ, C-μ, D-μ, and G-μ, which were each present in two GCs (Fig. 2 and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20071006/DC1). The B-μ clone, found in GC1 and GC2 of LN3, comprised 14 subclones (each designated by additive lowercase letters, Ba–Bn), with amino acid sequence differences in their IgVH-CDR3. Of this major “B” clone, both IgM- as well as IgG-expressing variants were detected (see below and Figs. S2 and S3). In the two GCs, theBg-μ subclone was found.

In the 24 GCs isolated from two separate sections of LN1, three recurrent IgM-VH4 clones were identified, designated
shared mutations between the GC samples were found in four of the seven recurrent clones, i.e., the A-μ and H-μ clones of LN1 and the C-μ and G-μ clones of LN3 (Figs. S1 and S4).

GCs contain isotype-switch variants of individual B cell clones

Within individual GCs of LN1, LN2, and LN3, a total of 11 IgVH clones were identified of which both IgM and IgG variants were present. In 8 of these 11 clonotypic IgM/IgG sets, at least one replacement mutation in the IgVH gene was shared between the IgM and IgG transcripts (Fig. 4). Interestingly, in GC16 and GC19 of LN1, IgG transcripts of the A-μ clone were detected. Small amino acid sequence differences in the IgVH-CDR3 regions were observed between some of the clonal IgM/IgG sets, i.e., in GC16 (clone A-μ), GC19 (clone D), and in GC20 (clone E) of LN1 (Fig. 4 A) and in GC13 (clone F) of LN3 (Fig. 4 C). Of note, the IgM and IgG variants of the Bh subclone in GC1 of LN3 belong to the already mentioned large clone B of which the IgM-expressing Bg-μ subclone was found in GC1 and GC2. As judged by IgVH-CDR3 differences, we identified 11 IgM-, 4 IgG-, and 1 IgM/IgG-expressing variants of this major B clone, which were all found in GC1 and GC2 of LN3 (Fig. 4 C and Figs. S2 and S3).

Figure 3. Selection of the IgM-VH4 sequences and mutations therein of the recurrent A-μ clone in four GCs of LN1. Lollipop-shaped symbols indicate nucleotide differences as compared with the V4-30.4 germline IgVH gene. Replacement and silent mutations are indicated by closed and open circles, respectively. Gray shaded bars at codons 37 and 56 indicate identical somatic mutations found in all A-μ clones. The gray shaded mutation at codon 93 in GC3 and GC5 represents an identical mutation that differed from the codon 93 mutation found in all A-μ clones of GC11. The total number of clones in which a particular IgVH mutation pattern was found is indicated by (Nx).
Figure 4. IgVH sequences and mutations therein of related IgM and IgG clones found within individual GCs of LNs 1, 2, and 3. Lollipop-shaped symbols indicate nucleotide differences as compared with the respective germline IgVH genes. Replacement and silent mutations are indicated by closed and open circles, respectively. Gray shaded bars covering mutations of IgM and IgG clones indicate identical mutations. The total number of clones in which a particular IgVH mutation pattern was found is indicated by (Nx).
IgM- and IgG-expressing B cells have different mutation frequencies

Out of LN1, LN2, and LN3, we analyzed 48 different GCs, 18 FMs, and 3 TZs. Thus, a total of 739 IgM-V_H and 524 IgG-V_H sequences of the GC samples and 370 and 48 IgM-V_H sequences of the FM and TZ samples were obtained (Table I). The number of unique IgV_H clones detected per GC varied from 1 to as many as 14 (GC1 of LN2; Tables S1–S3, available at http://www.jem.org/cgi/content/full/jem.20071006/DC1). The average number of mutations of GC IgM clones and of GC IgG clones was 4.1 and 7.4 per IgV_H, respectively (Fig. 5). Of note, a mutation frequency difference was also found when IgM and IgG sequences, whether or not clonally related, of individual GCs were compared (Fig. 6). In a minority of GCs only, higher mean numbers of IgV_H mutations were observed in IgM clones as compared with the IgG clones, i.e., in GC1, 24, 25, and 27 of LN1, GC5 of LN2, and in GC10 and 13 of LN3 (Table I). As expected, the IgM-V_H clones isolated from the FM samples harbored hardly, if any, somatic mutations, i.e., 0.8 mutations per IgV_H on average. The IgM-V_H clones of the TZ samples of LN1 harbored an average 3.5 mutations per IgV_H (Fig. 5). When applying an arbitrary cut-off of one or fewer mutation per IgV_H, unmutated IgM-expressing B cell clones were identified in 24 of the 46 GCs examined (52%). Interestingly, unmutated IgG-expressing B cell clones were identified as well in 8 of the 33 GCs examined (24%). Moreover, applying the same cut-off criterion, we found overall 22% unique mutated IgM clones in the FMs, 61% mutated IgM and 90% mutated IgG clones in the GCs, and 56% mutated IgM clones in the TZs, respectively (Tables S1–S6).

The offspring of single hypermutated IgG B cell clones is present in multiple GCs

In addition to the IgM clones A-μ, H-μ, and I-μ of LN1 and B-μ, C-μ, D-μ, and G-μ of LN3, we also encountered three IgG-V_H-expressing clones in multiple GCs of all three LNs. In GC5, 7, 16, 17, and 20 of LN1, the offspring of a heavily mutated VH4-IgG-V_H clone termed C-γ was detected. In the five GCs mentioned, the daughter C-γ clones contained mean numbers of 29.0, 17.5, 16.6, 16.3, and 25.0 mutations, respectively. As many as nine mutations were shared between all the C-γ clones (Figs. 2 and 7). In GC4 and 8 of LN2, a recurrent VH4-IgG-V_H clone termed G-γ was found. All G-γ clones harbored one shared mutation. Similarly, in LN3 one recurrent hypermutated VH1-IgG-V_H clone, J-γ, was detected in GC10, 11, and 13. Except for two clones in GC10 (J-γ subclones A2 and A5), all the daughter clones of J-γ harbored two shared replacement mutations in IgV_H. In addition, some J-γ clones showed differences of their IgV_H-CDR3s as compared with the consensus CDR3 sequence (J-γ subclones A2 and A13 of GC10) (Figs. 2 and 7).

DISCUSSION

Our in situ analyses on the reactive human LNs point out that the B cell response is a highly dynamic process based on entrance and reentrance of single naive and memory B cell clones in multiple GCs. The RT-PCR approach to amplify IgV_H genes was chosen to be able to discriminate between IgM- and IgG-expressing B cells, information that cannot be obtained by using genomic DNA. We were aware of a potential bias due to intra-GC plasmacytoid cells as they produce disproportionately more Ig per cell. Immunohistochemical stainings indicated that in the GCs of LN1 and LN2, CD138 (syndecan-1)–expressing plasma cells were virtually absent, whereas they were abundantly present in the extrafollicular areas. In LN3, ~50% of the GCs did contain scattered plasmacytoid cells, weakly expressing CD138 (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20071006/DC1). Intra-GC plasmacytoid cells are, for several reasons, unlikely to be just random follicular immigrants but are rather maturing locally and thus are a direct reflection of the Ag-stimulated GC B cell population. Extrafollicular differentiating plasma cells up-regulate CD138 and down-regulate receptors that...
### Table I. IgV\(_{H}\) clones found in samples microdissected out of three reactive human LNs

| GC samples | Amplified Ig transcripts | No. of RT-PCR | Clones sequenced | Mean no. of IgVH mutations of all clones |
|------------|--------------------------|---------------|------------------|----------------------------------------|
| **LN 1**   |                          |               |                  |                                        |
| GC1\(^a\)  | VH4-IgM                  | 5             | 47               | 2.3                                    |
| GC1\(^b\)  | VH4-IgG                  | 1             | 15               | 0.5                                    |
| GC2\(^a\)  | VH4-IgM                  | 7             | 74               | 1.9                                    |
| GC3\(^a\)  | VH4-IgM                  | 3             | 22               | 4.3                                    |
| GC5\(^a\)  | VH4-IgM                  | 9             | 53               | 2.6                                    |
| GC5\(^b\)  | VH4-IgG                  | 2             | 5                | 29.0                                   |
| GC7        | VH4-IgM                  | 1             | 6                | 2.0                                    |
| GC7        | VH4-IgG                  | 1             | 6                | 5.8                                    |
| GC10       | VH4-IgM                  | 1             | 7                | 2.0                                    |
| GC10       | VH4-IgG                  | 1             | 7                | 3.4                                    |
| GC11       | VH4-IgM                  | 3             | 22               | 3.5                                    |
| GC11       | VH4-IgG                  | 2             | 18               | 6.8                                    |
| GC12       | VH4-IgM                  | 1             | 2                | 2.0                                    |
| GC13\(^a\) | VH4-IgM                  | 2             | 13               | 2.0                                    |
| GC16       | VH4-IgM                  | 1             | 15               | 2.4                                    |
| GC16       | VH4-IgG                  | 1             | 25               | 10.3                                   |
| GC17       | VH4-IgM                  | 1             | 13               | 0.3                                    |
| GC17       | VH4-IgG                  | 1             | 18               | 6.3                                    |
| GC18       | VH4-IgM                  | 1             | 14               | 4.9                                    |
| GC18       | VH4-IgG                  | 1             | 6                | 8.0                                    |
| GC19       | VH4-IgM                  | 1             | 17               | 2.1                                    |
| GC19       | VH4-IgG                  | 1             | 16               | 4.7                                    |
| GC20       | VH4-IgM                  | 1             | 5                | 1.8                                    |
| GC20       | VH4-IgG                  | 1             | 14               | 4.3                                    |
| GC21       | VH4-IgM                  | 1             | 15               | 2.3                                    |
| GC22       | VH4-IgM                  | 1             | 15               | 1.7                                    |
| GC23       | VH4-IgM                  | 1             | 14               | 2.3                                    |
| GC24       | VH4-IgM                  | 1             | 14               | 1.9                                    |
| GC24       | VH4-IgG                  | 1             | 8                | 1.5                                    |
| GC25       | VH4-IgM                  | 1             | 11               | 3.1                                    |
| GC25       | VH4-IgG                  | 1             | 16               | 1.6                                    |
| GC26       | VH4-IgM                  | 1             | 14               | 2.6                                    |
| GC27       | VH4-IgM                  | 1             | 14               | 21.1                                   |
| GC27       | VH4-IgG                  | 1             | 12               | 1.3                                    |
| GC28       | VH4-IgM                  | 1             | 12               | 2.3                                    |
| GC29       | VH4-IgM                  | 1             | 9                | 2.7                                    |
| GC30       | VH4-IgM                  | 1             | 8                | 1.3                                    |
| FM1        | VH4-IgM                  | 1             | 23               | 0.2                                    |
| FM2        | VH4-IgM                  | 1             | 8                | 0.1                                    |
| FM3        | VH4-IgM                  | 1             | 27               | 0.0                                    |
| FM4        | VH4-IgM                  | 1             | 26               | 0.2                                    |
| FM5        | VH4-IgM                  | 1             | 26               | 1.6                                    |
| FM6        | VH4-IgM                  | 1             | 23               | 0.5                                    |
| FM7\(^a\)  | VH4-IgM                  | 2             | 26               | 1.5                                    |
| FM8\(^a\)  | VH4-IgM                  | 2             | 28               | 1.8                                    |
| FM9        | VH4-IgM                  | 1             | 6                | 0.8                                    |
| FM10       | VH4-IgM                  | 1             | 23               | 0.3                                    |
| TZ1        | VH4-IgM                  | 1             | 12               | 4.7                                    |
| TZ2        | VH4-IgM                  | 1             | 16               | 3.1                                    |
| TZ3        | VH4-IgM                  | 1             | 20               | 2.8                                    |
| **LN 2**   |                          |               |                  |                                        |
| GC1\(^a\)  | VH4-IgM                  | 4             | 51               | 2.8                                    |
| GC1\(^b\)  | VH4-IgG                  | 6             | 66               | 6.0                                    |
Table I. IgVH clones found in samples microdissected out of three reactive human LNs (Continued)

| GC samples | Amplified Ig transcripts | No. of RT-PCR | Clones sequenced | Mean no. of IgVH mutations of all clones |
|------------|--------------------------|---------------|------------------|----------------------------------------|
| GC2\(^b\)  | VH4-IgM                  | 2             | 15               | 2.1                                    |
| GC2\(^b\)  | VH4-IgG                  | 6             | 44               | 5.2                                    |
| GC3        | VH4-IgM                  | 1             | 9                | 1.6                                    |
| GC4        | VH4-IgM                  | 1             | 16               | 4.4                                    |
| GC4        | VH4-IgG                  | 1             | 7                | 6.9                                    |
| GC5        | VH4-IgM                  | 1             | 14               | 4.7                                    |
| GC5        | VH4-IgG                  | 2             | 21               | 2.9                                    |
| GC6        | VH4-IgM                  | 1             | 13               | 5.2                                    |
| GC6        | VH4-IgG                  | 2             | 17               | 6.1                                    |
| GC8        | VH4-IgM                  | 1             | 4                | 7.8                                    |
| GC8        | VH4-IgG                  | 1             | 25               | 7.9                                    |
| GC10       | VH4-IgM                  | 1             | 11               | 4.9                                    |
| FM1        | VH4-IgM                  | 1             | 16               | 1.1                                    |
| FM2        | VH4-IgM                  | 1             | 14               | 0.1                                    |
| FM3        | VH4-IgM                  | 1             | 8                | 0.3                                    |
| LN 3       |                          |               |                  |                                        |
| GC1\(^c\)  | VH1-IgM                  | 2             | 24               | 7.2                                    |
| GC1\(^c\)  | VH1-IgG                  | 2             | 26               | 12.7                                   |
| GC2        | VH1-IgM                  | 1             | 9                | 3.6                                    |
| GC2        | VH1-IgG                  | 1             | 10               | 5.1                                    |
| GC3        | VH1-IgM                  | 1             | 4                | 1.3                                    |
| GC3        | VH1-IgG                  | 1             | 14               | 7.0                                    |
| GC4        | VH1-IgM                  | 1             | 12               | 3.6                                    |
| GC4        | VH1-IgG                  | 1             | 15               | 3.6                                    |
| GC5        | VH1-IgM                  | 1             | 14               | 13.0                                   |
| GC7        | VH1-IgM                  | 1             | 12               | 2.8                                    |
| GC8        | VH1-IgM                  | 1             | 11               | 8.2                                    |
| GC8        | VH1-IgG                  | 1             | 10               | 21.3                                   |
| GC9        | VH1-IgM                  | 1             | 14               | 6.4                                    |
| GC9        | VH1-IgG                  | 1             | 14               | 8.0                                    |
| GC10       | VH1-IgM                  | 1             | 8                | 10.5                                   |
| GC10       | VH1-IgG                  | 1             | 14               | 9.2                                    |
| GC11       | VH1-IgM                  | 1             | 13               | 5.2                                    |
| GC11       | VH1-IgG                  | 1             | 13               | 8.1                                    |
| GC12       | VH1-IgM                  | 1             | 8                | 1.6                                    |
| GC12       | VH1-IgG                  | 1             | 12               | 16.3                                   |
| GC13       | VH1-IgM                  | 1             | 25               | 10.5                                   |
| GC13       | VH1-IgG                  | 1             | 8                | 5.4                                    |
| GC14       | VH1-IgM                  | 1             | 15               | 1.7                                    |
| GC14       | VH1-IgG                  | 1             | 8                | 9.4                                    |
| GC15       | VH1-IgM                  | 1             | 9                | 1.3                                    |
| GC16       | VH1-IgM                  | 1             | 5                | 2.4                                    |
| GC16       | VH1-IgG                  | 1             | 8                | 16.3                                   |
| GC17\(^d\) | VH1-IgM                  | 1             | 12               | 1.3                                    |
| FM1\(^d\)  | VH1-IgM                  | 1             | 15               | 2.0                                    |
| FM2\(^d\)  | VH1-IgM                  | 1             | 15               | 0.2                                    |
| FM3\(^d\)  | VH1-IgM                  | 1             | 14               | 2.8                                    |
| FM4\(^d\)  | VH1-IgM                  | 1             | 15               | 0.6                                    |
| FM5\(^d\)  | VH1-IgM                  | 1             | 57               | 0.3                                    |

\(^a\) Of GC2, GC3, GC5, GC13, MZ7, and MZ8, two microdissected samples were analyzed.
\(^b\) Of GC1 and GC2, four and two microdissected samples were analyzed, respectively.
\(^c\) Of GC1, two microdissected samples were analyzed.
\(^d\) The microdissected samples of GC17 and FM1–FM5 are indicated in Fig. S5.
are essential to enter GCs, i.e., membrane-bound Ig to interact with Ag and the follicle-attracting chemokine receptor CXCR5 (20). Moreover, they up-regulate CXCR4 whose ligand (CXCL12/SDF) is highly expressed in the medullary cords (20). Finally, the fact that in several GCs isotype-switch variants of individual B cell clones were detected underscores the solidity of the experimental strategy.

The number of unique VH4- or VH1-expressing clones identified per GC varied between 1 and 14 (GC1 of LN2; Tables S1–S3). Others have reported numbers of unique clones in human GCs, ranging between 4 and 13 and 1 and 16 (12–14), whereas in immunization studies with T cell–dependent Ags in the mouse, averages of 3–6 clones per GC have been documented (11). These combined results are suggestive for a more diverse B cell repertoire in GCs in man, which still is an underestimation (11). Moreover, they up-regulate CXCR4 whose ligand is essential to enter GCs, i.e., membrane-bound Ig to intercalate into the follicle membranes (20). The solidity of the experimental strategy.

In LN1 and LN3, we detected seven distinct IgM-expressing clones that were each present in at least two separate GCs (Figs. 1–3 and Figs. S1 and S2). Most remarkable was the IgM-VH4 A-μ clone that was traced in 19 of 24 GCs in two consecutive sections of LN1 (Fig. 2). It is emphasized that although the A-μ clone was detected in the majority of the GC samples, it was found only in 1 of the 10 analyzed samples from adjacent mantle zones and not at all in randomly collected samples from the TZs of LN1. All the A-μ clones contained two identical mutations, and in 14 GCs daughter clones with unique additional mutations were detected (Fig. 3 and Fig. S4). The finding of the widely disseminated A-μ clone in LN1 is in accordance with studies in mice showing that after a primary immunization with the hapten arsonate, some clonotypic B cells expanded and subsequently populated different follicles in which the daughter cells underwent their own clonal evolution (15). Extracellular proliferation has also been demonstrated in mice immunized with (4-hydroxy-3-nitrophenyl)acetyl coupled to chicken gamma globulin. In this system, unmutated and mutated daughter cells of a B cell clone were found in an extracellular plasma cell focus and in a neighboring GC, respectively (21). Thus, two scenarios may explain the presence of IgM clones with shared mutations in multiple GCs, such as the A-μ clone: (a) Ag activation of a naive IgM precursor B cell, induction of SHM, and, after limited proliferation, migration into multiple primary follicles; and (b) extracellular reactivation and proliferation of one or more members of a mutated memory IgM B cell and subsequent seeding into various follicles.

As expected, hypermutated IgVH14 clones were found mainly in the GC samples. In contrast, the mean number of mutations of the IgM clones from FMs did not exceed 0.7 per IgVH14. Still, the FM samples contained 22% of mutated IgM clones, and, conversely, in the GCs as many as 39% of the IgM clones and 10% of the IgG clones were unmutated. These findings are compatible with previous microdissection studies on human LNs (12, 13) and are explained by recent intravitral two-photon microscopy studies. In the latter studies, it was demonstrated that trafficking of naive B cells is not completely restricted to the mantle zones as they frequently surpass the GC borders (22, 23). We found means of 3.2, 4.2, and 4.8 mutations for IgM clones and 6.4, 5.8, and 9.9 mutations for IgG clones in LNs 1, 2, and 3, respectively (Fig. 5). For tonsillar IgM and IgG B cells, average mutation loads of 5.7 and 9.5 have been reported (24). Interestingly, we identified 11 clones of which IgM and IgG isotype variants were present within individual GCs, providing formal proof for active CSR in this environment in man. Shared replacement mutations were identified in 8 out of the 11 paired IgM/IgG clones (Fig. 4). Again, the IgG clones generally contained more mutations as compared with the corresponding IgM clones (Fig. 6). This finding is a priori not expected noticing that the 11 isotype-switch variants each originate from single Ag-responsive precursor cells that had resided for equal times in their particular GCs. Isotype-related mutation differences within GCs can, hypothetically, be explained by a nonrandom process of CSR that is more due at higher IgV affinities and thus stronger BCR signals. However, the finding of unmutated IgG clones in 8 of the 33 GCs examined indicates that such an affinity threshold is not absolute.

In three LNs, recurrent hypermutated IgG clones were identified, i.e., in LN1 the IgG-VH4 C-γ clone in five GCs, in LN2 the IgG-VH4 G-γ clone in two GCs, and in LN3 the IgG-VH1 J-γ clone in three GCs. Importantly, as many as nine mutations were shared among the C-γ clones found in the five GCs, whereas in the J-γ clones retrieved from the three GCs, two replacement mutations were common (Fig. 7). This, together with the fact that no corresponding IgM variants were traced, indicates earlier GC passage(s) of precursor clones of C-γ, G-γ, and J-γ. These recurrent IgG-expressing clones are thus to be considered as reactivated memory B cell clones. Repeated GC engagement has been proposed to occur in mice as well. Secondary response to phosphorylcholine-KLH yielded B cells that were more heavily mutated as compared with primary response B cells, whereas most of the mutations appeared to be shared among the clones. This latter observation suggested mere expansion of memory B cells within GCs with minimal additional SHM (25). Recent intravitral two-photon microscopic experiments in mice also indicated that memory B cells are able to join an existing GC, provided they have a competitive advantage in Ag binding affinity (23). Repetitive passing of memory B cells through
GCs is compatible with (a) the fact that peripheral blood memory B cells on average harbor higher mutation loads as compared with GC B cells in LN and tonsil (24, 26); (b) the positive correlation between mean IgVH mutation frequencies of memory B cells and age (in young and aged humans, respective mean mutation numbers of 9.7 and 11.5 for IgM and 17.3 and 22.5 for IgG memory B cells have been reported (17)); and (c) the reported difference in replication history of memory B cells in children and adults, having undergone ~8 and 11 cell divisions, respectively (27). In this respect, it can be envisaged that the relative contribution of memory B cells to GC responses increases with age.

It is generally believed that B cells expanding in GCs are at increased risk of genetic derangement. The facts that lymphomas are in majority of B cell origin, of GC or post-GC phenotype, and carry hypermutated IgVH genes support the notion that most lymphomas arise during this turbulent differentiation phase. The processes of SHM and CSR, both accompanied by single- and double-stranded DNA breaks, imply genetic instability and are potentially dangerous because they may act also beyond the Ig locus (28–30). Indeed, ample evidence is now available that many of the chromosomal translocations specific for the various B cell lymphoma entities are byproducts of these two processes (31, 32). So far, the implicit presumption has been that the genetic hits necessary for cellular transformation have to occur during the relatively brief proliferation phase of a single GC reaction. Knowing now that memory B cells reenter secondary follicles, most likely upon renewed Ag challenge, an alternative scenario of B cell lymphomagenesis can be envisaged. Hence, the transforming genetic hits do not have to occur during the first and only GC passage but can, in parallel to the gain of IgV mutations, gradually accumulate in memory B cells during successive recall responses throughout life. This scenario would explain why the peak incidence of B cell non-Hodgkin’s lymphoma is not early in life, when most primary responses occur, but at late adulthood long after establishing the memory B cell repertoire. This pathogenetic course is also in accordance with the high IgVH mutation frequencies found in all (post) GC B cell lymphomas, i.e., being in the range of those found in peripheral blood memory B cells rather than those of primary GC B cells (17, 24, 26, 33–36). Finally, if true one would expect that B cells belonging to expanded memory clones specific for common pathogens would be most at risk and therefore overrepresented among the various B cell lymphomas. This antigenic bias should be reflected in the Ig repertoire of (post) GC B cell lymphomas.

MATERIALS AND METHODS

Patient material. All LNs were fresh-frozen in liquid nitrogen shortly after surgical resection. LN1 was a cervical LN removed from a 4-yr-old male suffering from sustained lymphadenopathy, and LN2 originated from the artery hepatica communis region and had been removed from a 75-yr-old male suffering from pancreatic carcinoma. LN3 was a cervical LN resected out of a 46-yr-old woman suffering from chronic sialadenitis. All LNs contained reactive lymph follicles. Of note, LN2 was purely reactive and did not contain carcinoma cells.

The study was performed in accordance with the ethical standards and approved by the research code committee on human experimentation of our institute.

Immunohistochemistry. The immunohistochemical stainings were performed on acetone-fixed cryostat sections using the Prowessvision detection system (ImmunoVision Technologies). Endogeneous peroxidase activity was blocked with 0.1% NaN3, 0.3% H2O2 in PBS. Visualization of antibody binding was performed with 3-amino-9-ethylcarbazole (Sigma-Aldrich), 0.03% H2O2 in sodium acetate, pH 4.9. A monoclonal antibody specific for Ki-67 (MIB-1; DakoCytomation) was used.

Laser-aided microdissection and cDNA synthesis. 10-μm frozen tissue sections were mounted on polyethylene membranes (PALM) and briefly stained with hematoxylin for 1 min, followed by gentle rinsing with tap water and finally with distilled water. After air drying, microdissection was performed using the PALM system. Using the 20× objective, tissue pieces with a diameter of ~50 μm were cut out and catapulted in the cap of a microfuge tube containing 20 μl of RT reaction mix. Next, the tubes were incubated, upside down, in direct contact with a heating block at 42 °C for 1 h, followed by an inactivation at 95 °C for 10 min. The RT mix contained the following: 0.1 mmol/l Pd(NH)2 random primers, 8 U/ml molony murine leu- menia virus RT (Invitrogen), 1 mmol/l of each dNTP, and 1.2 U/μl RNase inhibitor (Roche) in 1× first strand buffer (Invitrogen).

IgVH amplification by RT-PCR, cloning, and sequencing. IgM-VH and IgG-VH transcripts were amplified using VH family-specific leader primers for the VH1, VH3, and VH4 families of the IgVH genes in combination with Cα and Cγ primers, respectively (37). In some experiments, a fluorescencelabeled Cα primer was used to enable automated detection of PCR products by gene scanning with capillary sequencing equipment (18). The PCR was performed with 1 μl cDNA in a 25-μl volume and started with 4 min at 95 °C, followed by 10 cycles of 1 min at 95 °C, 30 s at 57 °C, and 1 min at 72°C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55°C, and 1 min at 72°C. The reaction was terminated for 6 min at 72°C. The PCR was performed in 1.5 mmol/l MgCl2 using Platinum Taq polymerase and PCR buffers (Invitrogen) according to the manufacturer’s description. In each RT-PCR run, water controls were included, which were in all cases negative. Moreover, three control samples of the polyethylene membrane were also tested and turned out to be negative. VH1/VH4-IgVH RT-PCR products were cloned into pTOPO-TA vectors and transformed into TOP10 bacteria (Invitrogen) to generate molecular IgVH clones. Sequencing on both strands was performed using the big dye terminator cycle sequencing kit (Applied Biosystems). To identify the IgVH germline gene used and the somatic mutations therein, the sequences were compared with published germline IgVH genes using the Vbase database (38) and DNAplot online (http://www.mrc-ucp.cam.ac.uk).

The Taq error rate in our 50-cycle RT-PCR was experimentally determined in an RT-PCR specific for CD20 using two FM samples of LN1. By sequencing 29 clones, the Taq error rate was found to be ~0.3 bp per 300 bp.

Online supplemental material. Fig. S1 shows IgVH sequences and mutations therein of recurrent IgM clones found in the GCs of LN1 (H-μ and I-μ) and LN3 (B-μ, C-μ, D-μ, and G-μ). In Fig. S2, IgVH sequences of all B-μ subclones found in GC1 and GC2 of LN 3 are shown. Fig. S3 shows IgVH-CDR3 amino acid sequences of all B subclones found in GC1 and GC2 of LN3. Fig. S4 shows IgVH sequences of the recurrent A-μ clone found in 19 GCs of LN1, and Fig. S5 shows microdissected samples of GC17 and FM1-FMS in an additional section of LN3. In Fig. S6, immunohistochemical detection of CD138 in LNs 1–3 is shown. In Tables S1–S3, IgVH rearrangements of all IgM and IgG clones found in the GCs of LNs 1–3 are listed. Tables S4–S6 show IgVH rearrangements of all IgM clones found in the FMs and TZs of LNs 1–3.

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REFERENCES
1. Kroese, F.G., W. Timens, and P. Nieuwenhuis. 1990. Germinal center reaction and B lymphocytes: morphology and function. Eur. J. Immunol. 20:103–148.

2. MacLennan, I.C.M. 1994. Germinal centers. Annu. Rev. Immunol. 12:117–139.

3. Liu, Y.-J., G. Grouard, O. de Bouteiller, and J. Banchereau. 1996. Follicular dendritic cell and germinal centers. Int. Rev. Cytol. 166:139–179.

4. Berek, C., and C. Milstein. 1988. The dynamic nature of the antibody repertoire. Immunol. Rev. 105:5–26.

5. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. Cell. 67:1121–1129.

6. Jacob, J., G. Keboe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. Nature 354:389–392.

7. Lindhout, E., G. Koopman, S.T. Pals, and C. de Groot. 1997. A general marker for somatically mutated (memory) B cells. J. Exp. Med. 181:1319–1331.

8. Liu, Y.-J., J. Zhang, P.J.L. Lane, E.Y.T. Chan, and I.C.M. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur. J. Immunol. 21:2951–2962.

9. Roers, A., M.L. Hansmann, K. Rajewsky, and R. Küppers. 1998. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur. J. Immunol. 21:2951–2962.

10. Jacob, J., J. Przybyła, C. Miller, and G. Keboe. 1993. In situ study of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for pertiarteriolar lymphoid sheath-associated loci and germinal centers. J. Exp. Med. 194:45–56.

11. Jacob, J., and G. Keboe. 1992. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for pertiarteriolar lymphoid sheath-associated loci and germinal centers. J. Exp. Med. 176:679–687.

12. Allen, C.D., T. Okada, H.L. Tang, and J.G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. Science. 315:528–531.

13. Schwickert, T.A., R.L. Lindquist, G. Shakhar, G. Livni, D. Skokos, M.H. Kosco-Vilbois, M.L. Dustin, and M.C. Nussenzweig. 2007. In vivo imaging of germinal centres reveals a dynamic open structure. Nature 446:83–87.

14. Pascual, V., J.-Y. Liu, A. Magalhães, O. de Bouteiller, J. Banchereau, and J.D. Capra. 1994. Analysis of somatic mutation in five B cell subsets of human tonsil. J. Exp. Med. 180:329–339.

15. Miller, C., J. Stedra, G. Keboe, and J. Cerny. 1995. Facultative role of germinal centers and T cells in the somatic diversification of IgVH genes. J. Exp. Med. 181:1319–1331.

16. Klein, U., K. Rajewsky, and R. Küppers. 1998. Human immunoglobulin (Ig)M/IgD+ peripheral 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. J. Exp. Med. 188:1679–1689.

17. van Zelm, M.C., T. Szczepanski, M. van der Burg, and J.J. van Dongen. 2007. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J. Exp. Med. 204:645–655.

18. Shen, H.M., A. Peters, B. Baron, X. Zhu, and U. Storb. 1998. Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. Science. 280:1750–1752.

19. Pasqualucci, L., A. Migliazza, N. Fragiacomo, C. William, A. Neri, L. Baldini, R.S. Chaganti, U. Klein, R. Küppers, K. Rajewsky, and R. Dalla-Favera. 1998. BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. Proc. Natl. Acad. Sci. USA. 95:11816–11821.

20. Bende, R.J., W.M. Aarts, E.J. Steenbergen, P.M. Kluin, E.C.M. Ooms, W.M. Aarts, R.J. Bende, C.J.L.M. Meijer, S.T. Pals, and C.J.M. van Noesel. 2005. Among B cell non-Hodgkin's lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid factor reactivity. J. Exp. Med. 201:1229–1241.

21. Klein, U., T. Goossens, M. Fischer, H. Kanzer, A. Braeuninger, K. Rajewsky, and R. Küppers. 1998. Somatic hypermutation in normal and transformed human B cells. Immol. Rev. 162:261–280.

22. Tsujii, M., S. Yurasov, K. Velinzon, S. Thomas, M.C. Nussenzweig, and H. Wardemann. 2006. A checkpoint for autoreactivity in human IgM memory B cell development. J. Exp. Med. 203:393–400.

23. Aarts, W.M., R. Willemze, R.J. Bende, C.J.L.M. Meijer, S.T. Pals, and C.J.M. van Noesel. 1998. VH gene analysis of primary cutaneous B-cell lymphomas: evidence for ongoing somatic hypermutation and isotype switching. Blood 92:3857–3864.

24. Cook, G.P., and I.M. Tomlinson. 1995. The human immunoglobulin VH repertoire. Immunol. Today. 16:237–242.