Somatic Hypermutation in Low-Grade Mucosa-Associated Lymphoid Tissue-Type B-Cell Lymphoma

By Yufen Qin, Axel Greiner, Marcus J.F. Trunk, Bernd Schmausser, M. Michaela Ott, and Hans Konrad Müller-Hermelink

The origin of low-grade mucosa-associated lymphoid tissue (MALT)-type B-cell lymphoma is still unclear. Using a novel two-step procedure, we have sequenced the Ig V\(\gamma\) genes expressed by cells from four patients with gastric low-grade MALT-type lymphoma. The nucleotide sequences of the complementarity determining region 3 (CDR3) of the genomic DNA were first amplified using consensus oligonucleotide primers, then sequenced. Based on the CDR3 sequence amplified from each MALT lymphoma, individual tumor-specific primers were synthesized and used directly in the polymerase chain reaction (PCR) to analyze the sequences of their Ig heavy-chain variable region. When compared with the germ-line sequence, many nucleotide substitutions, mainly in the CDRs, were found in the variable gene sequences of the four MALT lymphomas. The mutations showed a high replacement-to-silent ratio and were distributed in a way which suggested that the tumor cells had been positively selected through their antigen receptor. Our findings indicate that the MALT-type lymphoma B cells are hypermutated postgerminal center lymphocytes that have undergone antigen selection.

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MUCOSA-ASSOCIATED lymphoid tissue (MALT)-type lymphoma of the stomach is characterized by a diffuse infiltrate of centrocyte-like cells in the stomach, immediately beneath the mucosal epithelium, and the development of a lymphoepithelial lesion. The malignant B cells share a number of features with normal B cells in the splenic marginal zone. Morphologically and phenotypically, the two cell populations are very similar. They both express CD19, and marginal zone centrocyte-like lymphoma B cells are postgerminal center B cells and that many are memory B cells generated during T-cell-dependent antibody responses. Recent studies have provided indirect evidence that antigen may play a role in the pathogenesis of low-grade tumors of the MALT type. However, both the origin of the MALT-type lymphoma and the role of antigen in its clonal expansion are still unclear. It has been shown in both rodents and humans that somatic hypermutation of B-cell Ig variable region genes occurs during B-cell differentiation which, when coupled to antigen selection in germinal centers, results in the production of antibodies of increased affinity. Somatic hypermutation of Ig V region genes is generally believed to occur in the germinal centers. Mutation seems to occur mainly in the framework regions (FRs) and complementarity determining regions (CDRs) of the Ig variable regions, which often show a marked accumulation of replacement (R) mutations in their CDRs. CDRs are thought to constitute the antigen-binding site and the clustering of R mutations in CDRs has been used as an indicator of antigenic selection. On this basis, it might be possible to study the origin and the role of antigen in clonal selection of a B-cell clone of lymphoma by analyzing Ig gene mutation. Somatic mutations have been found to occur during the rearrangement of Ig variable region genes in follicular lymphoma and multiple myeloma, but not in mantle-zone lymphoma, B-cell acute lymphocytic leukemia, B-cell chronic lymphocytic leukemia, and Burkitt’s lymphoma. However, somatic hypermutation analysis has not yet been applied to study MALT-type lymphoma.

In this report, we used a novel two-step procedure to analyze the IgH V region genes of biopsy samples from four patients with low-grade MALT-type lymphomas. The CDR3 regions from tumor tissue were first amplified using the polymerase chain reaction (PCR) technique, then sequenced. Tumor-specific primers were then synthesized on the basis of the corresponding CDR3 sequence. Together with primers for distinct VH families, the VH genes from tumor cells were amplified and sequenced. Analysis of the somatic mutations for each tumor-derived VH gene indicates that these centrocyte-like lymphoma B cells are postgerminal center lymphocytes that have undergone antigen selection.

MATERIALS AND METHODS

Extraction of DNA and RNA from tumor tissue. Biopsy specimens from four low-grade MALT-type lymphomas were used as a source of tumor DNA and RNA; control DNA was obtained from normal tonsil and lymph node material. Genomic DNA and total RNA were extracted using previously described procedures. Nested amplifications with consensus primers were performed in a GeneAmp PCR system (Perkin Elmer-Cetus, Norwalk, CT). Samples of genomic DNA (250 ng) were initially amplified in a final volume of 50 \(\mu\)L reaction buffer [50 mmol/L TRIS-HCL, pH 9.0 at 25°C; 20 mmol/L (NH\(\text{4}\))\(\text{2}\)SO\(\text{4}\); 3.0 mmol/L MgCl\(\text{2}\)] containing 2 \(\mu\)L of recombinant Taq Polymerase (GIBCO BRL, Gaithersburg, MD), 20 pmol of an upstream primer (V\(\text{\alpha}\)-ACACGCGTGTG-TATT-3’), designed using a sequence of framework region 3, and 20 pmol of downstream primer (L\(\text{\beta}\)-ACCTGAGGAGACGGT-3’), designed to match the 3’ ends of the six JH segments. After 5 minutes of denaturation at 94°C, samples were amplified for 40 cycles, each cycle consisting of 50 seconds at 94°C, 1 minute at 55°C, and 40 seconds at 72°C. Reamplification of a 1-\(\mu\)L aliquot
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(%) of the initial PCR product with 20 pmol primers Vcon and VLJH (5'-GGTGACCGGTTCCCTGAGCCG-3') was performed for 35 cycles (cycle conditions: denaturation step at 94°C for 30 seconds; and an annealing/extension step at 58°C for 40 seconds). Aliquots of the final reaction product were analyzed by electrophoresis in a 1.5% agarose gel (Sigma) containing ethidium bromide. PCR products were purified by electrophoresis and recovered from the gel by treatment with gelase (Epicentre Tech., WI). The recovered DNA was ligated into the pGEM T vector (Promega, La Jolla, CA), which was used to transfect Escherichia coli DH5α. Six to 12 white colonies were picked at random and grown overnight in 3 mL of LB (Luria-Bertani) medium. The double-stranded DNA template from the colonies was sequenced by the method of Sanger et al.25

The most common sequence was considered as being derived from the malignant B cells. Based on the complete sequence, oligonucleotides specific for each MALT-type lymphoma-derived CDR3 region were designed. The specificity tested by amplifying the VH family-specific leader primers was tested using the PCR technique and upstream consensus primers of framework region 2 (5'-TGGATCCGCCACGCTTCCTCAGG-3'), plus the nested primers for the J regions described above.

Amplification and sequencing of the MALT-type lymphoma VH genes. cDNA was synthesized from 2 μg of total RNA from MALT-type lymphomas, using an oligo d(T) primer and a modification of the protocol of Gubler and Hoffman.26 The rearranged VH genes were amplified by incubating the first-strand cDNA reaction with a mixture of six 5' VH family-specific leader primers (VNL1: 5'-CCATGGACTG GACCTGGAGG-3', VNL2: 5'-ATGGACA- TACTTTGTTCCAGC-3', VNL3: 5'-CCATGGAGTTTGGGCT- GAGC-3', VNL4: 5'-ATGAAAACACCTGTGGTTClT-3', VNL5: 5'-ATGGGTCACCGCGCAT CCT-3', VNL6: 5'-ATGTCAGTGCTTCCCTCCTCAT-3'), plus a different 3' tumor clone-specific primer (N-Dt-N region) in a reaction volume of 50 μL. The final concentrations of reagents in the solution were the same as described above. After 5 minutes' denaturation at 94°C, the samples were amplified for 40 cycles, each cycle consisting of 1 minute at 94°C, 30 seconds at 59°C, and 30 seconds at 72°C, and the amplified VH product sequenced as described above. All sequences were confirmed by sequencing in both orientations.

Assignment of mutations. Mutations, identified by comparing each sequence with germline sequences using the FASTA program and the GenBank Database (Genetics Computer Group of the University of Wisconsin), were defined on the basis of nucleotide changes in the VH segment, with any variability at the joining sites of the VH, D, and Jh gene segments not being classified as mutations, because they might result either from the insertion of N regions or from mutation. Two nucleotide exchanges in a single codon were scored as one replacement mutation.

RESULTS

Preparation of malignant B-cell–specific CDR3 primers. To determine the nucleotide sequence of tumor clone-spe-
Table 2. Predominant CDR3 Region Nucleotide Sequence and D Segment Usage of Each MALT B Lymphoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Identical Clone</th>
<th>VH</th>
<th>N</th>
<th>D</th>
<th>N</th>
<th>JH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/6</td>
<td>TGGGAGA</td>
<td>GAGGGGA</td>
<td>AGGGGGGT</td>
<td>TATAGGGGCTTGGCA</td>
<td>GGGGGG</td>
</tr>
<tr>
<td>2</td>
<td>7/9</td>
<td>TGGGAGA</td>
<td>G</td>
<td>ATCGTGAGTCGGCACGCTTG</td>
<td>C</td>
<td>TGGACTACTGGGG</td>
</tr>
<tr>
<td>3</td>
<td>5/7</td>
<td>TGGGAGA</td>
<td>A</td>
<td>GCAGTGGGCTTGGGGCTTGGG</td>
<td>ACTTTGCGACTACTGGGG</td>
<td>(JH4)</td>
</tr>
<tr>
<td>4</td>
<td>7/7</td>
<td>TCGGAGA</td>
<td>GGGGTCTAACACTCGT</td>
<td>T</td>
<td>CTACCTTGACTCTGGGG</td>
<td>(JH4)</td>
</tr>
</tbody>
</table>

Sequences of CDR3 region from four low-grade MALT-type B lymphomas are grouped and subdivided into VH, N, D, N, and JH regions. Name of the germline D and JH genes with maximum homology to the segments used in the VDJ joining are shown in the parentheses on the appropriate position. The underlined sequences were synthesized and used as tumor specific primers used in the study.

The three germlines used by the MALT lymphomas, hv1263, hv3005 and Vti 4-21, were found to be closely related to the gene segments that have been reported as potentially encoding autoreactive antibodies (anti-DNA, anti-Sm, and anti-thyroglobulin) in the VH gene, all resulting in amino acid differences. The R:S ratios in the CDRs and FRs were 6 and 0.8, respectively. The Vti gene sequence in case 2 was 95% identical with the human hv35 gene; which is more than 98% identical with the “fetal” S1P gene; it contained 17 substitutions, 6 occurring in the CDRs and all resulting in amino acid substitutions. The ratios of replacement (R) to silence (S) mutations in the CDRs and FRs were 6 and 0.8, respectively. The Vti gene sequence in case 3 displayed the highest degree of similarity (94%) with the germline sequence Humanhv2162 gene, which is more than 98% identical with the “fetal” S1P gene; it contained 17 substitutions, 6 occurring in the CDRs and all resulting in amino acid substitutions. The R:S ratios in the CDRs and FRs were 6 and 0.4, respectively. The Vti gene sequence in case 4 was 94% identical with the germline sequence, Vti 4-21 gene, which is 91% identical to the “fetal” gene, S8P1; it contained 16 substitutions, distributed throughout the sequence. The R:S ratios were 4 and 1.25 in the CDRs and FRs, respectively.

The sequences of the expressed D segments were compared with those of the published germline D and DIR segments. Sequence similarities between the expressed and germline D genes were found in all four cases. No apparent preferential utilization of any particular D1 gene segment was seen. The clones from all four patients appeared to contain two D1 gene segments: Xp’1 in the inverse orientation, rearranged to DIR-2c in the forward orientation (case 1); DLR1 combined with DNI (case 2); DLR2 combined with DIR-2c (case 3); and DA1/4 rearranged to DN4 (case 4).

Analysis of the pattern of somatic mutation. The combined R:S ratios for the FRs and CDRs domains, derived from the sum of all mutated codons in the four Vti sequences of tumors assignable to the germline gene segment, are shown in Table 3. Taken together, in cases 1, 3, and 4.

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Fig 3. Tumor-specific sequences of VH gene segments and translated peptides. The nucleotide (A) and deduced amino acid (B) sequences of VH germline gene segments are shown in comparison to germline Humanhv1263* (case 1), germline hv35* (case 2), germline hv3005* (case 3), and VH-lV4-21* (case 4). Conventionally defined CDR are indicated by an overscore. Dashes, identity to the reference sequence; only differences are shown by using uppercase letters for replacement mutations and lowercase letters for silent mutations.

the average R:S ratio in the framework regions was 0.8, as expected for a part of the antibody molecular structure essential for the overall maintenance of protein structure. However, the average R:S ratio in CDR1 and CDR2 was 4.6. This pattern is consistent with the notion of an antigen-driven selection of antibodies with high-affinity antigen-binding sites.

**DISCUSSION**

Progress in the understanding of the origin of malignant B cells and the potential role of antigens in clonal selection of MALT-type lymphomas has been hampered by the lack of experimental systems for correlating the stage of differentiation with that of normal B-cell populations of mucosa-associated lymphoid tissue. In this study, we have used a novel two-step procedure. The CDR3 gene fragment of the MALT lymphoma was first amplified using consensus oligonucleotide primers, then sequenced. Based on the predominant sequence of the amplified products, individual tumor-specific CDR3 primers were synthesized to isolate VH genes expressed by the MALT-type lymphomas. This approach takes advantage of the clonal specific diversity of CDR3, resulting from a combination of junctional diversity, D-inversion, D-D fusion, gene replacement and the addition of nucleotide sequences, termed N region. The V, D, and J segment combination is generally conserved within a clone. To verify this, the specificity of each tumor-specific CDR3

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**Table 3. Vh, D, and Jh Gene Composition**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>VH Gene Family</th>
<th>Germline VH Member</th>
<th>Closest VH Member</th>
<th>No. of Nucleotide Difference (total in)</th>
<th>R:S</th>
<th>Closest Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vhi</td>
<td>hv1263</td>
<td>51P1</td>
<td>94%</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Vhi</td>
<td>hv35</td>
<td>20P3</td>
<td>95%</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Vhi</td>
<td>hv3005</td>
<td>65P1</td>
<td>95%</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Vhi</td>
<td>Vh-4-21</td>
<td>68P1</td>
<td>94%</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

* The complete sequences of the genomic germline VH genes have been reported in the following studies: hv1263*; hv35*; hv3005*; and Vh-4-21. For complete sequences of the expressed fetal 51P1, 20P3, 56P1, 68P1 VH genes, please refer to Sanz et al.31

† The complete sequences of Dw genes have been reported by Dersimonian et al.32 and Merk et al.33

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primer was confirmed by showing that (1) they amplified the VH gene of their own tumor specimen, but not those of tumor specimens from other individuals; (2) similar CDR3 combinations were not detectable either in randomly selected tonsils or in lymph nodes, shown to be tumor free by histologic criteria.

Analyses of the genes encoding the VH regions showed that three different VH families were used. In cases 1 and 2, the VH family was used, whereas in cases 3 and 4 the VhIII and VhIV families, respectively, were used, indicating multiple-VH family usage by MALT-type lymphomas.

Positive selection of clones bearing somatic mutations, resulting in a net increase in affinity of the surface receptor for antigen, has been shown to be the primary mechanism underlying the process of affinity maturation during a specific antibody response. The somatically mutated status of Ig V genes can be readily determined by comparison of the expressed V gene sequences with the corresponding germ-line V gene sequences. Compared with the germline, the VH gene of each tumor clone from four patients with low-grade MALT lymphomas contained at least 14 substitutions, distributed in a pattern characteristic of antigen-driven affinity maturation, i.e., the somatic mutations were highly concentrated in the CDRs or FR regions, with a clustering of replacement (R) mutations in the FRs, but only a few in the FRs. In three of four cases of MALT lymphomas (cases 1, 3, and 4), the R:S mutation ratios in the CDRs (4.6) and FRs (0.8) were comparable with those in the V genes of high-affinity murine antibodies and autoantibodies and were significantly higher, or lower, than the theoretical R:S value of a protein ~2.9, calculated for somatic mutations occurring randomly in a gene encoding a protein whose structure need not be preserved. A higher CDR R:S mutation ratio reflects the positive selective pressure of an antigen on those gene products that come into close contact with antigen, whereas a lower FR R:S mutation ratio reflects the negative pressure for mutant selection applied to structural components that need to be conserved. An alternative explanation could be that these mutations may result from polymorphic variation of the Ig-VH gene, although VH 4.21 is known to be relatively nonpolyorphic. However, this seems unlikely. We have successfully amplified the entire gene segments of VH gene families and germlines from cases 1, 2, and 4. Comparison with the appropriate published germ fragment sequence shows that the sequence determined for case 1 differs by only a single nucleotide (position 148, A to G) from that for vh1263; the sequence for case 2 differs by two nucleotides (position 102 G to A and 209, T to G; respectively) from that for v35 and the germline of case 4 contains a gene segment showing 100% identity with the published VH 4.21 sequence. We also found that the sequence from case 1 germline segments showed 100% identity with the published sequence 51P1. Because the VH gene segment used by the lymphoma B cells in case 1 showed the highest degree of similarity (96%) with 51P1, the possibility cannot be excluded that the VH gene segment used by lymphoma B cells may be derived from the "fetal" 51P1 gene. The results demonstrate that, even when taking allelic polymorphism into account, the Ig VH genes of MALT-type lymphoma clearly show the somatic hypermutation. Our data indicate that the tumor clone from each MALT-type lymphoma must have undergone antigen-mediated selection. In case 2, a higher number of R mutations was also found in FRs. A similar phenomenon has been seen in the rearrangement of the human Ig Vh genes coding for high-affinity antirabies virus antibodies. Further evidence for antigen-driven selection can be seen in the D-D recombination. In all four cases, D-element–resembling motifs were found in Xp[1] in the inverse orientation and rearranged to DIR-2c in the forward orientation (case 1); DLR1 combined with DN1 (case 2); DLR2 combined with DIR-2c (case 3); or DA1/4 rearranged to DN4 (case 4). More importantly, this type of D-D recombination event has also been reported in response to hapten-antigen complex and the random ter-polymer GAT in mice and represents an important event in determining idioype expression and antigen-binding affinity.

Previous reports have shown that a single R mutation in the CDR1 region is able to increase the antibody binding affinity. This raises the question of the importance of the CDR1 region in contributing to antigen-initiated somatic hypermutation. Our results showed that, in the four cases of MALT-type B-cell lymphoma studied, the substitutes occurred in CDR1 region all resulted in amino acid substitutions. This was especially the case 4, with three of four R mutations occurring in CDR1 regions. These data suggest that the CDR1 region may also be an important element for study of somatic mutation in MALT-type lymphomas.

Although the nature of the selecting antigen is unknown, it could be an exogenous antigen, an autoantigen, or a regulatory autologous anti-idiotypic antibody. We favor the idea that autoantigen-mediated positive selection may be applied to the cell-surface Ig receptor. Autoimmune phenomena can occur in association with several clonal B-cell disorders, such as idiopathic cold agglutinin disease, chronic lymphocytic leukemia, and non-Hodgkin's and Hodgkin's disease. In autoimmune reactions involving the production of autoantibodies, such as rheumatoid factors or anti-DNA antibodies, positive selection for autoreactive clones has been observed. It is interesting that three of four genes, hv1263, hv3005, and VH4-21, are frequently found in a variety of autoantibodies, such as cold agglutinins, rheumatoid factors, and anti-DNA antibodies. These findings are consistent with our own results and those of other groups, suggesting that autoimmunity may be involved in the pathogenesis of MALT-type lymphoma.

From the current data, it is impossible to know if the constant presence of antigen is required for tumor evolution. Additional genetic or epigenetic events could result in antigen-independent malignant clonal evolution and expression. Unifying chromosomal aberrations and the rearrangements of certain oncogenes or Epstein-Barr virus infection have been suggested as possible first steps in the development of nodal lymphomas, but have not been detected in the case of MALT lymphomas. It is possible that antigen may be necessary for the continuous maintenance of the MALT-type lymphoma clone.

One issue that makes MALT-type lymphomas an attractive system to study is that they share many similarities with marginal zone B cells, whose cellular origin is also uncertain.
Here, we have provided direct evidence that MALT-type lymphoma B cells have undergone somatic mutation in their Ig VH genes, indicating that they may represent germinal center derived memory B cells. It will be interesting to determine if normal splenic marginal zone B cells contain mutated Ig V genes.

ACKNOWLEDGMENT

We are grateful to Drs M. Kneba and H. Martiner-Valdez for technical advice. We thank Drs A. Schimml, Y.P. Zhang, Y.J. Liu, A. Iglesias, and S. Czub for stimulating discussion and comments. We thank Dr T. Barkas for critically reading of this manuscript.

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