Despite increasing identification of concurrent gastric and intestinal lymphomas of mucosa-associated lymphoid tissue (MALT), the clonal relationship between the two tumors and their sequential development are poorly understood. It is also unknown whether the development of these concurrent tumors is closely associated with direct antigen stimulation, which is thought to play an important role in the clonal expansion of low-grade MALT lymphomas. To investigate these, we have studied six cases of concurrent gastric and intestinal MALT lymphomas by polymerase chain reaction (PCR) amplification, cloning, and sequencing of the rearranged Ig gene, a strategy that has been widely used for analysis of clonality and antigen-driven properties of B-cell malignancies. In each case, an identical or nearly identical complementarity determining region (CDR) 3 sequence was observed between the dominant clones of concurrent gastric and intestinal MALT lymphomas. In four of six cases examined, sufficient Ig variable region sequence information was obtained to permit analysis of somatic mutations. The mutation patterns in one case suggest that the intestinal lesion is secondary to the gastric tumor, and the mutation patterns in two cases indicate that the gastric and intestinal lesions are derived from different tumour subclones, which emerge after expansion of a common early tumor clone. Furthermore, three of four cases showed ongoing Ig mutations among different PCR clones at each site. These results show that concurrent gastric and intestinal MALT lymphomas are derived from the same clone and suggest that the intestinal lesions result from dissemination of gastric tumors. Antigen stimulation may play a role in tumor evolution, particularly at an early stage.

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MATERIALS AND METHODS

Tissue samples. Formalin-fixed tissue samples from six concurrent gastric and intestinal B-cell MALT lymphomas (cases no. 1, 2, 3, 5, and 6 were low grade; case no. 4 was high grade) were retrieved from the archives of the Department of Histopathology, University College London Medical School (London, UK). The diagnosis of low-grade B-cell MALT lymphoma in these cases was established by the characteristic histologic appearance together with immunophenotyping and clonal analysis of the rearranged Ig heavy chain gene. The high-grade case was diagnosed by its histologic appearance and identification of residual foci of low-grade MALT lymphoma.

DNA preparation. Genomic DNA was prepared from tissue sections of gastric and intestinal MALT lymphomas in each case as described previously. Polymerase chain reaction (PCR) amplification of the rearranged Ig heavy chain genes. In each case, PCR was performed to amplify

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- **Intestinal Dissemination of Gastric Mucosa-Associated Lymphoid Tissue Lymphoma**
  - By Ming-Qing Du, Chun-Fang Xu, Tim C. Dias, Huai-Zheng Peng, Andrew C. Wotherspoon, Peter G. Isaacson, and Lang-Xing Pan

**GASTRIC B-CELL LYMPHOMA** of mucosa-associated lymphoid tissue (MALT) is characterized by an indolent clinical course and generally remains localized at the site of origin for prolonged periods. However, the tumor may disseminate preferentially to other mucosal sites without systemic spread. Concurrent gastric and intestinal MALT lymphomas have increasingly been identified. Despite this, the clonal relationship between the tumors at each site and their sequential development are poorly understood. It is unknown whether the development of these concurrent tumors is associated with direct antigen stimulation, which is thought to play an important role in the clonal expansion of low-grade MALT lymphomas.

The most important function of B cells is production of specific antibodies. This is largely achieved through B-cell development by recombination of the Ig heavy chain variable (V), diversity (D), and joining (J) segments and hypermutation of the rearranged gene. The rearranged Ig genes of a mature B cell record much of its evolutionary history. Several important properties of B-cell lymphomas can now be studied by analysis of the rearranged Ig gene. Firstly, disseminated lesions and residual disease can be identified by examination of the unique complementarity determining region (CDR) 3 sequence. Secondly, the developmental stage (relative to the germinal center reaction) of lymphoma precursor cells can be deduced by analysis of somatic mutations of the variable (Vh) region. Thirdly, because somatic mutations may accumulate in the Ig gene during lymphoma development, examination of mutation patterns among different lesions of a lymphoma can be used to delineate its sequential development. Finally, the role of antigen stimulation in lymphoma growth can be assessed by analysis of ongoing Ig mutations, which are the characteristic feature of B cells in persistent response to direct antigen stimulation normally in a germinal center environment.

In this study, we have cloned and sequenced the rearranged Ig genes from six cases with concurrent gastric and intestinal MALT lymphomas. By analysis of the rearranged Ig gene, we have determined the clonal relationship between MALT lymphomas at each site and their sequential development and assessed the role of antigen stimulation during tumor evolution.

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the rearranged Ig heavy chain gene containing as much V region sequence as possible. The fragment from the framework (Fr) 1 to the joining (Jn) region of the Ig gene was PCR-amplified using individual Fr1 family specific (Vn1-Vn6) and Jn consensus primers, whereas the Fr2-Jn and Fr3-Jn regions were amplified using semi-nested protocols as described previously. All PCR reactions were performed using a hot start procedure and appropriate positive and negative controls (without template DNA) were included in each experiment. All samples were analyzed in duplicate. PCR products were analyzed on 6% (Fr1-JH and Fr2-JH products) or 10% (Fr3-J11 products) polyacrylamide gels. For each tumor, PCR products from two separate reactions were used for cloning and sequencing. Products were purified on Sephacryl S-400 columns (Pharmacia, St Albans, UK), ligated to pCRT II. and transfected into One Shot competent cells (INV®) according to the manufacturer’s protocol (Invitrogen, Leek, The Netherlands). The transfected cells were plated onto LB-ampicillin agar plates containing X-gal. White colonies were transferred to a fresh LB-ampicillin agar plate containing X-gal and grown overnight for secondary selection. Confirmed white colonies were then transferred into 150 µL of LB medium containing 50 µg/mL ampicillin and cultured at 37°C for 4 hours. The cultures (100 µL) were centrifuged, resuspended in 20 µL of water, and heated at 98°C for 10 minutes. After centrifugation, 1 µL of the resulting supernatants was used for PCR with Sp6 and T7 primers. The PCR products showing the expected insert size were sequenced using the ABI sequencer with dye terminators (Perkin Elmer, Warrington, UK). In each case, at least six PCR clones from each tumor site were sequenced. All clones of Fr1-Jn and Fr2-Jn PCR products were sequenced in both orientations.

Sequence analysis. Analysis of sequences was performed using Wisconsin GCG software (provided by the Human Genome Mapping Project, Cambridge, UK) and the GenBank Database. Mutations in the variable region were identified by comparing the consensus sequence of each tumor with the closest published germlines. Two nucleotide exchanges in one codon were recorded as one replacement mutation. Diversity (D) and joining (J) regions were also identified from published germline Dn and Jn sequences, respectively. Somatic mutation analysis. For n random mutations, the number of expected replacement (R) and silent (S) mutations in the CDR regions should be n × CDRn and n × (1 − CDRn), respectively (Rf, replacement frequency of total possible mutations that result in amino acid replacement), whereas the expected R and S mutations in the Fr regions should be n × Frn and n × (1 − Frn), respectively. Without selection, the R and S mutations should be distributed among the various Vn regions according to their respective sizes, ie, RCDRn = n × CDRn × CDR(CDRn), RS = n × (1 − CDRn) × CDR(CDRn), RFRn = n × Frn × Fr(CDRn), and SRS = n × (1 − Frn) × Fr(CDRn), relative size of the CDRs or Frs. The probability (P) that the observed R mutations in the CDRs were by chance was calculated using a binomial mutation model:

\[ P = \frac{n!}{k! (n-k)!} \times q^k \times (1-q)^{n-k} \]

where k is the number of observed R mutations in the CDRs; q = probability that an R mutation will localize to CDRs. Somat...
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Table 1. Use of \( V_\text{H}, D, \) and \( J_\text{H} \) Genes in MALT Lymphoma

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Region of Amplification</th>
<th>Closest Germline ( V_\text{H} )</th>
<th>Similarity (%)</th>
<th>Closest D Segment</th>
<th>Closest ( J_\text{H} ) Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fr1-JH</td>
<td>DP49(( V_\text{H} ))3</td>
<td>96.4</td>
<td>DN4</td>
<td>J5</td>
</tr>
<tr>
<td>2</td>
<td>Fr2-JH</td>
<td>( V_\text{H}, 4.21 )</td>
<td>94</td>
<td>Dirk-4*</td>
<td>J5</td>
</tr>
<tr>
<td>3</td>
<td>Fr2-JH</td>
<td>DP8(( V_\text{H} ))1</td>
<td>97</td>
<td>DXP-1, Dir-4*</td>
<td>J4</td>
</tr>
<tr>
<td>4</td>
<td>Fr2-JH</td>
<td>DP51(( V_\text{H} ))3</td>
<td>89</td>
<td>Dir-3*</td>
<td>J4</td>
</tr>
<tr>
<td>5</td>
<td>Fr3-JH</td>
<td>-</td>
<td>-</td>
<td>D-new</td>
<td>J5</td>
</tr>
<tr>
<td>6</td>
<td>Fr3-JH</td>
<td>-</td>
<td>-</td>
<td>D21-7</td>
<td>J5</td>
</tr>
</tbody>
</table>

* In the reverse orientation.

RESULTS

Amplification of the rearranged Ig heavy chain gene. In case no. 1, amplification of the Fr1-JH region was successful, whereas, in cases no. 2 through 4, the Fr2-JH region and, in cases no. 5 and 6, the Fr3-JH region were the largest \( V_\text{H} \) fragment amplified.

Clonal relationship between concurrent gastrointestinal MALT lymphomas. All cases examined showed PCR bands of the same sizes (1 band in cases no. 1, 2, 3, 4, and 6; 2 bands in case no. 5) between concurrent gastric and intestinal tumors in separate experiments (Fig 1). Cloning and sequencing of the PCR products in these cases (in case no. 5, only the upper PCR band was analyzed) showed a dominant clone in each tumor. In each of these cases, both the dominant clones of the gastric and intestinal tumors were identical or nearly identical in the CDR3 sequence (Fig 2), indicating the involvement of two sites by a single tumor clone.

Somatic mutations and intraclonal variations. These analyses were performed in cases no. 1 through 4, in which the Fr1-JH or Fr2-JH region was amplifiable. To analyze somatic mutations, the \( V_\text{H} \) sequence from the most representative PCR clone in each tumor was used to search for its germline from the GenBank Database. The closest germline

![Fig 2. Variation of Ig sequence in case no. 1. The individual Fr and CDR regions are indicated according to Kabat et al. Identity with the germline sequence is shown by dashes, replacement mutations are shown by uppercase letters, and silent mutations are shown by lowercase letters. The sequences of PCR primers (Fr1 and JH) are not included. These sequence data are available from GenBank under accession no. U70087-U70099.](www.bloodjournal.org)
germline the tumor clone was derived from an unknown germline.

3 displayed the closest homology to germline genes from gastric and intestinal tumors showed both common and

distinct mutations from the corresponding germline \( V_h \) genes. In case no. 1, the gastric and intestinal tumors displayed 5 mutations in common together with 3 and 4 distinct mutations, respectively. However, in case no. 2, the gastric and intestinal lesions showed 3 common along with 6 and 7 distinct mutations, respectively. Thus, the corresponding gastric and intestinal tumors in cases no. 1 and 2 were derived from a common early tumor clone but genotypically represented two different tumor subclones. In case no. 3, both gastric and intestinal tumors displayed the common mutations from the corresponding germline.

The patterns of replacement and silent mutations in the \( V_h \) region were analyzed and are shown in Table 2. In case no. 1, both the gastric and intestinal tumors showed a high R/S ratio in the CDRs, suggesting that the tumor had been

\[ V_h \] genes and their degree of similarity to each tumor clone are shown in Table 1. Cases no. 1 and 4 displayed the closest homology to germline genes from \( V_h^3 \), and cases no. 2 and 3 displayed the closest homology to germline genes from the \( V_h^1 \) and \( V_h^3 \) family, respectively.

Because of the highly polymorphic nature of the \( V_h^3 \) family and a relatively low homology with its closest known germline DP51(\( V_h^3 \)) in case no. 4, it cannot be excluded that the tumor clone was derived from an unknown germline \( V_h \) gene. Therefore, mutation analysis of rearranged \( V_h \) genes was confined to cases no. 1 through 3. In comparison with the corresponding germline, the rearranged \( V_h \) gene of each lymphoma examined showed frequent somatic mutations (Figs 3 through 5). In cases no. 1 and 2, the corresponding gastric and intestinal tumors showed both common and

\[ CD2 \]

\[ CD3 \]

\[ CD4 \]

\[ CD5 \]
**INTESTINAL DISSEMINATION OF GASTRIC MALT LYMPHOMA**

Table 2. Distribution of Mutations in Tumor-Derived V\textsubscript{H} Sequences

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Region</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G</td>
<td>CDRs</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Frs</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>1SI</td>
<td>CDRs</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frs</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2G</td>
<td>CDRs</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frs</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2SI</td>
<td>CDRs</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frs</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>CDRs</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frs</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: G, gastric lesion; SI, small intestinal lesion.

* P is the probability of obtaining the number of R mutations found in the CDRs by chance.

positively selected by antigen (Table 2). However, this feature was not observed in cases no. 2 and 3.

In addition to the extensive sequence variations between the corresponding gastric and intestinal tumors in cases no. 1 and 2, sequence differences among different PCR clones from the same tumor site were observed in all cases (Figs 3 through 6). The mutation frequency is summarized in Table 3. The gastric lesion of cases no. 1 and 2 and the intestinal lesion of cases no. 2 and 3 showed mutation frequencies above the rate of PCR errors (<0.2%) in the cloning and sequencing system used, suggesting ongoing mutation events in these low-grade lesions. However, both the gastric and intestinal lesions of case no. 4, a high-grade MALT lymphoma, showed a very low level of mutations.

**Use of D and J\textsubscript{H} genes.** The D segment of the rearranged Ig genes was examined in each case by comparison with the published germline D segments. The probable germline D segments used are shown in Fig 1 and Table 1.

Comparison of tumor-derived Ig with the germline J\textsubscript{H} sequence showed that cases no. 3 and 4 used J\textsubscript{H}4 and that all others used J\textsubscript{H}5 (Table 1).

**DISCUSSION**

By cloning and sequencing the rearranged Ig genes, we have confirmed the clonal link between concurrent gastric and intestinal MALT lymphomas in each case examined. Several strands of evidence suggest that the gastric tumors preceded the intestinal lesions. Firstly, the rearranged D segment in case no. 1 was in a germline configuration in the gastric tumor but mutated in the intestinal lesion, indicating an accumulation of somatic mutations during tumor progression. Secondly, the tumor cells from the intestinal lesion of case no. 1 (A. Dogan and P.G. Isaacson, unpublished results) have been shown to proliferate in response to gastric pathogen *Helicobacter pylori*-mediated stimulation, which is a characteristic feature of gastric MALT lymphoma. Moreover, the same strain of *H. pylori* was found to be responsible for the growth response of both gastric and intestinal tumors in this case. Finally, the incidence of gastric MALT lymphoma is much higher than that of intestinal MALT lymphoma.

Sequence analysis of the rearranged Ig genes also shows genetic evidence in the form of somatic mutation patterns, which can be used to delineate aspects of tumor evolution. The finding of sets of shared and distinctive mutations between the concurrent gastric and intestinal tumors in cases no. 1 and 2 suggests that the two lesions are formed by two different tumor subclones derived from a common precursor clone, which should only bear the common mutations. It is possible that, during clonal expansion of the early tumor clone of these cases, several subclones have been generated and one of the subclones predominated at the gastric site, whereas another subclone disseminated to the small intestine and formed the intestinal lesion.

Analysis of intraclonal variations of the rearranged Ig gene has been used to depict the role of antigen stimulation in lymphoma expansion. Because somatic mutations of the Ig gene occur specifically in B cells during the germinal centre reaction, which is an antigen-driven event, the finding of ongoing mutations in lymphoma Ig genes suggests that tumor cells still respond to direct antigen stimulation and, consequently, their clonal expansion may be dependent on antigen drive. There was a dramatic accumulation of Ig gene mutations during the evolution from the early tumor clone to tumor subclones in cases no. 1 and 2, suggesting an antigen-mediated tumor expansion. A low level of ongoing mutations was found within the low-grade gastric or intestinal lesions.
which is consistent with the findings of a recent study. Therefore, direct antigen stimulation may play an important role in the evolution of low-grade MALT lymphomas, particularly at an early stage. This is consistent with the frequent observation of follicular colonization of lymphoid follicles by MALT lymphoma cells, which indicates access to the germinal center environment. This speculation is also supported by the observation that the growth of low-grade MALT lymphoma cells in vitro responds to the stimulation by antibodies against the tumor idiotype. However, unlike low-grade tumors, high-grade lesions examined in both the present and previous studies did not show ongoing mutations. As suggested previously, the lack of ongoing mutations in high-grade tumor cells may reflect a loss of the ability to respond to direct antigen stimulation, which may result from the accumulation of additional genetic defects. However, this is unconfirmed, because the number of high-grade cases examined is small.

The epitope responsible for direct stimulation of MALT lymphoma cells is probably self antigen. The germline V<sub>H</sub> genes [DP49(V<sub>H</sub>3), V<sub>H</sub>4.21, and DP8(V<sub>H</sub>1)] used by these tumors have been found to be frequently employed by auto-antibodies, which is in accordance with previous observations. This is also supported by the finding that the Igs derived from gastric MALT lymphoma recognize single distinct autoantigens. Moreover, MALT lymphomas always develop from a background of chronic inflammatory diseases such as H. pylori gastritis or from Hashimoto's thyroiditis or Sjögren's syndrome, which are autoimmune in nature. Therefore, MALT lymphomas may result from the transformation of autoreactive B-cell clones.

The pattern of common Ig V<sub>H</sub> mutations found in a lymphoma has been used to infer the status of its cell of origin in relation to the germinal center reaction. However, this inference may not always be reliable. There are significant differences in the pattern of common mutations between the ultimate tumor subclones and their postulated cells of origin in cases no. 1 and 2. Therefore, the mutation pattern found in a lymphoma may actually indicate the current status of the tumor clone rather than that of cell of origin. Nevertheless, in the two cases in which a prediction can be made, both putative cells of origin showed frequent somatic mutations in their rearranged V<sub>H</sub> segments, with one case displaying a mutation pattern of positive antigen selection (P < .04). Thus, it is pertinent to conclude that MALT lymphoma Ig genes are mutated. Together with the phenotypic similarities of MALT lymphoma to the marginal zone B cells, which are known to be memory B cells, this supports the notion that the tumor is derived from postgerminal center B cells.

Unlike previous studies in which all MALT lymphomas examined showed an Ig gene mutation pattern indicating positive antigen selection, two of three cases examined in the present study did not show this feature but rather displayed R/S values substantially lower than expected. This may reflect the nature of cells of origin of these lymphomas. Because MALT lymphomas are thought to be derived from autoreactive B cells, which are constantly under surveillance by negative selection mechanisms, the observation of MALT lymphomas without a history of positive antigen selection is therefore expected. The heterogeneous findings in the mutation pattern of MALT lymphomas may reflect differences in their autoimmune background. Nevertheless, the consistently observed ongoing mutations in MALT lymphoma suggest that Ig expression may be important for lymphoma survival. Similarly, antigen stimulation probably plays an important role in the clonal expansion of nodal lymphomas showing both positive and negative selection features.

Our results show that concurrent gastric and intestinal MALT lymphomas are derived from the same tumor clone and suggest that the intestinal tumors are the result of dissemination from gastric lesions. Antigen stimulation may play a role in the expansion of low-grade MALT lymphomas, particularly at an early stage of tumor expansion.

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Intestinal dissemination of gastric mucosa-associated lymphoid tissue lymphoma

MQ Du, CF Xu, TC Diss, HZ Peng, AC Wotherspoon, PG Isaacson and LX Pan