

Brief Report

**MOLECULAR ANALYSIS OF THE
PROGRESSION FROM *HELICOBACTER
PYLORI*-ASSOCIATED CHRONIC
GASTRITIS TO MUCOSA-ASSOCIATED
LYMPHOID-TISSUE LYMPHOMA
OF THE STOMACH**

EMANUELE ZUCCA, M.D., FRANCESCO BERTONI, M.D.,
ENRICO ROGGERO, M.D., GIOVANNA BOSSHARD,
GIOVANNI CAZZANIGA, B.Sc., ENNIO PEDRINIS, M.D.,
ANDREA BIONDI, M.D., AND FRANCO CAVALLI, M.D.

SEVERAL lines of evidence suggest a link between chronic gastritis due to *Helicobacter pylori* and mucosa-associated lymphoid-tissue (MALT) lymphoma of the stomach.^{1,2} A close epidemiologic association has been reported.³⁻⁵ The microorganism can be found in the gastric mucosa in nearly all cases, and regression of low-grade gastric MALT lymphoma has been demonstrated after the eradication of *H. pylori*.^{6,7}

The suggested progression from *H. pylori*-associated chronic gastritis to overt MALT lymphoma⁸ has not been formally demonstrated, although if it exists, such a mechanism may have important implications for the treatment of *H. pylori*-associated gastritis. Each B cell contains immunoglobulin heavy-chain genes in a novel configuration that provides a marker for small populations of monoclonal B cells, which can be identified in pathological specimens by the polymerase chain reaction (PCR).^{9,10} We describe two patients with histories of chronic *H. pylori*-associated gastritis and subsequent gastric MALT lymphoma in whom gastric-biopsy samples obtained several years before the onset of lymphoma were molecularly analyzed with a patient-specific PCR approach. We were able to show that the lymphomas arose from a B-cell clone at the site of chronic gastritis.

From the Servizio Oncologico Cantonale, Ospedale San Giovanni, Bellinzona, Switzerland (E.Z., F.B., E.R., G.B., F.C.); Clinica Pediatrica dell'Università di Milano, Ospedale San Gerardo, Monza, Italy (G.C., A.B.); and Istituto Cantonale di Patologia, Locarno, Switzerland (E.P.). Address reprint requests to Dr. Cavalli at the Servizio Oncologico Cantonale, Ospedale San Giovanni, 6500 Bellinzona, Switzerland.

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CASE REPORTS

Patient 1

Patient 1 was a 36-year-old man with a six-month history of epigastric pain for which he underwent upper gastrointestinal endoscopy, with random biopsy specimens obtained, in 1981 (Table 1). Benign gastric peptic ulcer with chronic gastritis of the antrum and the body was diagnosed. The patient was treated with antacids and remained well until 1985, when the gastric symptoms recurred. Further endoscopic gastric biopsy confirmed the diagnosis of chronic gastritis associated with *H. pylori* infection. Treatment with ranitidine was given, with good relief of symptoms. Follow-up endoscopy in March 1990 revealed lymphoepithelial lesions and plasma-cell infiltrates in both the gastric body and the duodenum. A histologic diagnosis of low-grade MALT lymphoma was made. Routine staging procedures¹² revealed stage I disease (lymphoma confined to the gastric mucosa). The symptoms resolved with the resumption of ranitidine treatment, and the patient declined to undergo gastrectomy. His condition was monitored with regular endoscopy and gastric biopsy, which showed no changes in histologic findings. In September 1993, he received a 10-day course of amoxicillin (750 mg three times daily), metronidazole (500 mg three times daily), and omeprazole (40 mg daily) to eradicate *H. pylori*. Repeated gastric biopsies from 1994 to 1996 documented the eradication of *H. pylori* but the persistence of multifocal lesions of low-grade MALT lymphoma.

In October 1996 endoscopy revealed a clear progression of the low-grade lymphoma in the stomach, with diffuse and deep infiltration of the gastric wall and duodenal invasion with stenosis of the duodenal bulb. A computed tomographic scan showed abdominal adenopathy; the results of a bone marrow biopsy were normal. He was treated with six cycles of a regimen consisting of doxorubicin, cyclophosphamide, vincristine, prednisone, and methotrexate. Complete remission was documented in June 1997. The patient was still in remission at the most recent follow-up visit in December 1997.

Patient 2

Patient 2 underwent selective proximal vagotomy in 1976, at the age of 40, followed eight years later by partial gastrectomy with a Billroth II anastomosis (Table 2). She had had severe peptic ulcer disease since the early 1970s. Pathological examination of the stomach specimens obtained at resection showed diffuse gastritis and peptic ulcer, without neoplastic tissue. In June 1988 a diffuse, mixed, predominantly large cell lymphoma (group F, according to the working formulation for the classification of lymphoma) in the gastric stump was diagnosed by endoscopic biopsy, and the patient was referred for staging and treatment. She reported increasing dysphagia, back pain, regurgitation, fever, night sweats, and a substantial weight loss in the preceding six months. Her *H. pylori* status was not known. Abdominal ultrasonography and computed tomographic scanning showed a large mass in the remnant gastric wall; multiple paragastric, mesenteric, and retroperitoneal lymphadenopathies; a focal liver lesion; and splenomegaly. Radiographs and computed tomographic scans of the chest showed no abnormalities. Analysis of a bone marrow aspirate and trephine biopsy showed infiltration from a large-cell lymphoma.

The patient was treated with six courses of a regimen consisting of doxorubicin, cyclophosphamide, vincristine, prednisone, and high-dose methotrexate with leucovorin rescue, alternating every 22 days with etoposide, bleomycin, procarbazine, and cytarabine.¹³ She had a complete remission, but she had a relapse identified in bone marrow in January 1990 (the results of a gastric-stump biopsy were suggestive of relapse but not conclusive). She had a second complete remission after two cycles of salvage treatment¹⁴ with a regimen consisting of etoposide, methylprednisolone, high-dose cytarabine, and cisplatin and subsequently underwent autologous bone marrow transplantation in September 1990. At the most recent follow-up visit in December 1997, she had no evidence of lymphoma.

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENT 1 AND RESULTS OF MOLECULAR ANALYSES.

| DATE OF STOMACH BIOPSY | ENDOSCOPIC FEATURES | HISTOLOGIC FINDINGS | | | CLONALITY ON CDR3 PCR | ALLELE-SPECIFIC PCR | |
|------------------------|---|-----------------------------|--------|-------------------------|-----------------------|---------------------|----------|
| | | DIAGNOSIS | SCORE* | <i>H. PYLORI</i> STATUS | | CLONE 1 | CLONE 2 |
| November 1981 | Ulceration | Active chronic gastritis | 3 | Positive | Oligoclonal | Positive | Positive |
| September 1985 | Antral erythema and multiple small erosions | Low-grade MALT lymphoma (?) | 4 | Positive | Monoclonal | Positive | Positive |
| March 1990 | Antral erythema | Low-grade MALT lymphoma | 5 | Positive | Monoclonal | Positive | Positive |
| May 1992 | Gastritis | Low-grade MALT lymphoma | 5 | Positive | Monoclonal | Positive | Positive |
| September 1993 | Gastritis | Low-grade MALT lymphoma | 5 | Positive | Monoclonal | Positive | Positive |
| September 1994 | Gastritis | Low-grade MALT lymphoma | 5 | Negative | Monoclonal | Negative | Positive |
| June 1995 | Gastritis | Low-grade MALT lymphoma | 5 | Negative | Monoclonal | Negative | Positive |

*The scoring system of Wotherspoon et al.¹¹ was used, in which a score of 0 indicates normal gastric mucosa and a score of 5 typical low-grade MALT lymphoma.

TABLE 2. CLINICAL CHARACTERISTICS OF PATIENT 2 AND RESULTS OF MOLECULAR ANALYSES.

| DATE AND SITE OF BIOPSY | ENDOSCOPIC FEATURES | HISTOLOGIC FINDINGS | | | CLONALITY ON CDR3 PCR | ALLELE-SPECIFIC PCR |
|---|---------------------|---|--------|-------------------------|-----------------------|---------------------|
| | | DIAGNOSIS | SCORE* | <i>H. PYLORI</i> STATUS | | |
| August 1984; stomach (before surgery) | Ulceration | Active chronic gastritis; focal intestinal metaplasia | 2 | Positive | Oligoclonal | Positive |
| November 1984; stomach (at gastrectomy) | Ulceration | Active chronic gastritis; focal intestinal metaplasia | 2 | Positive | Oligoclonal | Positive |
| June 1988; gastric stump | Neoplastic mass | Diffuse large-cell lymphoma, with areas of low-grade MALT lymphoma | 5 | Positive | Monoclonal | Positive |
| July 1988; bone marrow | — | Focal large-B-cell lymphoma infiltration | — | — | Monoclonal | Positive |
| January 1990; stomach | Erythema | Chronic gastritis; focal, dense, small lymphocytic infiltrates (residual low-grade lymphoma?) | 4 | Negative | Monoclonal | Positive |
| March 1990; stomach | Erythema | Chronic gastritis | 3 | Negative | Oligoclonal | Negative |
| October 1992; stomach | Normal findings | Chronic gastritis | 1 | Negative | Oligoclonal | Negative |
| May 1996; bone marrow | — | No evidence of lymphoma | — | — | Polyclonal | Negative |

*The scoring system of Wotherspoon et al.¹¹ was used, in which a score of 0 indicates normal gastric mucosa and a score of 5 typical low-grade MALT lymphoma.

METHODS

Histologic Analysis

All slides were reviewed according to the criteria of Isaacson and Norton¹⁵ and graded according to the histologic scoring system proposed by Wotherspoon et al.¹¹ In this system a score of 0 indicates normal gastric mucosa; a score of 1, chronic active gastritis with small clusters of lymphocytes in the lamina propria, without lymphoid follicles or lymphoepithelial lesions; a score of 2, chronic active gastritis with prominent lymphoid follicles and surrounding mantle-zone and plasma cells without lymphoepithelial lesions; a score of 3, a probable reactive lymphoid infiltrate,

with lymphoid follicles surrounded by small lymphocytes that diffusely infiltrate the lamina propria and occasionally the epithelium; a score of 4, a probable neoplastic lymphoid infiltrate composed of lymphoid follicles surrounded by centrocyte-like cells that diffusely penetrate the lamina propria and epithelium in small groups; and a score of 5, low-grade MALT lymphoma with dense infiltration of centrocyte-like cells in the lamina propria and prominent lymphoepithelial lesions. A modified Giemsa stain was used to identify *H. pylori*.¹⁶ The slides were reviewed by one pathologist before and after the completion of the molecular analysis. For the second review the pathologist was unaware of the results of the molecular analysis. The histologic diagnoses were confirmed by a second pathologist.

Consensus-Sequence PCR

DNA was extracted from paraffin-embedded tissue sections that were 10 μm thick, as previously described.¹⁷ A 268-bp segment of the β -globin gene was amplified by PCR with the PCO4 (5'CAACTTCATCCACGTTACCC3') and GH20 (5'GAAGAGCCAAGGACAGGTAC3') primers (Perkin-Elmer, Norwalk, Conn.). We amplified the complementarity-determining region 3 (CDR3), which is the most variable region of the immunoglobulin heavy-chain gene,⁹ using semi-nested PCR with the FR3A primer (5'ACACGGCC[TC]GTGTATTACTGT3') for the conserved-framework-3 segment of the variable region and the LJH (5'TGAGGAGACGGTGACC3') and VLJH (5'GTGACCAGGG-TNCCTTGGCCCCAG3') primers for the joining region. PCR reactions were performed in accordance with the methods described by Diss et al.¹⁰ Samples from the patients were analyzed in duplicate, and DNA from the Raji cell line of human B-cell lymphoma was used as a positive control. A negative control containing all PCR reagents without any template DNA was also used. The amplification products were analyzed on 10 percent nondenaturing polyacrylamide gels and visualized with ultraviolet light after staining with ethidium bromide. The presence of a distinct single band (with or without other less intense bands, reflecting the formation of heteroduplexes) was considered to indicate monoclonality, the presence of a few intensely stained distinct bands oligoclonality, and the presence of a smear of amplified products polyclonality.^{18,19}

Cloning and Sequencing of PCR Products

Monoclonal bands from at least two different lymphoma-containing samples from each patient were excised from the polyacrylamide gel. DNA was extracted^{19,20} and purified with Sephadryl MicroSpin S-400 HR columns (Pharmacia, Uppsala, Sweden). Cloning and sequencing of CDR3 were performed with the pMOSBlue T-vector kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) and a Sequenase DNA Sequencing kit (version 2.0, Amersham), according to the manufacturer's instructions.

Allele-Specific PCR

Allele-specific oligonucleotides were designed for each patient from the CDR3 sequences of the predominant clones (i.e., identical clones representing at least three of six recombinants examined from each patient) and used as 5' primers to perform nested PCR in DNA from biopsy samples showing gastritis in the two patients. The CDR3 sequences have been assigned GenBank accession numbers AF016207 and AF016208 in the case of the two clones in Patient 1 and AF016215 in the case of the predominant clone in Patient 2. The first round of amplification was carried out in the same manner as the PCR for the consensus sequence of CDR3; in the second round the allele-specific oligonucleotide was used as a primer in conjunction with the consensus VLJH primer. DNA from the sequenced lymphoma sample from each patient was used as a positive control. In order to assess the specificity of the lymphoma allele-specific oligonucleotide, DNA from a disease-free specimen from the patients and DNA from the Raji cell line were analyzed. Reaction products were analyzed on 10 percent nondenaturing polyacrylamide gels as described above.

RESULTS

Patient 1

In Patient 1, chronic gastritis (histologic score, 3) was confirmed by histologic review only in the initial sample (Fig. 1A), obtained in 1981. This sample had an oligoclonal pattern of B cells on PCR assay of the CDR3 segment of the immunoglobulin gene. Low-grade MALT lymphoma (histologic score, 4 or 5)

was diagnosed in biopsy specimens obtained from 1985 onward (Fig. 1C and 1D); a monoclonal rearrangement of the immunoglobulin heavy-chain gene with a similar band size was detected in all samples (Table 1).

Sequencing analysis of the monoclonal band in the specimens of low-grade MALT lymphoma that were obtained in 1990 and 1992 showed two major lymphoma clones of 97 bp and 100 bp. Because the two differed by only 3 bp, they could not be clearly distinguished on gel electrophoresis. Both CDR3 sequences of these clones were used for allele-specific PCR, and both clones were present in the initial (1981) sample as well as in the biopsy specimens obtained in 1985 (Table 1 and Fig. 2). After 1993, one of the two B-cell clones was no longer detectable (Table 1). Both CDR3 sequences were in the correct reading frame for the production of a potentially functional protein. As a result of allelic exclusion, a B-cell clone usually produces only a single immunoglobulin. The fact that both sequences were in the correct reading frame suggests the presence of a B-cell clone usually produces only a single immunoglobulin. The fact that both sequences were in the correct reading frame suggests the presence of a B-cell clone usually produces only a single immunoglobulin.

Patient 2

Histologic review of the gastric-biopsy specimens obtained three months before gastric resection in 1984 and at gastrectomy confirmed the absence of lymphoma (Fig. 1B). The pathological features were those of active gastritis, with lymphoid follicles, focal intestinal metaplasia, and chronic peptic ulceration (histologic score, 2). The presence of *H. pylori* was documented in specimens obtained in 1984 and 1988. The patient never received antibiotics with the specific aim of eradicating *H. pylori*. However, during and after chemotherapy, she received a variety of antibiotics to treat infections. These treatments may have eradicated *H. pylori*.

Review of samples from the gastric-stump biopsy performed in 1988 confirmed the presence of diffuse, aggressive non-Hodgkin's lymphoma, which was reclassified as a large-cell lymphoma with focal areas of low-grade MALT lymphoma (Fig. 1E and 1F). The presence of large lymphoma cells was also substantiated in the bone marrow after histologic review of the specimen obtained by trephine biopsy. The histologic examination of the endoscopic-biopsy specimens obtained immediately before the relapse of lymphoma in the bone marrow showed focal suspicious-appearing dense, small lymphocytic infiltrates (histologic score, 4) that may have been related to the presence of residual low-grade MALT lymphoma. The gastric-biopsy specimen obtained after transplantation showed no such B-cell infiltration.

PCR amplification of the immunoglobulin gene with consensus primers demonstrated an oligoclonal pattern in the samples obtained at the time gastritis was diagnosed and a monoclonal band of the same

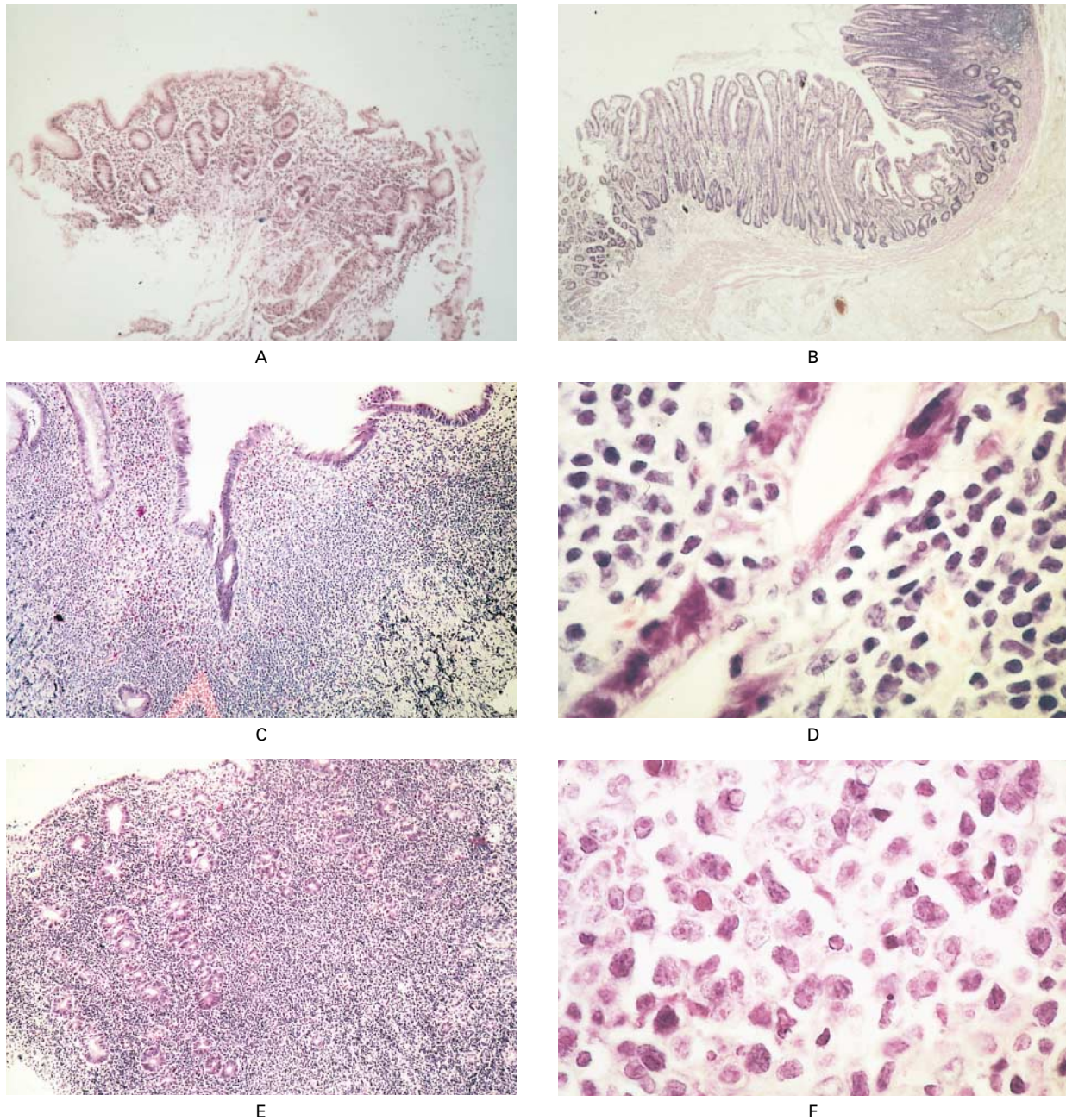


Figure 1. Histologic Patterns in the Evolution from Chronic Gastritis to Gastric Lymphoma.

Chronic gastritis is evident in biopsy specimens obtained in 1981 from Patient 1 (Panel A) and in 1984 from Patient 2 (Panel B) ($\times 100$). Panel C shows low-grade MALT lymphoma in the 1990 biopsy specimen from Patient 1 ($\times 200$), and Panel D shows a lymphoepithelial lesion from this specimen ($\times 1000$). A 1988 biopsy of the gastric stump in Patient 2 revealed areas of low-grade MALT lymphoma (Panel E, $\times 200$) within a high-grade, predominantly large cell lymphoma (Panel F, $\times 1000$). All specimens were stained with hematoxylin and eosin.

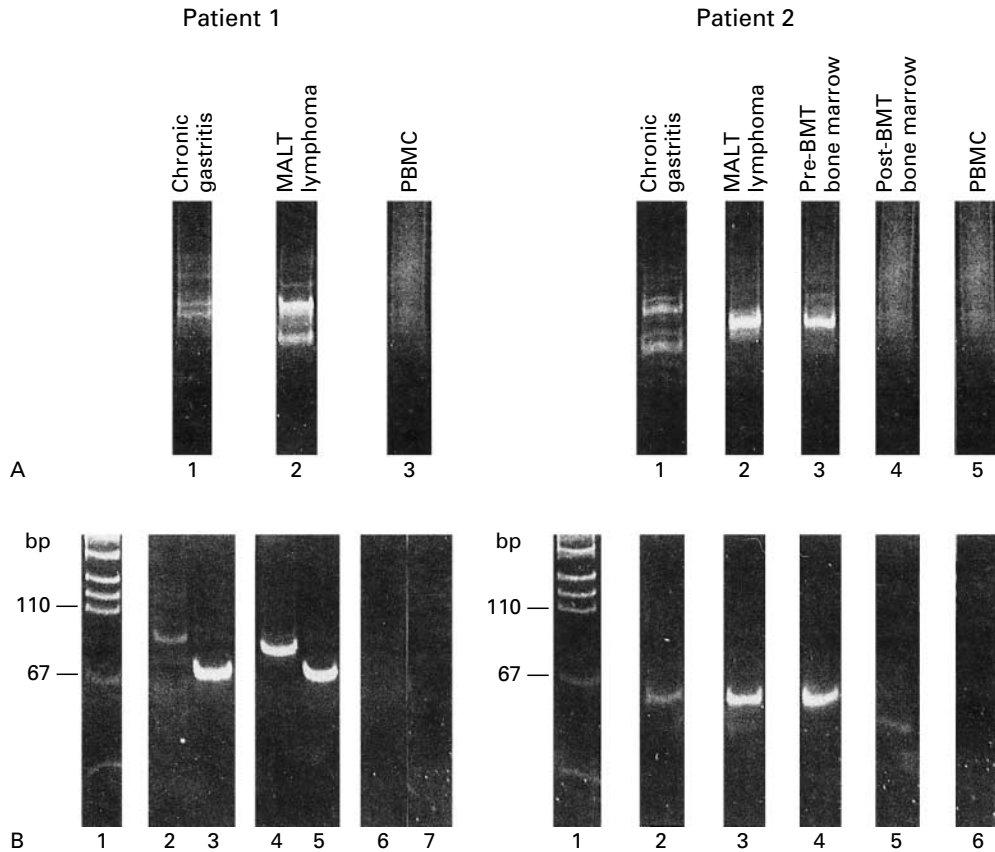


Figure 2. Results of Polyacrylamide-Gel Electrophoresis of the Immunoglobulin Heavy-Chain CDR3 PCR Products from the Two Patients.

Panel A shows the results of PCR analysis of B-cell clonality with consensus primers for the conserved-framework-3 segment of the variable region and for the joining region of the immunoglobulin heavy-chain genes. In stomach-biopsy specimens obtained at the time chronic gastritis was diagnosed (1981 in Patient 1 and 1982 in Patient 2), multiple faint bands are evident, indicating oligoclonality, whereas samples obtained at the time MALT lymphoma was diagnosed (1990 in Patient 1 and 1988 in Patient 2) show a monoclonal B-cell population. In Patient 2, a similar monoclonal pattern was evident in the bone marrow sample obtained before autologous bone marrow transplantation (BMT) in 1988, but it was no longer detectable after transplantation in 1996. In both patients, peripheral-blood mononuclear cells (PBMC) showed a smear of amplified products, indicating B-cell polyclonality. Sequencing of the lymphoma bands showed a single predominant clone in Patient 2 and two major clones in Patient 1. The amplified CDR3 sequences of each clone were used to design allele-specific primers. Panel B shows results of allele-specific PCR analysis. In each case, lane 1 shows a DNA size marker. In Patient 1, the gastritis specimens show the two B-cell clones (lanes 2 and 3) that predominated after the transformation to malignant lymphoma (lanes 4 and 5). In Patient 2, the lymphoma clone (lane 3) is evident in the gastritis specimen (lane 2), as well as in the bone marrow specimen obtained before transplantation (lane 4), but not in that obtained afterward (lane 5). In both patients PBMC were analyzed to assess the specificity of allele-specific PCR (Patient 1, lanes 6 and 7; Patient 2, lane 6).

size in all the samples of both low-grade and high-grade lymphoma obtained in 1988 and 1990 before autologous bone marrow transplantation. An oligoclonal or polyclonal pattern continued to be present after transplantation (Table 1). One predominant B-cell clone was sequenced from a paraffin-embedded lymphoma specimen, and its CDR3 sequence was used to make an allele-specific primer. Allele-specific PCR demonstrated the presence of this

clone in the gastritis specimen and in all subsequent biopsy samples until bone marrow transplantation in 1990, after which this clone became undetectable (Fig. 2).

DISCUSSION

Epidemiologic,^{4,5} experimental,²¹ and clinical^{6,7,15,22} data support a strong association between primary MALT lymphoma of the stomach (a clonal B-cell

cancer) and gastric *H. pylori* infection. In our study, we used a sensitive, patient-specific molecular technique — allele-specific PCR — to show that histologically confirmed *H. pylori* gastritis harbors the clonal B cell that eventually gives rise to MALT lymphoma.

A spectrum of lymphoproliferations, from polyclonal to monoclonal, may be involved in the transformation from benign to overtly neoplastic forms in the stomach.^{23,24} The scoring system proposed by Wotherspoon et al.¹¹ reflects this continuum from a histologic point of view. Tumor progression is a multistep process, and defining the point at which a non-neoplastic lesion becomes neoplastic is difficult.²⁵ Some genetic lesions have a clonal abnormality that remains responsive to normal regulators of growth and differentiation.^{25,26} Recent studies²⁷⁻²⁹ have shown antigenic selection and clonal expansion in B-cell clones of MALT lymphoma. The proliferation of these clones might be subclinical or of minimal clinical significance until additional genetic changes occur and the process becomes irreversible.

Sequencing analysis of DNA from Patient 1 showed two major lymphoma clones apparently arising from a biclonal proliferation of B cells. Allele-specific PCR based on these sequences was positive in the gastritis samples and in the subsequent lymphoma samples. One clone disappeared after several years, after antibiotic treatment, whereas the other did not. The persistent clone may have progressed to a stage of independence from *H. pylori*. The development of biclonal or oligoclonal lymphomas has also been documented in other circumstances, such as after organ transplantation.³⁰⁻³² In lymphomas induced by Epstein-Barr virus and associated with organ transplantation, a similar process of clonal B-cell proliferation is seen, and withdrawal of immunosuppression may suppress the proliferation and halt the progression of lymphoma.^{31,32} Withdrawal of the *H. pylori* antigenic stimulus at an early stage of clonal B-cell proliferation — such as by eradicating the microorganism — may reverse the malignant process before it becomes irreversible.

H. pylori is a very common bacterial infection in humans, present in almost half of all people.³³ Gastric MALT lymphoma, however, will develop in only a very small percentage. The estimated incidence of this lymphoma in the United States is between 1 in 30,000 and 1 in 80,000.³⁴ A higher incidence (13 in 100,000) has been reported in northeastern Italy.⁴ Additional environmental or host-related factors may be needed to sustain gastric lymphomagenesis.

The point at which *H. pylori* infection should be treated is controversial. There is general agreement only that it should be eradicated in patients with gastric or duodenal peptic ulcer.^{35,36} Our findings could be seen as supporting the use of antibiotic therapy in all symptomatic patients with *H. pylori*

gastritis. However, considerations of cost as well as the high cure rate of MALT lymphoma^{37,38} and the increasing resistance to antibiotics^{36,39} weigh against the indiscriminate use of antibiotics to treat all people with *H. pylori* gastritis. Moreover, there are insufficient data to demonstrate that eradicating *H. pylori* prevents the progression of gastritis to cancer or lymphoma.

Nevertheless, our findings suggest that analysis of B-cell clonality by PCR may help physicians to care for patients with *H. pylori* gastritis, particularly those with suspicious-appearing lymphoid infiltrates at histologic examination. In patients with a monoclonal B-cell population, which might represent an early, reversible step in the process of lymphomagenesis, we believe that antibiotic treatment of gastritis should be considered in order to reduce the risk of lymphoma.

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