Immunoglobulin G1 Antibody Response to *Helicobacter pylori* Heat Shock Protein 60 Is Closely Associated with Low-Grade Gastric Mucosa-Associated Lymphoid Tissue Lymphoma

E. ISHII,1 K. YOKOTA,1 T. SUGIYAMA,2 Y. FUJINAGA,1 K. AYADA,1 I. HOKARI,2 S. HAYASHI,3 Y. HIRAI,1 M. ASAKA,2 AND K. OGUMA1*

Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho Okayama, 700-8558,1
Department of Gastroenterology, Hokkaido University Graduate School of Medicine, N15, W7, Kita-ku, Sapporo, 060-0815,2 and Department of Microbiology, Jichi Medical School, 3311-1 Yakushiji Minamikouchi-cho Kawauchi-Gun, 329-0498,3 Japan

Received 5 April 2001/Returned for modification 24 May 2001/Accepted 23 July 2001

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is related to *Helicobacter pylori* infection. Specifically, it has been pointed out that pathogenesis of MALT lymphoma involves the 60-kDa heat shock protein (hsp60). To investigate humoral immune responses to the *H. pylori* hsp60 in patients with gastroduodenal diseases and patients with MALT lymphoma, the hsp60 of *H. pylori* was expressed with a glutathione S-transferase fusion protein and was purified (recombinant hsp60). Sera were obtained from *H. pylori*-positive patients with gastroduodenal diseases (MALT lymphoma, *n* = 13; gastric ulcer, *n* = 20; duodenal ulcer, *n* = 20; gastritis, *n* = 20) and from *H. pylori*-negative healthy volunteers (*n* = 9). Sera from patients with MALT lymphoma were also obtained at two times: before and after eradication therapy. Antibodies to hsp60 and *H. pylori* were assessed by enzyme-linked immunosorbent assay. The levels of immunoglobulin G (IgG) antibodies to the hsp60 of *H. pylori*-positive patients with gastroduodenal diseases were significantly elevated compared to those in the controls. The levels of IgG1 antibodies to hsp60 were elevated and correlated with the levels of anti-*H. pylori* antibodies in patients with MALT lymphoma. Humoral immunity against hsp60 may be important and relevant to gastroduodenal diseases induced by *H. pylori* infection.

*MATERIALS AND METHODS*

**Patients.** Sera were obtained from 73 *H. pylori*-positive patients, including 13 patients with gastric MALT lymphoma (5 women and 8 men; mean age, 56.6 years), 20 patients with gastric ulcer (3 women and 17 men; mean age, 46.3 years), 20 patients with duodenal ulcer (10 women and 10 men; mean age, 30.4 years), and 20 patients with gastritis (6 women and 14 men; mean age, 41.5 years). Sera were also obtained from nine *H. pylori*-negative volunteers (six women and three men; mean age, 37.9 years old). Informed consent was obtained from each patient and healthy volunteer. In nine patients with gastric MALT lymphoma who were treated with eradication therapy (a 1-week course of omeprazole at 40 mg/day, amoxicillin at 1,500 mg/day, and clarithromycin at 400 mg/day), endoscopic findings and histopathology were assessed 6 months after therapy and serum samples were collected. All sera were collected at the Hokkaido University Hospital and were stored at −80°C.

**Diagnosis.** The histology of MALT lymphoma was assessed according to the Working classification. All the lymphomas were diagnosed as low-grade MALT lymphoma. Diagnosis of the other diseases was determined according to the symptoms and by endoscopy. Infection with *H. pylori* was confirmed by culture, the rapid urease test, and histology of gastric biopsy specimens and by the presence of serum antibodies to *H. pylori* as detected by enzyme-linked immunosorbent assay (ELISA) (14). *H. pylori* was detected in the gastric mucosa of all patients whose sera were positive for anti-*H. pylori* antibodies. In the healthy control volunteers, negativity for *H. pylori* infection was defined by serology.

**Cloning, expression, and purification of recombinant protein.** The hsp60 protein of *H. pylori* was expressed as a glutathione S-transferase fusion protein by PCR cloning. PCR primers were designed on the basis of sequences published in GenBank. To facilitate cloning, restriction endonuclease cleavage sites were included in the PCR primer sequence. The PCR primer pair of GGAGGATCC

---

* Helicobacter pylori is now recognized as a cause of gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (9, 19). Several *H. pylori* pathogenic factors, including CagA and VacA, may contribute to the gastric mucosal damage (6, 7). In recent years, it has also been accepted that host immune reactions play an important role in the pathogenesis of *H. pylori* infection. Although *H. pylori* is a noninvasive bacterium and is restricted to gastric epithelial cells, infection with *H. pylori* induces humoral and cellular immune responses in the gastric mucosa (16, 22). Bacterial attachment causes induction of interleukin-8 (IL-8) release, and Cag proteins evoke activation of nuclear factor-κB and release of IL-8 that may support leukocyte attachment during inflammation (8, 17, 21). However, the bacterial antigens associated with inflammation are still not clear.

Heat shock proteins (hsp’s) are immunodominant antigens in various diseases including *H. pylori* infection (4, 26). Our previous studies have shown that the 60-kDa hsp (hsp60) is expressed in the follicular dendritic cells of the gastric mucosa in patients with gastric MALT lymphoma (15) and that antibodies to human hsp60 can be detected in MALT lymphoma patients (14). These results indicate that hsp60 may be associated with the host immune reaction in *H. pylori* infection, specifically, the pathogenesis of gastric MALT lymphoma.

In the present study, *H. pylori* hsp60 recombinant protein was expressed, and the levels of immunoglobulin G (IgG), IgG1, IgG2, IgM, and IgA antibodies to hsp60 in sera were measured in patients with gastric ulcer, duodenal ulcer, gastritis, and gastric MALT lymphoma to demonstrate the immunological role of hsp60 in *H. pylori*-infected patients.

---

* Corresponding author. Mailing address: Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho Okayama, 700-8558, Japan. Phone and fax: 81-86-235-7162. E-mail: kuma@med.okayama-u.ac.jp.
CCATGGCGAAGAGAATACCAATTT and GGCGGTCGACCATCAGGCGC
CCATGGCTTC was used to amplify the open reading frame from purified
H. pylori ATCC 43504 genomic DNA.

The PCR was performed in a 20-μl volume containing ExTaq
polymerase (Takara), 5 nM sense and antisense oligonucleotide primers,
and 500 ng of H. pylori genomic DNA. The cycling conditions were
25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The
amplicon was cut with BamHI and Sall restriction endonucleases and cloned
into the vector pGEX-5X3 (Amersham Pharmacia) by standard molecular
biology techniques, and the resultant plasmid was transformed into
Escherichia coli DH-5α. Recombinant protein was expressed at 25°C
in Luria-Bertani broth containing 2% glucose and ampicillin (100 μg/ml).
At an optical density (OD) of 540 nm of 0.6 to 0.8, the culture was induced
with 0.1 mM isopropyl-β-D-thiogalactopyranoside and was incubated at
37°C for 3 h. The cells were harvested by centrifugation at 7,000 × g for 10 min
and resuspended in ice-cold phosphate-buffered saline (PBS). The
bacterial suspension was frozen at −70°C and thawed, and the cells were
disrupted by sonication on ice for 2 min with a probe sonicator (Astron) set
to full power. Soluble fusion protein expressed by the glutathione S-transferase–hsp clone (hsp60) was purified by
glutathione-Sepharose 4B (Amersham Pharmacia) affinity chromatography
according to the manufacturer’s instructions.

ELISA for detection of antibodies to hsp60 and H. pylori. Serum antibodies
to hsp60 and H. pylori were measured by ELISA. Ninety-six-well microtiter
plates were coated with hsp60 fusion protein (10 μg/ml) or H. pylori lysate (50 μg/ml)
in 100 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C.
After the wells were blocked with PBS containing 10% skim milk, the plates
were incubated with sera, at a 1:100 dilution for hsp60 antibody or 1:1,000 for
H. pylori antibody, for 2 h and washed with PBS containing 0.05% Tween 20.
Peroxidase-labeled rabbit anti-human IgA, IgM, or IgG antibody (DAKO Japan)
was then added, and the plates was incubated for 2 h. After the plate was washed,
the wells were reacted with 1 mg of o-phenylenediamine (Wako Pure Chemical)
per ml in citrate buffer (pH 5.5). The ODs were measured at 490 nm on an ELISA plate
reader (Bio-Rad). Cutoff ODs were determined as the mean plus 2 times
the standard deviation for the sera obtained from negative controls.

For the detection of IgG1 or IgG2 antibodies, the contents of the wells of the
microtiter plates were reacted with human sera as described above. Anti-human
IgG1 or IgG2 monoclonal antibodies (Pharmingen) were then added, and
the plates were incubated for 2 h, followed by washing. The wells were continuously
reacted with an anti-mouse antibody conjugated with horseradish
peroxidase (DAKO Japan). After the plates were washed, the enzyme reaction
described above was used for final color development.

Statistical analysis. Differences in the ODs of the antibodies among
the disease groups were evaluated by the Student t test. Correlation between anti-
bodies to H. pylori and hsp60 were evaluated with Pearson’s correlation
coefficient. P values of <0.05 were considered to represent a significant difference.

RESULTS

IgG, IgM, and IgA antibodies to hsp60. The levels of IgG antibodies to
H. pylori whole antigens and hsp60 in the sera of patients with
gastroduodenal diseases were significantly higher
than those in the sera of healthy control (Fig. 1A and B). The
cutoff ODs of the ELISA for anti-whole-antigens and hsp60
antibodies were 0.59 and 1.20 respectively. The sensitivity and
specificity of IgG antibodies to whole antigens were 100
and 91.8%, respectively and the sensitivity and specificity of
IgG antibodies to hsp60 were 100 and 69.9%, respectively.
The levels of IgM antibodies and IgA antibodies were elevated,
but they were not significantly different from those in the control
group (Fig. 1C and D).

Correlation coefficient between IgG and IgGl antibodies to
hsp60 and H. pylori. IgG isotype antibodies were measured in
patients with gastroduodenal diseases. The levels of IgG1 antibodies
to H. pylori and hsp60 were significantly elevated in patients with
gastroduodenal diseases compared with the levels in the
c Control group. However, the levels of IgG2 antibodies to
both H. pylori whole antigens and hsp60 were increased in
patients, but the difference was not statistically significant (data
not shown). We statistically analyzed correlations between IgG
antibodies to H. pylori and IgG1 antibodies to hsp60 in the
patients. The patients with MALT lymphoma had a high cor-
relation coefficient, although patients with other disease had
only low correlation coefficients (Fig. 2).

IgG antibodies after eradication therapy in patients with
MALT lymphoma. We studied the change in total IgG anti-
body levels after eradication therapy in nine patients with
MALT lymphoma. Eradication therapy resulted in histological
and endoscopic remission in six MALT lymphoma patients;
however, three patients did not have a regression 6 months
after eradication therapy. IgG antibody levels to whole
H. pylori and hsp60 in the patients with regression were signi-
icantly decreased (Fig. 3A and B). One levels of IgG antibodies
to hsp60 in patients with or without regressions were decreased
following eradication therapy, and at pretreatment levels of
IgG antibody to hsp60 in the six patients with regressions were
significantly higher than those in the three patients without
regressions (Fig. 3B).

DISCUSSION

We have reported on the measurement of the levels of
immunoglobulin antibody against hsp60 in H. pylori-infected
patients. Specifically, the IgG1 response against hsp60 is
closely associated with MALT lymphoma in H. pylori-infected
patients. IgG1 antibodies are produced by B cells activated
under the T-helper 2 (Th2) cytokine IL-4. Th1 cells that produce gamma interferon are able to promote the production of IgG2 opsonizing and complement-fixing antibodies. An IgG subclass response to *H. pylori* in patients with chronic active gastritis and duodenal ulcer was reported by Bontkes et al (5). They indicated that the titers of IgG1 antibodies to the bacteria were raised in patients with *H. pylori* infection and that the titers of IgG2 antibodies in duodenal ulcer patients were higher than those in gastritis patients. In this study, there was a strong correspondence between total serum anti-*H. pylori* and anti-hsp60 IgG levels in patients with gastritis; however, the correspondence for IgG1 and IgG2 antibodies individually was weak. The anti-hsp60 IgG1 antibodies of patients with MALT lymphoma had a strong correspondence with anti-*H. pylori* IgG1 antibodies. The results indicate that a long-standing *H. pylori* infection induced the Th-2 reaction against hsp60 in patients with gastric MALT lymphoma.

We have reported that autoimmunity through human hsp60 is involved in patients with MALT lymphoma (14, 15) and that it may be caused by a long-term Th-2 reaction against bacterial hsp60. Infection with *H. pylori* also leads to an IgG response against the bacteria and hsp60 in patients with peptic ulcer and gastritis. We assessed the anti-hsp60 IgG antibody titers in a few patients with peptic ulcers and found that total levels of IgG to hsp60 were decreased by antibacterial eradication therapy (data not shown). However, the responses of Th-1 (IgG2) and/or Th-2 (IgG1) were diverse in patients with gastritis and peptic ulcer; hence, gastritis and ulcer diseases may be characterized by the development of cellular or humoral immune responses at various stages.

Some markers related to the pathogenesis of gastric MALT lymphoma have been investigated. Homozygous p16 tumor suppressor gene deletion was found in the gastric mucosae of patients with high-grade MALT lymphoma but were not found in those of patients with gastritis (18). The cell cycle regulatory gene (edc2/edk1) might play an important role in the modulation of cellular death, proliferation, and translation during the evolution of chronic gastritis to MALT lymphoma (3). PCR detection of IgH rearrangement in gastric biopsy specimens could be used to monitor MALT lymphoma regression during treatment (1). These observations about host epithelial cell growth and/or lymphocyte regulations indicated that some bacterial antigens were involved in the progression of the gastric lesion of MALT lymphoma; however, the bacterial antigen was not clearly detected. The present study showed that the levels of IgG antibodies against hsp60 are markedly decreased during eradication therapy and may possibly be used as a marker of the host immune reactions in patients with MALT lymphoma.

Bacterial virulence factors, like CagA and VacA s1, show a strong association with duodenal ulcer disease (2, 20, 23). Serological study of patients indicated that MALT lymphoma is associated with *H. pylori* strains expressing the CagA protein.
On the other hand, a large variety of H. pylori strains could be considered responsible for the induction of MALT lymphoma, as judged from the heterogeneity of their antigenic properties and the fact that CagA did not seem to be a virulence marker associated with the disease in Japan (13). H. pylori infection induces class II major histocompatibility complex expression and CD4-positive T-cell activation (11); however, antigens associated with lymphocyte activation were not detected. We have reported that local immunoglobulin against hsp60 is associated with infiltrating cell numbers in the gastric mucosa of patients with gastritis (12) and have also reported that anti-hsp60 antibodies are relevant to gastric inflammation in patients with peptic ulcer during eradication therapy (25). In addition, the level of expression of CD40L on the peripheral blood mononuclear cells (PBMCs) of patients with MALT lymphoma was increased by cytokines (granulocyte-macrophage colony-stimulating factor and IL-4) or by cytokine plus hsp60 stimulation compared to the level of expression on PBMCs of patients with gastritis or healthy volunteers. The production of IL-4 in PBMC cultures was also increased by cytokines and/or hsp60 stimulation in patients with MALT lymphoma. In patients with MALT lymphoma, however, the level of production of gamma interferon was at the low end (24). Hence, hsp60 may be an important antigen for the production of antibodies and T-cell activation in patients with H. pylori infection.

In conclusion, the present study indicated that H. pylori infection induced an IgG1 response (humoral Th-2 immunity) against H. pylori in patients with MALT lymphoma, and that may in infection induced an IgG1 response (humoral Th-2 immunity) in patients with MALT lymphoma. In patients with MALT lymphoma, however, the level of production of gamma interferon was at the low end (24).

Cover, T. L., and M. J. Blaser. 1992. Purification and characterization of the vacuolating toxin from Helicobacter pylori. J. Biol. Chem. 267:10570–10575.

Crawford, J. E., A. Covacci, S. M. Farmery, Z. Xiang, S. Tompkins, S. Perry, I. J. Lindley, and R. Rappuoli. 1995. Helicobacter induced interleukin-8 expression in gastric epithelial cells is associated with CagA-positive phenotype. J. Clin. Pathol. 48:414–419.

Dunn, B. E., H. Cohen, and M. J. Blaser. 1997. Helicobacter pylori. Clin. Microbiol. Rev. 10:720–741.

Eck, M., B. Schmausser, R. Hass, A. Greiner, S. Czuh, and H. K. Muller-Hermelink. 1997. MALT-type lymphoma of the stomach is associated with Helicobacter pylori strains expressing the CagA protein. Gastroenterology 112:1482–1486.

Fan, X., S. E. Crowe, S. Behar, H. Gunasena, G. Ye, H. Haeberlie, N. Van Houten, W. K. Gourley, P. B. Ernst, and V. E. Reyes. 1998. The effect of class II major histocompatibility complex expression on adherence of Helicobacter pylori and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. J. Exp. Med. 187:1659–1669.

Hayashi, S., S. Sugiyama, K.Hisano, T. Awakura, I. Kurokawa, A. Yachi, H. Isogai, E. Isogai, K. Yokota, Y. Hirai, K. Oguma, and N. Fujii. 1996. Quantitative detection of secretory immunoglobulin A to Helicobacter pylori in gastric juice: antibody capture enzyme-linked immunosorbent assay. J. Clin. Lab. Anal. 10:74–77.

Ito, Y., T. Azuma, S. Ito, H. Miyai, M. Hirai, Y. Yamazaki, F. Sato, T. Kato, Y. Kusihara, and M. Kurimoto. 1997. Analysis and typing of the vacA gene from cagA-positive strains of Helicobacter pylori isolated in Japan. J. Clin. Microbiol. 35:1710–1714.

Kawahara, Y., K. Yokota, M. Mizuno, N. Yunoki, T. Uetsu, H. Okada, K. Komatsu, Y. Hirai, K. Oguma, and T. Tsujii. 1999. Antibodies to human gastric epithelial cell and heat shock protein 60 in Helicobacter pylori-positive mucosa associated lymphoid tissue lymphoma. Gut 45:20–23.

Kobayashi, K., K. Yokota, T. Yoshino, Y. Kawahara, A. Dey, Y. Hirai, K. Oguma, and T. Akagi. 1998. Detection of Helicobacter pylori-associated antigen and heat shock protein 60 on follicular dendritic cells in the germinal centres of low grade B cell lymphoma of gastric mucosa associated lymphoid tissue (MALT). J. Clin. Pathol. 51:396–398.

Mattsson, A., M. Quinding-Jarbrink, H. Hoonrooth, A. Hamlet, I. Ahlstedt, and A.-M. Svensson. 1998. Antibody-secreting cells in the stomachs of symptomatic and asymptomatic Helicobacter pylori-induced subjects. Infect. Immun. 66:2705–2712.

Mori, N., A. Wada, T. Hirayama, T. P. Parks, C. Stratowa, and N. Yamamoto. 2000. Activation of intercellular adhesion molecule 1 expression by Helicobacter pylori is regulated by NF-eB in gastric epithelial cancer cells. Infect. Immun. 68:1806–1814.

Neumeister, P., G. Hoeffler, C. Beham-Schmid, H. Schmidt, U. Apfelbeck, H. Schaidler, W. Linkos, and H. Sill. 1997. Deletion analysis of the p16 tumor suppressor gene in gastrointestinal mucosa-associated lymphoid tissue lymphomas. Gastroenterology 111:2181–1875.

Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. Helicobacter pylori infection and gastric lymphoma. N. Engl. J. Med. 330:1267–1271.

Rudi, J., C. Kolb, M. Maivald, D. Kuck, A. Sieg, P. R. Galle, and W. Stremmel. 1998. Diversity of Helicobacter pylori vacA and cagA genes and their relation to the vacA and CagA protein expression, cytokine production, and associated diseases. J. Clin. Microbiol. 36:944–948.

Sharma, S. A., M. K. Tummuru, M. J. Blaser, and L. D. Kerr. 1998. Activation of IL-9 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. J. Immunol. 160:2401–2407.

Sommer, F., G. Faller, P. Konturek, T. Kirchner, E. G. Hahn, J. Zeus, M. Rohlffinghoff, and M. Lobohf. 1998. Antrum- and corpus mucosa-infiltrating CD4+ lymphocytes in Helicobacter pylori gastritis display a Th1 phenotype. Infect. Immun. 66:5543–5546.

van Doorn, L. J., C. Figueiredo, R. Sanna, A. Plaisier, P. Schneeberger, W. de Boer, and W. Quint. 1998. Clinical relevance of the cagA, vacA, and iceA status of Helicobacter pylori. Gastroenterology 115:58–66.

Yamazaki, R., K. Kobayashi, K. Yokota, S. Takada, E. Ishii, H. Okada, T. Yoshino, K. Oguma, and T. Akagi. 2000. Study of CD40 ligand expression by antigen stimulation with H. pylori-HSP60. Gut 47(Suppl. I):A40–A41.

Yunoiki, N., K. Yokota, M. Mizuno, Y. Kawahara, M. Adachi, M. Ogada, S. Hayashi, Y. Hirai, K. Oguma, and T. Tsujii. 2000. Antibody to heat shock protein can be used for early serological monitoring of Helicobacter pylori eradication treatment. Clin. Diagn. Lab. Immunol. 7:574–577.

Zugel, U., and S. H. Kaufmann. 1999. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin. Microbiol. Rev. 12:19–39.