Recurrence of Gastric Ulcer Dependent Upon Strain Differences of Helicobacter pylori in Urease B Gene

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To evaluate the role of different strains of Helicobacter pylori on the recurrence of gastric ulcer, we divided H. pylori into four types (I, II, III, and IV) according to the urease B gene using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The relationship between the recurrence of gastric ulcer and the prevalence of H. pylori types was studied in 32 patients with benign open gastric ulcers using upper gastrointestinal endoscopy. The rate of recurrence was significantly lower in patients with type II than in patients with types I, III, and IV ($P < 0.05$). Using Mongolian gerbils, an animal model of H. pylori infection, we also showed that the occurrence of gastric ulceration following restraint water-immersion stress was significantly lower in type II compared with types I and III. These data indicate that in the context of ulcer recurrence, it is not necessary to eradicate H. pylori during infection with type II.

**KEY WORDS:** Helicobacter pylori; urease B gene; polymerase chain reaction–restriction fragment length polymorphism; nonrecurrent gastric ulcer; Mongolian gerbils.

*Helicobacter pylori* is now recognized as the major etiological cause of chronic gastritis. *H. pylori* infection plays an important role in the pathogenesis of peptic ulceration and is a risk factor for the development of gastric adenocarcinoma and MALT lymphoma (1, 2). The eradication of *H. pylori* is associated with a marked reduction of ulcer recurrence and ulcer complications (3, 4).

On the other hand, many patients with persistent *H. pylori* infections do not develop peptic ulcers. Some of these patients receive maintenance doses of H$_2$-receptor antagonists, whereas others without any specific therapy do not develop peptic ulcers. On one hand, these facts emphasize the role of H$_2$-receptor antagonist therapy to block peptic ulceration following *H. pylori* infection, and they also indicate that the strain diversity of *H. pylori* may influence the genesis of peptic ulceration.

In this study, we have divided the *H. pylori* strain into four types according to the urease B gene (ureB) by using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), and examined the relationship between recurrence of gastric ulcers and restriction digest types. To further study this relationship, we inoculated Mongolian gerbils with the different types of *H. pylori* and evaluated the occurrence of gastric ulceration following restraint water-immersion stress. The pathogenic and protective capacities of different types of *H. pylori* and their clinical implications are also discussed.

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The patients as well as the standard strain from ATCC were housed in an air-conditioned biohazard room and fed sterilized water with the sterilized rodent diet (CE-2, Clea Japan Inc., Tokyo, Japan) and sterilized water ad libitum. All animal experiments were approved by the animal experimental committee, Ehime University School of Medicine.

Bacterial Strains and Culture. *H. pylori* strains from patients as well as the standard strain from ATCC were evaluated. The *H. pylori* strains were obtained from the gastric mucosa of the patients by upper gastrointestinal endoscopy. Antral biopsy specimens were cultured under microaerobic conditions (5% O₂, 15% CO₂ and 85% N₂) in seven-wk-old male Mongolian gerbils (*Meriones unguiculatus*). The resulting fragments were separated on 3% agar and stained with ethidium bromide and photographed. Primer 1 (5'AGAGTTTGATCCTGCGACT3') and primer 2 (5'-CTACACAAACCGCTTCATTCA-3') were designed for the urease B gene according to the report by Labigne et al (5). Two microliters each of primer 1 (10 μM) and primer 2 (10 μM), 0.5 units of Taq DNA polymerase (Takara, Shiga, Japan), 3.2 μl of 1.25 mM dNTPs, and 6 μl of DNA solution were added to 2 μl of a 10-fold PCR reaction solution and made up to 20 μl with sterile distilled water. The samples were amplified through 30 cycles in a thermal cycler (Gene Amp 2400, Perkin-Elmer Cetus, Norwalk, Connecticut). Each cycle consisted of denaturation (1 min at 94°C), annealing (1 min at 56°C), extension (2 min at 72°C). A band at 713 bp indicated the presence of *H. pylori*. To type the PCR products into four restriction digest patterns, reaction mixtures were digested with the restriction enzyme *Hae*III (Takara, Tokyo, Japan). The resulting fragments were separated on 3% agar gel (Bio-Rad Lab.). The bands were visualized with ethidium bromide and photographed.

**Materials and Methods**

**Subjects.** A retrospective study was conducted at the Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan, between July 1995 and August 1998. Patients with benign open gastric ulcers were treated with a full dose of H₂ receptor antagonists. The patients who developed scars within three months of the initial therapy were enrolled in this study. A total of 32 patients were followed for the next two years; their clinical profiles are given in Table 1. Written consent was obtained from all patients after explaining the nature and purpose of the study.

**Patients.** Multiple specimens were taken from the antrum and body of the stomach. Histological examination, culture, and the rapid urease test confirmed the existence of *H. pylori* in all cases. Initial therapy consisted of a full dose of H₂ receptor antagonists and mucosal protective agents. Three months later, upper gastrointestinal endoscopy revealed the presence of scar. The maintenance therapy, with a half-dose of H₂-receptor antagonists and mucosal protective agents, was then administered for the entire two-year study period. The follow-up study was performed by upper gastrointestinal endoscopy in all patients over two years to monitor the recurrence of open ulcers.

**Animals.** Seven-week-old male Mongolian gerbils (*M. unguiculatus*) were inoculated with different types of *H. pylori*. The animals were housed in an air-conditioned biohazard room and fed with the sterilized rodent diet (CE-2, Clea Japan Inc., Tokyo, Japan) and sterilized water ad libitum. All animal experiments were approved by the animal experimental committee, Ehime University School of Medicine.

**Bacterial Strains and Culture.** *H. pylori* strains from patients as well as the standard strain from ATCC were evaluated. The *H. pylori* strains were obtained from the gastric mucosa of the patients by upper gastrointestinal endoscopy. Antral biopsy specimens were cultured under microaerobic conditions (5% O₂, 15% CO₂ and 85% N₂) in 7% horse blood agar plates (basic medium, heart brain infusion agar; Eiken Chemical Co., Ltd.) at 37°C for three days. A sweep of multiple colonies was transferred to a new agar plate to obtain a pure culture. Isolates were confirmed to be *H. pylori* by positive urease, catalase, and oxidase test results, and a typical appearance on Gram stain. The organisms were maintained at –80°C in *Brucella* broth containing 15% glycerol. *H. pylori* ATCC43504 (American Type Culture Collection, Rockville, Maryland) was used as the standard strain.

**DNA Extraction.** Chromosomal DNA was extracted from isolated pure cultures of *H. pylori* from patients and the standard *H. pylori* from ATCC sequentially with sodium dodecyl sulfate, proteinase K and hexadecyltrimethyl ammonium bromide. The crude DNA was purified by phenol chloroform isoamylalcohol extraction and ethanol precipitation and was then dissolved in TE buffer. The spectrophotometric absorbencies were measured at 260 nm to indicate DNA concentrations.

**PCR-RFLP Analysis.** PCR was performed using two primers. Primer 1 (5'-GAGATTGGGGGATCAGACT-3') and primer 2 (5'-CTACACAAACCGCTTCATTCA-3') were designed for the urease B gene according to the report by Labigne et al (5). Two microliters each of primer 1 (10 μM) and primer 2 (10 μM), 0.5 units of Taq DNA polymerase (Takara, Shiga, Japan), 3.2 μl of 1.25 mM dNTPs, and 6 μl of DNA solution were added to 2 μl of a 10-fold PCR reaction solution and made up to 20 μl with sterile distilled water. The samples were amplified through 30 cycles in a thermal cycler (Gene Amp 2400, Perkin-Elmer Cetus, Norwalk, Connecticut). Each cycle consisted of denaturation (1 min at 94°C), annealing (1 min at 56°C), and extension (2 min at 72°C). A band at 713 bp indicated the presence of *H. pylori*. To type the PCR products into four restriction digest patterns, reaction mixtures were digested with the restriction enzyme *Hae*III (Takara, Tokyo, Japan). The resulting fragments were separated on 3% agar gel (Bio-Rad Lab.). The bands were visualized with ethidium bromide and photographed.

**Bacterial Inoculation of Mongolian Gerbils and Restraint Water-Immersion Stress.** Mongolian gerbils were divided into four groups, each consisting of 10 gerbils. Three groups were inoculated with *H. pylori* types I, II, and III, and the fourth group was kept unmanipulated [control (n = 7 in each group)]. Three Mongolian gerbils in each group were used for confirming the infection with *H. pylori*. Typing of *H. pylori* was done according to their ureB gene type.

*H. pylori* ATCC43504 (*H. pylori* type II) and two isolates from patients (type I and III) were grown in *Brucella* broth (BBL, Cockeysville, Maryland) supplemented with 10% heat-inactivated horse serum (Nacali Tesque, Kyoto, Japan) for 24 hr at 37°C in a 10% CO₂ environment on a rotary shaker at 140 rpm.

After each Mongolian gerbil had been fasting for 24 hr, 500 μl of the *H. pylori* suspension (4.0 x 10⁸ CFU/ml) was administered directly into the stomach using a feeding needle. After eight weeks, *H. pylori* infections were confirmed by a positive serum anti-*H. pylori* antibody test (Kyowa Med. Co., Ltd., Tokyo, Japan). After 12 weeks, three Mongolian gerbils from each group were killed and the presence of *H. pylori* was confirmed by establishing a culture from the stomach, and by Gram staining. Then, seven Mongolian gerbils in each group (three groups having received *H. pylori* type I, II, and III, one as the control) were restrained and immersed in water at 22°C for 45 min. The area of erosion and ulcers was measured and expressed in square millimeters.