Helicobacter pylori Heat Shock Protein A: Serologic Responses and Genetic Diversity

ENDERS K. W. NG,1,2* STUART A. THOMPSON,1 GUILLERMO I. PÉREZ-PÉREZ,1 IMAD KANSAU,3 ARIE VAN DER ENDE,4 AGNÉS LABIGNE,3 JOSEPH J. Y. SUNG,5 S. C. SYDNEY CHUNG,3 AND MARTIN J. BLASER1,6

Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine,1 and Veteran Affairs Medical Center, Nashville, Tennessee6; Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris, France3; Department of Medical Microbiology, Academic Medical Center, Amsterdam, The Netherlands4; and Department of Medicine and Therapeutics5 and Department of Surgery,2 Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong

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Helicobacter pylori synthesizes an unusual GroES homolog, heat shock protein A (HspA). The present study was aimed at an assessment of the serological response to HspA in a group of Chinese patients with defined gastroduodenal pathologies and determination of whether diversity is present in the nucleotide sequences encoding HspA in isolates from these patients. Serum samples collected from 154 patients who had an upper gastrointestinal pathology and the presence of H. pylori defined by biopsy were tested for an immunoglobulin G (IgG) serologic response to H. pylori HspA by an enzyme linked immunosorbant assay. HspA-encoding nucleotide sequences in H. pylori isolates from 14 patients (7 seropositive and 7 seronegative for HspA) were analyzed by PCR and direct sequencing of the PCR products. The sequencing results were compared to those of 48 isolates from other parts of the world. Of the 154 known H. pylori-positive patients, 54 (35.1%) were seropositive for HspA. The A domain (GroES homology) of HspA was highly conserved in the 14 isolates tested. Although the B domain (metal-binding site unique to H. pylori) resembled in the known major variant, particular amino acid substitutions allowed definition of an HspA variant associated with isolates from East Asia. There were no associations between patient characteristics and HspA seropositivity or amino acid sequences. We confirmed in this study that the clinical outcomes of H. pylori infection are not related to HspA antigenicity or to sequence variation. However, B-domain sequence variation may be a marker for the study of the genetic diversity of H. pylori strains of different geographic origins.

Helicobacter pylori is now recognized as an important organism associated with peptic ulcer disease, gastric adenocarcinoma, and gastric mucosal-associated lymphoid tumor-type lymphoma (9, 16, 17, 23). Although putative virulence factors like cytotoxins (3), adhesins (12), and flagella (6) have been identified, the mechanisms by which H. pylori contribute to these diverse clinical outcomes remain poorly understood. Recently, H. pylori has been shown to synthesize two heat shock protein homologs with differing antigenic characteristics. Heat shock protein A (HspA) is a 13-kDa protein of the GroES class, and heat shock protein B (HspB) is a GroEL homolog of 58 kDa (5, 13, 22); the genes encoding these two proteins form a bicistronic operon (22). While HspB and the first 90 amino acids of HspA (A domain) are highly homologous to other bacterial heat shock proteins (11, 20), HspA contains a unique 27-amino-acid histidine-rich carboxyl terminus (B domain). Experimental studies have shown that this histidine-rich region is involved in urease activity, presumably secondary to nickel binding (22). While HspA is essentially a cytoplasmic protein, H. pylori cells often lyse and expose the internal antigens.

Although all H. pylori strains studied possess hspa, in two previous studies only 40% of persons infected with H. pylori had detectable levels of serum antibody against this protein (19, 22). These two studies involved North American and European patients, but the immunologic responses to H. pylori HspA among Asian populations have not been determined. In one of the studies, an association between HspA seropositivity and proximal gastric carcinoma was found, but this also could have reflected the advanced age of these patients (19).

H. pylori is highly diverse at the genomic level (1, 2, 14). Kansau et al. (10) demonstrated diversity in the deduced hspa-encoded peptide sequences among 32 strains studied. They reported nucleotide polymorphism for the region encoding the HspA B domain, but the diversity of this region for strains from non-European populations has not been explored. The aims of this study were to investigate whether in Asian patients the anti-HspA serologic responses are also heterogeneous and whether variation correlated with clinical outcome. A secondary aim was to examine the extent of variation in the nucleotide and amino acid sequences of HspA among H. pylori strains collected from different geographic locales and to determine whether this variation might help explain differences in host responses.

MATERIALS AND METHODS

Patients studied. Between January 1994 and December 1996, 179 Hong Kong patients who were of Chinese descent and who presented with upper digestive tract symptoms were enrolled in this study after written informed consent was obtained. All were examined by esophagogastroduodenoscopy for investigation of symptoms, and demographic data were recorded. The presence of H. pylori was determined by culture and/or histological examination of the gastric mucosal biopsy specimens (15). In total, 154 patients (mean age, 52.3 ± 17.0 years; 94 males and 64 females) were confirmed to be carrying H. pylori. The diagnoses among these patients, were as follows: duodenal ulcer, n = 60; gastric ulcer, n = 29; gastric adenocarcinoma, n = 29; and unremarkable endoscopy, n = 36. For

* Corresponding author. Mailing address: Department of Surgery, Prince of Wales Hospital, Shatin, N.T., Hong Kong. Fax: (852) 26350075. E-mail: endersng@netvigator.com.
the remaining 25 (14%) patients (mean age, 45.6 ± 13.4 years; 17 males and 8 females), neither of the presence of *H. pylori* nor any endoscopic abnormality was detected.

**Serologic methods.** Recombinant HspA produced as a fusion protein with the maltose binding protein MalE (MBP-HspA) or MalE alone (MBP) were harvested from DH5α-*Escherichia coli* strains carrying pILL933 or pMAL-2, respectively, as described previously (10). The cells were induced with isopropyl-

β-D-thiogalactopyranoside and lysed by passage through a French pressure cell, and the recombinant proteins were purified to homogeneity by large-scale affinity chromatography. The presence of anti-HspA immunoglobulin G (IgG) in patient sera diluted 1:100 was determined in parallel enzyme-linked immunosorbent assays (ELISAs) as described previously (19). Goat anti-human IgG conjugated with horseradish peroxidase was used as the secondary antibody and was used at a dilution of 1:4,000. For each patient, the optical density (405 nm) that resulted from the serologic reaction with MBP alone was subtracted from that obtained from MBP-HspA to calculate a net optical density. The ratio of the net optical density of the tested serum samples to that of a standard positive control specimen on the same plate was defined as the optical density ratio (ODR). The cutoff ODR for seropositivity was 0.182, which was determined for a group of 40 asymptomatic volunteers known to be *H. pylori* negative. The cutoff value was equivalent to 3 standard deviations above the mean ODR for these 40 serum samples (data not shown). The presence of serum IgG antibodies to *H. pylori* CagA was also determined by ELISA, as reported previously (18).

**Characterization of *H. pylori* isolates from Hong Kong patients.** *H. pylori* isolates from 14 Hong Kong patients were used to analyze the *hspA* nucleotide sequence; 7 patients each were HspA seropositive or HspA seronegative and were randomly selected. After growth on Trypticase soy agar with 5% sheep blood under microaerobic conditions for 48 to 72 h, bacterial cells were collected for DNA extraction as described previously (25). A 487-bp segment containing the 334-bp *hspA* gene was amplified by PCR with the primers (5'-TGGCGTAT AGTTGTGTCGC and 5'-GCATCAGGAAATTGATTCTTTCG) described by Kansa et al. (10).

The PCR was conducted with a 50-μL mixture containing 100 ng of DNA, 5 μL of 10× PCR buffer (Qiagen, Hilden, Germany), 0.2 mM each deoxynucleotide (United States Biochemicals, Cleveland, Ohio), 2.5 U of Taq DNA polymerase (Qiagen), and 50 pmol of each primer. Gene amplification was carried out through 30 cycles of denaturation (94°C) for 1 min, primer annealing (52°C) for 1 min, and extension (72°C) for 2 min. PCR products were purified with the QiAquick PCR purification kit (Qiagen) and were submitted for direct sequencing with an Applied Biosystems 373A automated sequencer. The same PCR primers were also used for the sequencing of both strands of the PCR products. Identical methods were used to obtain *hspA* sequences from other strains used for comparison (see below).

**Characterization of other isolates.** For comparison with the Hong Kong isolates, we analyzed *hspA* nucleotide sequences from 48 other strains. We included the *hspA* sequences of 39 clinical isolates from France, whose amino acid sequences were reported previously (10), as well as from strain 26695, for which the genomic sequence was recently published (24). Two *H. pylori* isolates collected from each of three members of a Dutch family (27) were also studied. All six strains had previously been analyzed by PCR-based random amplified polymorphic DNA fingerprinting, which showed that all six strains were highly similar but not identical. The pairs of strains from each patient differed in their cagA status. Strains 2a, 3a, and 5a were cagA positive, while strains 2b, 3b, and 5d were cagA negative. In addition, the *hspA* sequences of two *H. pylori* isolates from rhesus monkeys, previously designated 31001 and ATCC 31407, respectively (4), were also determined.

The nucleotide and deduced amino acid sequences of the 14 Hong Kong strains were compared with those of all 48 strains described above by using the PHYLIP program of the GCG package (version 7.3). A phylogenetic tree was constructed by the neighbor-joining method with PHYLIP (version 3.5) (26). The stability of the tree was tested by performing 1,000 bootstrap replicates.

**Statistical analysis.** The chi-square test, Fisher’s exact test, and Student’s *t* test were carried out with Epi-Info software, when appropriate. The actual values for the anti-HspA serologic responses of patients with different clinical entities were analyzed with the chi-square test, Fisher’s exact test, and Student’s *t* test, respectively, and were not significant (*P* = 0.51; one-way analysis of variance).

**RESULTS**

**Serologic responses to HspA.** As expected, none of the 25 patients who were not colonized with *H. pylori* showed an IgG serologic response to *H. pylori* HspA, with all ODR values being below 0.1. This finding confirmed the high specificity of the IgG HspA ELISA for this Asian population. Of the 154 serum samples collected from persons known to carry *H. pylori*, 54 (35.1%) were HspA seropositive (Table 1), a proportion close to those reported previously (19, 22). Although patients with either duodenal ulcer or gastric ulcer had seropositivity rates slightly higher than those for the other groups of patients, these differences were not statistically significant. Among the entire group (Fig. 1), a bimodal distribution of the anti-HspA serologic responses was observed, and the distribution was similar to that reported previously (19, 22). The mean (standard deviation) anti-HspA ODRs for patients with stomach cancer, duodenal ulcer, gastric ulcer, and nonulcer dyspepsia were 0.14 (0.30), 0.18 (0.28), 0.26 (0.28), and 0.20 (0.33), respectively, and were not significant (*P* = 0.51; one-way analysis of variance).

**TABLE 1. IgG serologic response against *H. pylori* HspA among 154 patients colonized with *H. pylori***

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of patients examined</th>
<th>Mean (SD) age (yr)</th>
<th>No. of males:no. of females</th>
<th>% Patients with anti-HspA IgG serum antibodiesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pathology</td>
<td>36</td>
<td>48.7 (16.6)</td>
<td>18:18</td>
<td>33.3</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>60</td>
<td>43.1 (12.5)</td>
<td>42:18</td>
<td>36.7</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>29</td>
<td>65.0 (15.8)</td>
<td>16:13</td>
<td>37.9</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>29</td>
<td>62.8 (13.2)</td>
<td>18:11</td>
<td>31.0</td>
</tr>
<tr>
<td>Total</td>
<td>154</td>
<td>52.3 (17.0)</td>
<td>94:60</td>
<td>35.1</td>
</tr>
</tbody>
</table>

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*a P = 0.97 (chi-square analysis).”

**FIG. 1. Distribution of serologic responses against *H. pylori* HspA.** For serum specimens from 154 patients known to be carrying *H. pylori* (black bars) and 25 patients not carrying the organism (white bars).
analysis of hspA sequences of the six strains from members of a multigeneration Dutch family (27) revealed complete identity. Neighbor-joining analyses for the sequences of the entire hspA gene and the B domain alone were performed and resulted in similar trees. The tree derived from the B domain alone was a consensus of 1,000 bootstrap replicates and suggested the geographic segregation of H. pylori strains (Fig. 2).

All except two of the Hong Kong strains were part of a major (East Asian) branch of the dendrogram (Fig. 2). Six strains isolated in France were also part of the East Asian branch. However, two of these strains (strains F27 and F30) had actually been isolated from patients of Far Eastern origin residing in France. The other three major branches consisted mainly of isolates from persons of European or North African origin; there was no obvious distinction between the isolates from persons in these two groups. However, it is noteworthy that the East Asian branch was not supported in a majority of bootstrap replicates, and the classification of a distinct East Asian clonal grouping awaits the characterization of larger or additional gene segments. Nevertheless, when the proportion of East Asian strains in the Asian cluster is compared with that of those outside the cluster, the difference is statistically significant (25 of 21 versus 9 of 31 [P < 0.001; two-tailed Fisher exact test]).

Comparison of HspA amino acid sequences. The translated amino acid sequences of HspA from all 14 Hong Kong strains examined were highly similar to that of the major variant reported previously (10). Although there was a high degree of conservation in domain A compared to the published sequences (10), variations were chiefly detected at residues 68, 75, and 90. There was greater variation in domain B, again almost exclusively confined to three residues: residues 98, 99, and 110 (Fig. 3). None of the HspA molecules had the signature (95ANSxxxxxHxxHA107) of the minor variant that was previously described to be present in 10% of the European isolates. Only 2 of the 14 Hong Kong strains had the same primary HspA sequences; the other 12 strains had different substitutions at one or more amino acids. There was no association between the variation within domain B of the H. pylori isolate and the presence of serologic responses by the 14 Hong Kong patients.

For the 14 Hong Kong isolates, the probability of an amino acid substitution was significantly (P < 0.001) higher in domain B (0.08) than in domain A (0.007). However, only one of these domain-B amino acid substitutions involved any of the eight histidine positions. The probabilities of replacement in domain B were 0.009 for the histidine residue and 0.11 for the nonhistidine residues (P < 0.001). Each of the substitutions except
of glmM of Dutch and Chinese H. pylori isolates, and are consistent with the multilocus enzyme electrophoresis analysis of 74 H. pylori isolates without geographic clustering by Go et al. (7). Further analysis with larger numbers of strains might better clarify the population structure. The segregation between Dutch and Chinese H. pylori isolates in a dendrogram on the basis of cagA sequences may be explained by the location of cagA on a genetic element with a different evolutionary background (28).

That the A domain is highly conserved at the amino acid level confirms previous findings (10). The near invariance of the B-domain histidines is also consistent with an important functional role, perhaps related to nickel binding (10). However, the remaining amino acids in the B domain, which show more than 10-fold variation compared with those for the A-domain and the B-domain histidines, are thus not as strongly conserved. The presence of substantial variation at different sites within a single gene suggests that selection varies at particular codons, reflecting different functional constraints. The high degree of similarity among hspA sequences from human and monkey strains suggests a very close relationship between H. pylori in humans and other primates, a finding consistent with an ancient origin for H. pylori.

In conclusion, we confirm and extend previous studies (10, 22) that the diversity of hspA does not account for the bimodal distribution of serologic responses to HspA among persons colonized with H. pylori. The basis for this phenomenon, although related to patient age, remains unexplained.
FIG. 3. Translated HspA amino acid sequences of 14 H. pylori strains from Hong Kong patients in comparison with that of reference strain 85P. The vertical line separates domains A and B between residues 90 and 91. The A domain represents the conserved portion of HspA that is homologous to GroES.

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