Detection of Serum Antibodies to CagA and VacA and of Serum Neutralizing Activity for Vacuolating Cytotoxin in Patients with *Helicobacter pylori*-Induced Gastritis

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Thirty patients with dyspepsia, with histological diagnosis of gastritis, and with endoscopic diagnosis of peptic ulcer disease (PUD) (n = 13) or nonulcer dyspepsia (NUD) (n = 17) were admitted to the study. *Helicobacter pylori* vacuolating cytotoxin-producing strains (Tox+) were isolated from 14 (46.7%) patients, whereas non-cytotoxin-producing (Tox-) *H. pylori* strains were isolated from the remaining patients. Of 30 patients studied, 20 (66.7%) had serum cytotoxin neutralizing activity in vitro. Fourteen patients with Tox+ *H. pylori* strains showed serum cytotoxin neutralizing activity and serum immunoglobulin G (IgG) and IgA antibodies reactive with both 87-kDa *H. pylori* vacuolating cytotoxin (VacA) and 128-kDa cytotoxin-associated gene product (CagA) by immunoblotting using native enriched preparations of VacA and CagA proteins from *H. pylori* culture supernatants as the antigens. A 94-kDa antigen cross-reacting with the 87-kDa VacA protein could be demonstrated in culture supernatant with immune sera from humans and animals. All patients (n = 10) lacking serum neutralizing activity were also negative for IgG or IgA against VacA antigen, whereas 6 of the 10 patients showed IgG serum antibody responses against CagA antigen. The prevalence of antibodies to VacA and CagA antigens was significantly (P < 0.001) higher in patients with gastritis (20 and 26 patients for VacA and CagA, respectively, of 30 patients) than in *H. pylori* culture-negative controls (0 of 27 for both VacA and CagA) and in randomly selected blood donors (17 and 21 for VacA and CagA, respectively, of 120 subjects). All patients with PUD had antibodies to CagA, whereas 13 of 17 (76.5%) patients with NUD had anti-CagA antibodies. Serum IgG antibodies to VacA were present in 9 (69.2%) patients with PUD of 13 patients and in 11 (64.7%) patients with NUD of 17 patients. Anti-CagA antibodies seemed to correlate better with PUD than anti-VacA antibodies.

*Helicobacter pylori* has been established as a major cause of chronic active gastritis and peptic ulcer disease (PUD), also associated with gastric cancer (2, 26). However, the clinical diagnosis of *Helicobacter* infection may be difficult due to the absence of either specific symptoms or specific endoscopic findings. In addition, the results of cultures, histological tests, and urease test on biopsies may be highly variable depending on the size and number of the specimens obtained. Therefore, immunological tests may be critical for the reliable diagnosis of *H. pylori* infections.

Several serological studies using enzyme-linked immunosorbent assay (ELISA) or Western immunoblotting (WB) have been performed to detect antibodies against *H. pylori*. Most of the available data have been obtained by ELISA for serum immunoglobulins against *H. pylori* (17, 22, 24), whole bacterial cells that have been used as the antigen. However, the presence of serological cross-reactions between *H. pylori* and other gram-negative bacteria such as *Pseudomonas aeruginosa*, *Campylobacter jejuni*, and *Haemophilus influenzae* (3, 19) has called into question the specificity of the results obtained by ELISAs using the whole bacterial cell as the antigen. More recently, recombinant CagA has been used as an antigen by ELISA for specific serological diagnosis of *H. pylori* infection in patients with PUD and nonulcer dyspepsia (NUD) (7, 13, 16).

Many studies have been also performed by WB technique with whole *H. pylori* cells as the antigen (1, 23, 32, 33). The high number of bands recognized by patients’ sera hampered reliable detection of specific *H. pylori*-reactive antigens. Very few studies have been performed with purified or partially purified major *H. pylori* antigens (12). Prominent *H. pylori* antigens detected by WB technique are represented by a 120- to 128-kDa CagA protein (11, 14, 31) and by a protein with vacuolating cytotoxic activity (VacA) (21) that migrates at 87 to 94 kDa under denaturing conditions (9, 35).

We now report on the detection of specific serum immunoglobulin G (IgG) and IgA responses against VacA and CagA of *H. pylori* in 30 patients with gastritis and in control subjects by WB technique, using as antigens enriched preparations from *H. pylori* broth culture supernatant that allow simple and clear detection of major *H. pylori* antigens. The relationships between serum antibodies against the VacA and CagA antigens and serum neutralizing activity of vacuolating cytotoxin have also been analyzed in both patients and controls.

**MATERIALS AND METHODS**

**Patients and clinical specimens.** Thirty patients (14 men and 16 females; mean age, 46 years; age range, 22 to 75 years) who underwent gastroduodenoscopy for dyspepsia and were positive for *H. pylori* by both culture and histology were admitted to the study. Gastric specimens were cultured for *H. pylori*, as previously described (29). Briefly, samples were homogenized and cultured on Columbia agar base (Oxoid, Milan, Italy) enriched with 7% horse blood and Dent’s

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selective supplement (Oxoid). The cultures were incubated in a microaerophilic atmosphere at 37°C in GasPak jars and Campy Pack II envelopes (BBL Microbiology Systems, Cockeysville, Md.), and isolates were identified as *H. pylori* by gram stain morphology and by positive urease, oxidase, and catalase tests. Histological sections of formalin-fixed biopsy specimens were stained with hematoxylin-eosin to evaluate the morphology and the presence of Helicobacter-like organisms. A serum sample was obtained by routine venipuncture for serological experiments.

In addition, 27 subjects (14 men and 13 females; mean age, 53 years; age range, 25 to 76 years) with chronic idiopathic dyspepsia negative for *H. pylori* by both culture and histology were included in the study (9). All patients were seronegative volunteers from the Danish National Furniture Council in the study. Septa were studied for the presence of serum cytotoxin neutralizing activity or a Tox gene (1). Lane b, *H. pylori* CCUG strain; lane c, ccgA-negative HPG21 strain; lanes d to n, 10 ccgA-positive isolates; lanes n to r, five isolates which gave no PCR product when amplified with primers for ccgA gene.

Finally, sera were also obtained from 120 blood donors to evaluate *H. pylori* serology.

**Concentration of *H. pylori* broth culture supernatant.** *H. pylori* strains were cultured at 37°C in brucella broth (Difco Laboratories, Detroit, Mich.) containing 5% fetal bovine serum (Amgen, Beverly, Mass.).

**Assay for cytotoxicity.** Concentrated supernatants from *H. pylori* strains were inoculated into HeLa cells in twofold dilutions from 1:2 to 1:160. Cell vacuolization was assessed by light microscopy after 48 h of incubation. Wells in which 50% or more cells were vacuolated were defined as showing cytotoxic effect.

**Neutralization of *H. pylori* cytotoxic activity.** Sera were heated to 50°C and diluted to Eagle’s minimal essential medium. Twofold-diluted sera (from 1:10 to 1:160) were incubated for 1 h at 37°C with an equal volume of concentrated *H. pylori* type strain CCUG 17874 (Culture Collection of the University of Göteborg, Göteborg, Sweden) culture supernatant. Adherent HeLa cells were incubated for 18 h at 37°C in 96-well plates with 50-μl volumes of serum and *H. pylori* supernatant plus 50 μl of Eagle’s minimal essential medium. The neutralization titer was defined as the highest dilution of a serum sample completely neutralizing vacuolization, assessed by light microscopy.

**SDS-PAGE and WB analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20) using 7.5% acrylamide gels. The WB procedure of Towne et al. (25) was as described previously (5). Briefly, after electrophoretic transfer, the blots were incubated for 12 h at room temperature with human, rabbit, or mouse sera diluted with Eagle’s minimal essential medium. Twofold-diluted sera (from 1:10 to 1:160) were incubated for 1 h at 37°C with an equal volume of concentrated *H. pylori* type strain CCUG 17874 (Culture Collection of the University of Göteborg, Göteborg, Sweden) culture supernatant. Antigen-antibody complexes were detected with anti-species peroxidase-labeled antibodies (DAKO, Copenhagen, Denmark) diluted 1:500 in phosphate-buffered saline and with 4-chloro-1-naphtol (Bio-Rad, Hercules, Calif.) as the enzyme substrate.

**Intrinsic labeling and autoradiography.** *H. pylori* CCUG 17874 was grown at 37°C in brucella broth (Difco) containing 5% fetal bovine serum in the presence of 5 μCi of 14C-labeled amino acid mixture (Amersham, Amersham, United Kingdom) per ml. After 48 h of incubation, the cell-free supernatants were sterilized by passage through a 0.22-μm pore-size filter and concentrated 15-fold with Centriprep-100 ultrafiltration units (Amicon, Beverly, Mass.).

Concentrated supernatants from 14 (46.7%) of 30 *H. pylori* isolates and from reference strains *H. pylori* CCUG 17874 and HPG21. The PCR protocol amplified 1,350-bp products of *H. pylori* ccgA gene (A) and 1,160-bp products of *H. pylori* vacA gene (B). Lane b, *H. pylori* CCUG strain; lane c, ccgA-negative HPG21 strain; lanes d to n, 10 ccgA-positive isolates; lanes n to r, five isolates which gave no PCR product when amplified with primers for ccgA gene. Lane a contains marker DNAs. Size markers for the expected products are indicated on the right. PCR products were visualized with UV light following staining with ethidium bromide.

RESULTS

Thirty patients who underwent gastroduodenoscopy for dyspepsia were admitted to the study. Endoscopic diagnoses were PUD (n = 13) and NUD (n = 17). Gastric tissue specimens from both antral and fundus mucosas were used for histological examination and *H. pylori* culture. The histological diagnoses were chronic active gastritis (n = 21) and chronic gastritis (n = 9). All patients were *H. pylori* positive by culture. Supernatants from 14 (46.7%) of 30 *H. pylori* isolates produced vacuolization of HeLa cells, whereas the remaining 16 did not. The supernatant dilution that produced vacuolization ranged from 1:5 to 1:160. Specific sequences of vacA and ccgA genes of *H. pylori* isolates were searched for by PCR: all the strains were vacA positive, whereas only 25 of 30 strains possessed the ccgA gene (Fig. 1). The relationships between genetic properties and in vitro vacuolating cytotoxic activity of *H. pylori* strains isolated from 30 patients with gastritis are shown in Table 1.

Serum neutralizing activity (serum titers ranging from 1:10 to 1:160) was present in 20 (66.7%) of the 30 *H. pylori* culture-positive patients. Ten (33.3%) patients did not present either serum neutralizing activity or a Tox gene (1) *H. pylori* strain. In addition to the above patients, 27 *H. pylori*-negative control subjects were studied for the presence of serum cytotoxin neutralizing activity: all sera were negative.

We next sought to determine by WB whether the presence of serum neutralizing activity was associated with the presence of serum IgG and IgA responses to CagA and VacA, using concentrated supernatant from *H. pylori* type strain CCUG 17874 broth culture as the antigen. Some experiments had been previously done to partially characterize the antigenic preparation used in WB. Radiolabeling of *H. pylori* CCUG strain grown in broth culture in the presence of 14C-labeled amino acid mixture allowed us to verify whether isotope-labeled *H. pylori*-derived products were present in the concentrated supernatant used as the antigen in WB and to compare
the protein profiles of isotope-labeled bacteria. Several radio-
labeled bands were present in the gel run with whole-bacte-
rium preparations, whereas only two major radiolabeled bands
of 87 and 40 kDa and two minor bands of 94 and 128 kDa were
observed in the gel performed with concentrated supernatant
preparation (Fig. 2).

Concentrated supernatant from H. pylori broth culture was
first tested with rabbit polyclonal monospecific antibodies
raised against the purified CagA and VacA proteins (34). Rab-
bit anti-CagA immune serum recognized the 128-kDa CagA
band, whereas anti-VacA immune serum recognized both a
major 87-kDa band (VacA) and a less intensely stained 94-kDa
band, which proved to be antigenically related to the 87-kDa
protein (Fig. 3B). Since H. pylori urease subunit polypeptide sizes (66 and 29.5
kDa) add up to an apparent molecular mass of 95.5 kDa (18),
which is very close to the 94-kDa mass of the antigen identified
by rabbit anti-VacA immune serum, we ruled out any cross-
reactivity of the immune serum by using mouse antiurease
immune ascitic fluid. As expected, the antiurease preparation
recognized only two bands of 66 and 29 kDa, when probed with
H. pylori cell extract (data not shown). When concentrated
supernatant from H. pylori broth culture was tested with sera
from patients with gastritis and positive for H. pylori by culture
of gastric biopsy, 20 of 30 serum samples recognized three
bands with apparent molecular masses of 128, 94, and 87 kDa,
whereas 4 serum samples were not reactive (Fig. 3A). The relationships
between serum neutralizing activity, serum IgG and IgA
against H. pylori VacA and CagA antigens, and in vitro cyto-
toxin production (Tox+) by H. pylori isolates in patients with
gastritis and with defined gastroduodenal pathology (NUD and
PUD) are shown in Table 1. Twenty patients with serum neu-
tralizing activity against vaculating cytotoxin showed specific
IgG against both VacA and CagA antigens, whereas 17 (85%)
of 20 were also positive for serum IgA against the same anti-
gens. It is interesting to point out that sera reactive with the
87-kDa band also recognized the 94-kDa band which is anti-
genically related to the 87-kDa protein. Of 10 patients with
gastritis lacking serum neutralizing activity for vaculating cyto-
toxin, 6 had serum IgG against CagA, whereas 4 were neg-
active. Of 13 patients with PUD (9), 9 (69.2%) had serum neutral-
izing activity and serum antibodies against VacA, whereas all
patients were positive for antibodies against CagA. Of 17 pa-
tients with NUD, 11 (64.7%) had serum neutralizing activity
and serum antibodies against VacA, whereas 13 (76.5%) were
positive for antibodies against CagA. Four patients with NUD
were serologically negative for H. pylori. The prevalences of
serum antibodies directed against both VacA and CagA anti-
gens in patients with gastritis, H. pylori-negative controls, and
blood donors are reported in Table 2. The prevalence of anti-
VacA and anti-CagA antibodies was significantly (P < 0.001)
higher in patients with gastritis than in the other subjects.
Cytotoxin neutralizing activity was studied in 60 random serum
samples from 103 blood donors and in 27 H. pylori-negative
control subjects: all samples were found negative.

**DISCUSSION**

The cytotoxin-associated protein CagA and the vaculating
cytotoxin VacA, which are thought to be the major H. pylori
virulence factors involved in the pathogenesis of H. pylori dis-
eases (15, 27), are produced by only a subset of clinical H. pylori
isolates, defined as H. pylori type I (35). Bacterial strains
which do not express CagA and cytotoxin form a group termed
H. pylori type II (35). Only type I H. pylori strains have been

**FIG. 3. Reactivities of H. pylori major antigens with immune sera from hu-

mans (A) and animals (B) by WB. (A) The following three patterns of reactivity
were observed when 30 serum samples from patients with gastritis were tested
with H. pylori concentrated supernatant: negative (lane 1); positive for one band
of 128 kDa (lane 2); and positive for three bands of 128, 94, and 87 kDa (lane 3).
(B) Rabbit immune serum raised against purified VacA protein recognized two
bands of 87 and 94 kDa (lane 1), whereas rabbit immune serum raised against
CagA recognized a 128-kDa band (lane 2).**

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**TABLE 1. Serological, phenotypic, and genetic properties of H. pylori isolates in 30 patients with gastritis and endoscopically defined gastroduodenal pathology**

<table>
<thead>
<tr>
<th>Total no. of patients</th>
<th>No. of patients with gastritis and:</th>
<th>Serologya</th>
<th>Phenotypic and genetic properties of H. pylori isolatesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUD</td>
<td>NUD</td>
<td>Serum neutralizing activity</td>
<td>Anti-VacA IgG IgA</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>+</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
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<td>3</td>
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<td>2</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0</td>
<td>−</td>
</tr>
</tbody>
</table>

a Symbols: +, possesses the activity, antibody, or gene; −, does not possess the
activity, antibody, or gene.
b Tox+, in vitro cytotoxin production.
associated with the most-severe gastroduodenal pathologies in humans (8, 33).

Serological experiments have shown that antibody titers to the 120- to 128-kDa protein CagA correlate with the severity of the disease and that CagA-producing strains are associated with PUD and gastritis (34). A strong correlation between the presence of IgG antibodies against the 87-kDa protein cytotoxin and cytotoxin-neutralizing activity has been also demonstrated in patients with H. pylori infection (10, 11).

The evaluation of phenotypic and genetic properties of H. pylori isolates and the serological results obtained in the present study from patients with gastritis showed a good correlation between anti-VacA IgG and serum neutralizing activity, whereas there was no detectable relationship between serology and in vitro cytotoxin production. In fact, of 30 patients with gastritis, only 14 showed an in vitro Tox’ strain as well as both serum neutralizing activity and serum antibodies against CagA and VacA. Six patients had similar serological responses with apparently Tox’ H. pylori isolates from gastric biopsies; in one patient, this response may reflect simultaneous infection with both Tox’ and Tox’ H. pylori strains, since the isolate from this patient was VacA positive and cagA negative; the remaining five patients may have been infected with H. pylori strains producing levels of cytotoxin undetectable in the in vitro assay but high enough to elicit an antibody response in vivo, since the isolates from these patients were indeed cagA and vacA positive. The fact that there were six patients with serum antibodies against CagA but without both antibodies against VacA and serum neutralizing activity may reflect infection with the H. pylori strain possessing the cagA gene but with no cytotoxin activity. Finally, four patients lacking serum neutralizing activity and antibodies against both VacA and CagA may reflect infection with the H. pylori strain possessing vacA but failing to express cytotoxin activity in vitro and very likely in vivo. In addition, the absence of expression of the vacA gene and the possible roles of other genes in the control of vacA gene expression (possibly cagA) also should be considered.

All sera reactive with the 87-kDa protein also reacted with a 94-kDa antigen, present in concentrated supernatant from H. pylori broth culture. This antigen, which was radiolabeled when H. pylori was grown in the presence of 14C-labeled amino acid mixture, was antigenically related to the 87-kDa protein, since it was recognized by rabbit polyclonal antiserum raised against VacA protein. The origin and the biological significance, if any, of the 94-kDa band require further investigation. However, it is interesting that the structural gene for cytotoxin, the vacA gene, encodes a true secretory protein of 136 kDa (25), whereas the purified protein has an apparent molecular mass of 87 kDa. At present, we do not know whether the active 87-kDa protein is generated by processing of the 136-kDa protein. If this were the case, the 94-kDa protein, antigenically related to the 87-kDa protein, could be derived by the same process.

The prevalence of serum antibodies directed against either VacA or CagA antigens was significantly higher in patients with gastritis than in H. pylori-negative controls or in blood donors, a better correlation with gastritis being found when considering anti-CagA antibodies. As far as the association between PUD and NUD with antibodies against CagA and VacA antigens is concerned, our results showed that all patients with PUD had antibodies to CagA, whereas 13 of 17 (76.5%) with NUD had anti-CagA antibodies. Antibodies to VacA antigen were present in 69.2% of patients with PUD and in 64.7% of patients with NUD. Therefore, anti-CagA antibodies seem to correlate better with PUD than anti-VacA antibodies, in agreement with previously reported data (7, 13).

In conclusion, the results suggest that a simple serological test such as the WB described is helpful in identifying subjects infected with H. pylori strains producing in vivo major bacterial virulence factors possibly involved in PUD and NUD. The use of a specific anti-H. pylori antibody assay, especially for the detection of specific serum IgG against CagA antigen, may help to better select patients for endoscopic and gastric biopsy.

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