Antibody to Cytotoxin in Infection by Helicobacter pylori

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Gastrointestinal disease and colonization by Helicobacter pylori were determined in 36 asymptomatic volunteers and 30 symptomatic individuals undergoing endoscopy and biopsy. Serum antibody immunoglobulin G (IgG) and IgA to H. pylori were measured by enzyme-linked immunosorbent assay. Serum antibody to a cytotoxin produced by H. pylori was detected with a neutralization assay. Serum IgG was 95% predictive of infection by H. pylori, and serum IgA was 88% predictive. Antibody to the cytotoxin was detected in 12 of 18 infected individuals. Antibody to the cytotoxin was a highly specific (96%), but not a very sensitive (67%), indicator of infection by H. pylori. The neutralization assay was 87% predictive of infection. These data confirm the diagnostic value of serum antibody to H. pylori for the detection of infection. The toxin-neutralizing activity of sera from individuals infected with H. pylori suggests that the cytotoxin is produced in vivo. It may therefore contribute to disease associated with H. pylori.

Colonization of human gastric tissue by Helicobacter pylori (formerly Campylobacter pylori; 5) is associated with gastritis and peptic ulceration (1). Bacteria colonize the gastric mucus layer and induce gastric inflammation with depletion of gastric mucus (13). The mechanism by which H. pylori produces gastric disease is not clear.

A cytotoxic activity is produced in vitro by H. pylori. Culture filtrates of broth-grown bacteria induce intracellular vacuolation of several cultured mammalian cell types (9). Fifty-seven percent of over 200 strains of H. pylori tested produced this activity. Intracellular vacuolation of gastric epithelial cells in biopsy specimens of gastritis patients (16) and experimentally infected germfree pigs (4) was reported. Vacuolation may be a nonspecific indicator of unhealthy or dying cells (3) or may be specifically induced by the toxin. Production of the cytotoxin in vivo or its contribution to disease associated with H. pylori has not been demonstrated.

Serum antibody to H. pylori is detectable in individuals infected with the bacterium (2, 8, 15). Measurement of specific antibody has diagnostic value for the detection of infection by H. pylori (6, 10, 12, 14). However, the utility of serological techniques to indicate the outcome of antimicrobial therapy for infection has not been clearly demonstrated.

The aim of this work was to determine whether the cytotoxin of H. pylori detected in vitro stimulates an immune response in infected individuals. The ultimate goal is to determine whether the cytotoxin is produced in vivo during infection and, therefore, whether it may be relevant to gastroduodenal disease associated with H. pylori.

MATERIALS AND METHODS

Clinical data and sera. Clinical data, sera, and bacterial isolates were collected at Mt. Sinai Hospital, Toronto, Ontario, Canada. All procedures were approved by the University of Toronto Review Committee on the Use of Human Subjects. A total of 36 healthy, asymptomatic volunteers and 30 individuals with symptoms of dyspepsia who were referred for endoscopy were examined at the University of Toronto. A serum sample was obtained by routine venipuncture. Gastric tissue samples were obtained by biopsy at endoscopy, and macroscopic endoscopic findings were noted. Gastric biopsies were cultured for H. pylori and processed histologically for assessment of inflammation. More-detailed procedures for these analyses are described elsewhere (7).

ELISA. Serum antibody to H. pylori was determined by enzyme-linked immunosorbent assay (ELISA). A pool of four strains of H. pylori (133C, originally isolated in Peru; 60190, from England; Tx30A, from the United States; and 11638, from Australia) was used as the antigen. Broth-grown bacteria (11) were enumerated by a standard plate count technique and fixed in 0.25% formaldehyde in saline. Equal numbers of bacteria of each of the four strains were mixed together and adjusted to 1 × 10^8 bacteria per ml for addition to wells of a 96-well microdilution plate (5 × 10^7 bacteria per well).

A standard ELISA procedure was used to quantitate serum antibody to H. pylori. Serial twofold dilutions of each serum (1/100 to 1/6,400) were tested. Rabbit antisera to human immunoglobulin G (IgG) (γ-chain specific) conjugated to alkaline phosphatase (A-3150, Sigma Chemical Co.) was used as the probe for detecting specific IgG. Rabbit antisera to human IgA (α-chain specific) conjugated to alkaline phosphatase (A-3400, Sigma) was used as the probe for specific IgA.

Disodium p-nitrophenyl phosphate (104-105, Sigma) was the substrate. Optical density at 405 nm was read with a Biomek 1000 W automated workstation (Beckman Instruments, Inc.).

Test sera were run in triplicate. Three serum samples of individuals from whom H. pylori was cultured and three samples of individuals from whom H. pylori could not be cultured were included as controls for each assay. The inclusion of wells without test serum established the background optical density to be <0.100. An optical density value of >0.200 was considered positive for a given dilution of test serum. The highest dilution of a test serum yielding a positive result defined the titer. An IgG titer of ≥1/1,600 and an IgA titer of ≥1/400 were considered to be indicative of infection. Antibody titers indicative of infection were determined in previous studies with 50 human serum samples. Of the 50...
serum donors, 24 were infected by *H. pylori* as determined by cultivation of *H. pylori* from gastric tissue, observation of spiral bacilli in Gram-stained sections of gastric tissue (n = 7), or both (n = 17). By the above ELISA procedure, an IgG titer of ≥1/1,600 and an IgA titer of ≥1/400 were most predictive of infection in these 50 individuals.

Assays for cytotoxin and neutralization. Cytotoxin was assayed as previously described (9) except that HeLa cells (ATCC CCL2) were used instead of Intestine 407 cells. Briefly, broth culture filtrates of *H. pylori* were concentrated at least 10-fold by precipitation with 50% saturated ammonium sulfate. Dilutions of broth culture filtrate were incubated with HeLa cells (10⁴ cells per well in a 24-well plate) for 48 h at 37°C in an atmosphere containing 5% CO₂. Intracellular vacuolation in ≥50% of the HeLa cells in a well indicated the presence of the cytotoxin.

Antibody specific for the cytotoxin was detected by neutralization. The complement in test sera was inactivated by heating to 56°C for 30 min. Sera were then serially diluted twofold (undiluted to 1/32) in cell culture medium in 30-μl amounts. To each dilution was added 30 μl of a preparation of cytotoxin previously determined to have a titer of 1/16 to 1/32. Diluent only, rather than cytotoxin, was added to additional dilutions of serum as controls for the effects of sera on HeLa cells. The mixtures were incubated for 60 min at 37°C in an atmosphere containing 5% CO₂. Samples (50 μl) of each mixture were then added to wells with HeLa cells (1 × 10⁴ to 1.5 × 10⁴ cells per well) in equal amounts of culture medium in a 96-well plate. Following overnight incubation at 37°C in a humidified atmosphere containing 5% CO₂, wells were observed by phase-contrast microscopy for cytotoxic effects.

Each serum was tested on at least two occasions for neutralization of cytotoxin prepared from two different isolates of *H. pylori*. The neutralization titer was defined as the highest dilution of a serum completely neutralizing vacuolation. Wells containing cytotoxin without serum served as controls for HeLa cell vacuolation. In addition, at least two neutralizing and two nonneutralizing control sera were included in each assay. Neutralizing human sera contained antibody specific for *H. pylori*, as determined by ELISA, and demonstrated consistent neutralization of cytotoxin at titers of 1/8 to 1/64. Neutralizing rabbit sera were prepared by immunization with formalized *H. pylori* (9) and neutralized at a titer of 1/8. Nonneutralizing human sera did not contain antibody specific for *H. pylori* and consistently failed to neutralize cytotoxic activity (neutralization titer, <1/2).

RESULTS

**Patient population.** The asymptomatic volunteer population included 19 males and 17 females ranging from 20 to 56 years of age (mean, 29 years). The symptomatic patient population included 14 males and 16 females ranging from 22 to 71 years of age (mean, 46 years). *H. pylori* was cultured from 19 of the 66 individuals, including 4 asymptomatic and 15 symptomatic persons. Gastric colonization by *H. pylori* was associated with histological gastritis in every case.

**ELISA.** The value of serum antibody detection as an indicator of infection by *H. pylori* is shown in Table 1. The ELISA for serum IgG was 90% sensitive and 98% specific for infection. The positive predictive value was 94%, and the negative predictive value was 96%. The ELISA for serum IgA was 68% sensitive and 96% specific for infection. The positive predictive value was 87%, and the negative predictive value was 88%. The presence of specific IgG or IgA was 95% sensitive and 96% specific for infection. The positive predictive value was 90%, and the negative predictive value was 98%.

The results of IgG and IgA measurement were concordant for 58 of 68 (88%) sera tested. From seven sera, IgG but not IgA specific for *H. pylori* was detected. From two sera, IgA but not IgG specific for *H. pylori* was detected.

**Neutralization.** Of 66 serum samples tested, 14 neutralized the cytotoxin of *H. pylori*. Neutralization titers ranged from 1/2 to 1/64, with most sera (11 of 14) having neutralization titers between 1/2 and 1/8. Neutralization could not be determined for three of the sera because of their cytolytic effect on HeLa cells which precluded observation of intracellular vacuolation.

Antibody to the cytotoxin, as measured by neutralization, correlated well with the presence of antibody to the bacterium, measured by ELISA (Table 2). Of 19 serum samples, 12 (63%) with antibody to the bacterium contained antibody to the cytotoxin. Of 44 samples, 42 (95%) without antibody to the bacterium did not contain antibody to the cytotoxin. In the sera of two individuals from whom *H. pylori* was not cultured and in which antibody to the bacterium was not detected, antibody to the cytotoxin was detected.

The diagnostic value of antibody to the cytotoxin for infection is shown in Table 3. Detection of antibody specific for the cytotoxin, as measured by neutralization, was 67% sensitive and 96% specific for infection by *H. pylori*.

**Cytotoxin.** Production of cytotoxin in vitro by isolates from the patients was measured directly by assessing the ability of broth culture filtrate to induce vacuolation of HeLa cells. This was compared with the ability of serum from the same patient to neutralize a standard preparation of cytotoxin. Neutralization of cytotoxin by serum from a patient correlated poorly with production of cytotoxin by the isolate of that patient (Table 4). For 14 patients studied, concordance was 50%. Considering both measures, evidence for the production of cytotoxin in vivo was present for 11 of 14 patients (79%).

**TABLE 1.** Relationship of detection of antibody in serum and infection by *H. pylori*

<table>
<thead>
<tr>
<th>Infection by <em>H. pylori</em></th>
<th>No. of sera with antibody to <em>H. pylori</em></th>
<th>IgG</th>
<th>IgA</th>
<th>IgG or IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td>17</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>46</td>
<td>2</td>
</tr>
</tbody>
</table>

* +, *H. pylori* isolated from gastric tissue (n = 19); −, *H. pylori* not isolated (n = 47).

**TABLE 2.** Correlation of antibody to the cytotoxin and antibody to the bacterium in infection by *H. pylori*

<table>
<thead>
<tr>
<th>Antibody to the bacterium</th>
<th>No. of sera with antibody to the cytotoxin</th>
<th>+</th>
<th>−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td>12</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>−</td>
<td></td>
<td>2</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>14</td>
<td>49</td>
<td>63</td>
</tr>
</tbody>
</table>

* +, IgG or IgA specific for *H. pylori* detected in the serum of the patient by ELISA; −, specific antibody not detected.

* +, Antibody to the cytotoxin detected in the serum of the patient by neutralization; −, neutralizing antibody not detected.
TABLE 3. Relationship of antibody to the cytotoxin and infection by *H. pylori*

<table>
<thead>
<tr>
<th>Infection by <em>H. pylori</em></th>
<th>No. of sera with antibody to cytotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
</tr>
</tbody>
</table>

*a, +, *H. pylori* isolated from gastric tissue; −, *H. pylori* not isolated.

*b, +, Antibody to the cytotoxin detected in the serum of the patient by neutralization; −, neutralizing antibody not detected.

DISCUSSION

Detection of serum antibody specific for *H. pylori* was an accurate indicator of infection. Overall, measurement of specific IgG was more predictive than measurement of specific IgA and was as predictive as consideration of both IgG and IgA results was. These data support the value of a noninvasive test for infection by *H. pylori* based on the detection of serum antibody (6, 10, 12, 14).

The presence of antibody to the cytotoxin, measured by neutralization, correlated well with the presence of antibody to the bacterium, measured by ELISA. This is consistent with neutralization of cytotoxic activity by specific antibody, as opposed to destruction of the cytotoxin by a nonspecific mechanism, such as degradation by a serum protease. Neutralization of cytotoxin by the sera of individuals infected with *H. pylori* suggests that the cytotoxin is produced in vivo. It may, therefore, be involved in the production of disease associated with *H. pylori*. Correlation of cytotoxin with either the symptoms or clinical outcome of disease is of interest.

Antibody to the cytotoxin was a highly specific indicator of infection by *H. pylori*, although it was not a sensitive indicator of infection. Measurement of antibody to the cytotoxin was less predictive of infection than was measurement of antibody to the bacterium. These results might be expected on the basis of the finding that not all isolates produce the cytotoxin in vitro. The neutralization assay is subjective and takes longer to complete than the ELISA. In addition, some sera were not evaluable by neutralization because of their effects on HeLa cells. Detection of antibody to the cytotoxin offers no advantage over detection of antibody to the bacterium as an indicator of infection by *H. pylori*.

In 14 individuals studied, neutralization of the cytotoxin correlated poorly with production of the cytotoxin by the homologous isolate. Antibody to the cytotoxin was frequently detected in patients from whom a nontoxicogenic strain was isolated. This might be explained by a loss of the ability to produce cytotoxin upon cultivation in vitro, an insensitive assay for the cytotoxin, or coinfection by toxicogenic and nontoxicogenic isolates. The absence of antibody to the cytotoxin in a patient infected by a toxicogenic isolate of *H. pylori* was not observed in this group of patients.

Antibody to *H. pylori* and to the cytotoxin in serum was investigated in these studies. Secretory antibody specific for *H. pylori* in infected individuals has been demonstrated (15). Whether secretory antibody specific for the cytotoxin is also produced is not currently known.

The question of how best to measure involvement of cytotoxin in disease associated with *H. pylori* remains. Cytotoxin from some isolates of *H. pylori* is detectable only after concentration of the broth culture filtrate (9). This suggests that direct assay for the cytotoxin in vitro is not very sensitive. Demonstration of antibody to the cytotoxin by neutralization is an indirect measure of the involvement of cytotoxin in infection. Use of both approaches suggests that more isolates of *H. pylori* produce the cytotoxin than is suggested by direct assay alone. A sensitive molecular probe for the cytotoxin or for its structural gene is required to determine accurately the percentage of isolates producing the cytotoxin.

TABLE 4. Relationship of cytotoxin production by an isolate of *H. pylori* and neutralization of cytotoxin by serum from the same patient

<table>
<thead>
<tr>
<th>Production of cytotoxin by isolate of <em>H. pylori</em></th>
<th>No. of sera with antibody to the cytotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>−</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
</tbody>
</table>

*a, +, Concentrated broth culture filtrate of the isolate from the patient induced vacuolation of HeLa cells; −, no vacuolation observed.

*b, +, Antibody to the cytotoxin detected in the serum of the patient by neutralization; −, neutralizing antibody not detected.

LITERATURE CITED


