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Characterization of and Human Serologic Response to Proteins in *Helicobacter pylori* Broth Culture Supernatants with Vacuolizing Cytotoxin Activity

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***Helicobacter pylori* infection is strongly associated with histologic gastritis and peptic ulcer disease. Broth culture supernatants from a subset of *H. pylori* strains induce vacuolization in cultured cells, a phenomenon that has been attributed to cytotoxin activity. Concentrated culture supernatants from 15 of 28 (53.6%) *H. pylori* strains tested induced vacuolization in HeLa cells in titers ranging from 1:10 to 1:180. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of supernatants from these 28 strains and 2 control strains demonstrated an 82-kilodalton (kDa) protein band in 3 of 16 supernatants with vacuolizing activity, but in none of 14 supernatants without vacuolizing activity. By immunoblotting with human sera, a 128-kDa band was recognized in all 16 supernatants with vacuolizing activity, compared with 9 of 14 (64%) supernatants without vacuolizing activity ($P = 0.014$). Serologic recognition of the 128-kDa band in *H. pylori* culture supernatants was more prevalent among persons infected with vacuolizing *H. pylori* strains than among persons infected with nonvacuolizing strains, but the difference was not statistically significant (80 versus 45%; $P = 0.079$); human serologic recognition of the 82-kDa band was less common. The 128-kDa band was recognized by 100% of 31 serum samples from *H. pylori*-infected patients with duodenal ulcer disease, compared with 60.8% of 74 serum samples from *H. pylori*-infected persons without peptic ulcer disease ($P = 0.0001$). These data indicate that antigenic 128- and 82-kDa proteins are present in *H. pylori* broth culture supernatants with vacuolizing activity and that serologic responses to the 128-kDa protein are more prevalent among *H. pylori*-infected persons with duodenal ulceration than among infected persons without peptic ulceration.**

Since the initial isolation of *Helicobacter pylori* (formerly known as *Campylobacter pylori*) (11) from gastric mucosa in 1983 (17), numerous studies throughout the world have demonstrated a strong association between the presence of this organism and antral gastritis (1, 3, 5, 12). Most patients with gastric or duodenal ulceration are also infected with *H. pylori* (3, 5, 12, 15, 18, 25). An increasing body of evidence indicates that *H. pylori* causes gastritis rather than secondarily colonizing damaged mucosa (7); however, little is known about the mechanisms whereby the organism may cause inflammation and tissue injury. Histologic and immunofluorescent studies have rarely demonstrated invasion of the gastric mucosa by *H. pylori* (6, 27). Thus, extracellular toxins or metabolic products may play a role in inciting mucosal damage. Urease (20) and a protease that degrades gastric mucus (26) have been proposed as possible virulence determinants. In addition, Leunk et al. (16) have demonstrated that approximately half of *H. pylori* isolates produce cytotoxins that induce vacuolization in cultured cells. In one study, cytotoxin-producing *H. pylori* strains were isolated more frequently from patients with peptic ulcer disease than from patients with chronic gastritis only (9). We sought to identify the proteins that are associated with the vacuolizing cytotoxin of *H. pylori* by comparing the proteins that were present in culture supernatants of cytotoxin-producing and non-cytotoxin-producing *H. pylori* strains. We then sought to determine whether human serologic recognition of cytotoxin-associated proteins was more prevalent among *H. pylori*-infected patients with peptic ulcer disease than among *H. pylori*-infected persons with histologic gastritis only.

MATERIALS AND METHODS

Source of strains. *H. pylori* 60190 and Tx30a, which have been previously reported to be cytotoxin-producing (Tox^+) and non-cytotoxin-producing (Tox^-), respectively (16), were obtained from Robert Leunk (Miami Valley Laboratories, The Procter & Gamble Co., Cincinnati, Ohio). Twenty-eight strains were clinical *H. pylori* isolates from dyspeptic patients treated at the Veterans Administration Medical Center (Denver, Colorado), Bangkok Christian Hospital (Bangkok, Thailand), or New York Medical College (Valhalla, N.Y.) (Table 1). All strains used in this study were identified as *H. pylori* by Gram stain morphology and by positive urease, oxidase, and catalase tests (19). The strains were maintained frozen at −70°C in brucella broth containing 15% glycerol.

Assay for cytotoxicity. *H. pylori* strains were cultured at 37°C in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) containing 5% defined fetal bovine serum (Hyclone, Logan, Utah). Broth cultures were incubated in an atmosphere of 7.5% CO_2–7.5% H_2–5% O_2–80% N_2 on a gyratory shaker at 100 rpm for 96 h. Cultures were centrifuged at 3,000 × *g* for 15 min, and the cell-free supernatants were stored at −70°C. After thawing, supernatants were concentrated 30-fold by using a 100-kilodalton (kDa) Omega

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TABLE 1. Source of *H. pylori* strains and human sera used in this study

Specimen	Source	No. of isolates
Paired strains and sera[a]	Dyspeptic patients, Denver, Colo.	19
	Dyspeptic patients, Bangkok, Thailand	6
	Dyspeptic patients, Valhalla, N.Y.	3
Sera alone (Los Angeles, Calif.)	*H. pylori*-infected persons	
	Patients with duodenal ulcer	31
	Patients with gastric ulcer	12
	Patients with non-ulcer dyspepsia	25
	Asymptomatic volunteers	49
	Uninfected volunteers	49

[a] For each patient, both serum and the *H. pylori* isolate were available.

series ultrafiltration membrane (Pharmacia Fine Chemicals, Piscataway, N.J.), and retentates were sterilized by passage through a 0.22-μm-pore-size filter. These concentrated culture supernatants (CCSs) were incubated with HeLa cells (obtained from Allison O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md.) in twofold dilutions from 1:10 to 1:320 as described previously (16), except that toxicity assays were performed in a total volume of 100 μl in 96-well microtiter plates (Falcon; Becton Dickinson and Co., Lincoln Park, N.J.). Cells were observed for 48 h for the development of intracellular vacuolization. Wells in which 50% or more cells were vacuolated were defined as showing a cytotoxic effect.

Source of human sera. Serum samples were obtained from 204 consenting individuals at the time of upper gastrointestinal endoscopy and were stored at −20°C. Both sera and *H. pylori* isolates were available for the 28 patients described above; in addition, 176 serum samples were obtained from individuals who were treated at the University of Southern California (Los Angeles, Calif.) (Table 1). *H. pylori*-infected patients were defined by either a positive culture for *H. pylori* or visualization of gastric *Campylobacter*-like organisms in endoscopic biopsy specimens; most patients met both criteria, but patients meeting only one criterion were also considered infected. All sera were tested for reactivity with a pool of sonicated *H. pylori* strains in an enzyme-linked immunosorbent assay (ELISA), as described previously (22, 23). Among the 176 Los Angeles patients, 3 patients with duodenal ulceration, 2 patients with gastric ulceration, 0 patients with non-ulcer dyspepsia, and 5 asymptomatic individuals were infected with *H. pylori* based on culture results but were seronegative for immunoglobulin G (IgG) antibodies in the ELISA, and were therefore excluded from further analysis. The characteristics of the remaining 166 Los Angeles patients are presented in Table 1. Of the 98 asymptomatic volunteers who underwent endoscopy, 49 were infected with *H. pylori* and 49 were uninfected. All 49 uninfected volunteers studied had cultures and stains of endoscopic biopsy specimens that failed to demonstrate *H. pylori* infection and were seronegative by the ELISA. Of the 49 *H. pylori*-infected volunteers studied, 47 (95.9%) had histologic gastritis, whereas no inflammation was present in the 49 volunteers without infection. A total of 26 of the 31 (83.9%) patients with duodenal ulcers and all of the patients with gastric ulcers had active ulceration visualized by endoscopy at the time of serum collection; previous duodenal ulceration had healed in 5 patients. Serum sample M1 was from an asymptomatic person whose sera have been consistently positive for IgG and IgA antibodies to *H. pylori* since 1978.

Histologic characterization of gastric biopsy specimens. Endoscopic biopsy specimens from the 49 asymptomatic *H. pylori*-infected volunteers described above were classified as demonstrating chronic gastritis or active chronic gastritis by previously published criteria (10). Biopsy specimens from the 19 *H. pylori*-infected patients in Denver were examined, and the degree of inflammation was classified by the criteria of Hazell et al. (13).

Preparation of antisera. Adult female New Zealand White rabbits were immunized with antigens from *H. pylori* 60190 (Tox^+) or Tx30a (Tox^-). Supernatant preparations of these strains were prepared by concentrating broth culture supernatants 10-fold with a 100-kDa molecular weight cutoff ultrafiltration membrane and by precipitating them with 50% (vol/vol) ammonium sulfate. The precipitate was suspended in and dialyzed overnight at 4°C against phosphate-buffered saline (pH 7.2) by using a 12- to 14-kDa molecular weight cutoff membrane tubing (Fisher Scientific Co., Pittsburgh, Pa.). Formalinized whole-cell preparations were prepared by suspending water-washed bacteria in 0.6% Formalin at a McFarland no. 5 density for 3 days. French press preparations were prepared from water-washed whole cells by using a French pressure cell (Aminco; SLM Instruments, Urbana, Ill.); the preparations were centrifuged at 3,000 × *g* for 15 min, and the supernatants were saved. The protein concentrations of the concentrated supernatants, formalinized whole cells, and French press preparations were 25, 0.15, and 0.3 μg/μl, respectively. Rabbits were immunized on both days 0 and 21 with four 0.5-ml subcutaneous injections of preparations consisting of 10 parts antigen, 4 parts hexadecane (Eastman Kodak Co., Rochester, N.Y.) and 1 part glyceryl monooleate (Pfaltz and Bauer, Inc., Watebury, Conn.); formalinized whole cells were used as immunogens without hexadecane or glyceryl monooleate. Two subcutaneous injections with 0.1 ml of undiluted antigen were given on both days 28 and 29, followed by a 1-ml intraperitoneal injection on day 30. Serum was collected 6 days later and was stored at −20°C. The reactivity of sera with antigens used for immunization was determined by ELISA (22). Sera from rabbits immunized with supernatants yielded optical density values that were more than 1,000-fold higher than those of preimmune sera when they were reacted with the respective supernatant preparations used as immunogens.

SDS-PAGE and immunoblotting with human and rabbit sera. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (4) by using a 4.5% stacking gel and a 7.0% separating gel. Samples containing 3 μg of protein were applied to each gel lane. After electrophoresis, gels were fixed and proteins were resolved by the modified silver stain method of Oakley et al. (21). Western blotting (immunoblotting) was performed as described previously (24). Portions of CCS containing 500 μg of protein were diluted in sample buffer and were layered onto the surface of a polyacrylamide gel in a Mini-PROTEAN II slab cell (Bio-Rad Laboratories, Richmond, Calif.). Following electrophoresis, proteins were transferred to nitrocellulose paper by electroblotting for 2.5 h at 300 mA. After nonspecific binding was blocked with milk-borate buffer, the nitrocellulose paper was incubated at room temperature for 1 h with 1:100 dilutions of serum samples. Peroxidase conjugates of goat anti-human IgG, IgA, and IgM (Tago, Inc., Burlingame, Calif.), in dilutions of 1:2,500, 1:1,000, and 1:5,000, respectively, or peroxidase-conjugated swine anti-rabbit immunoglobulins diluted

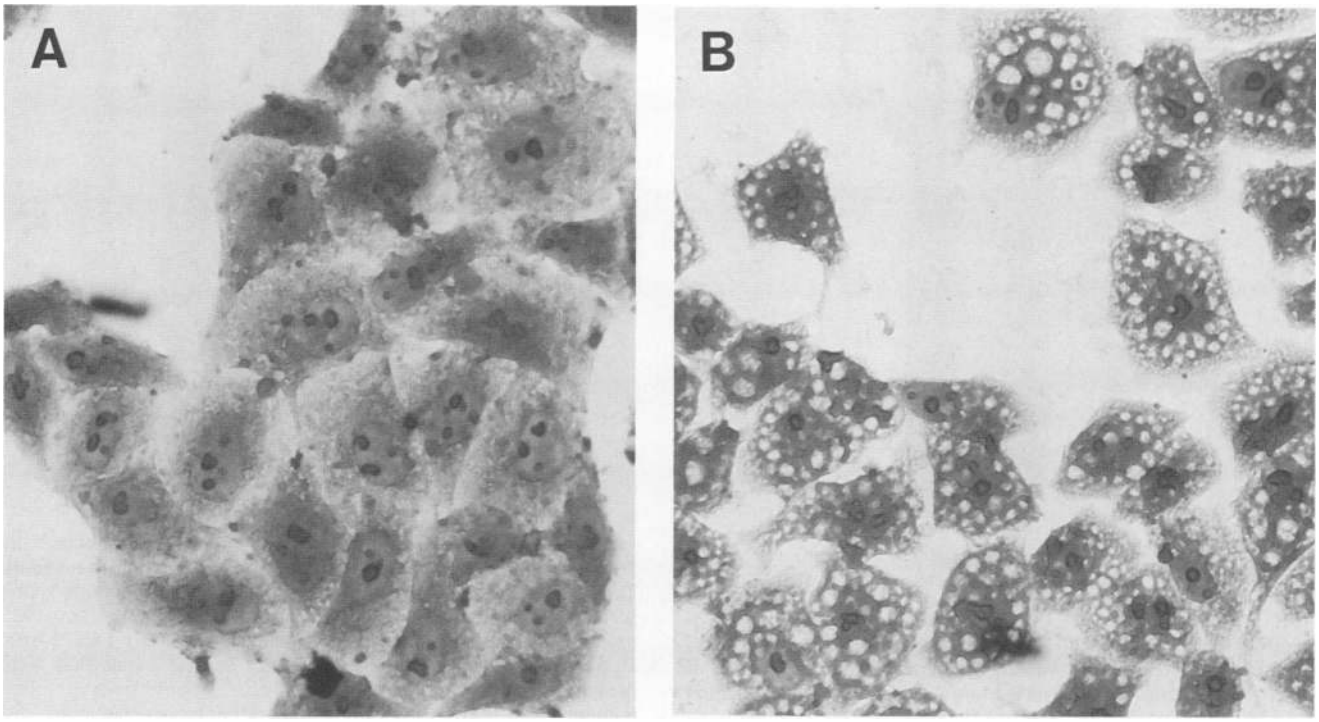

FIG. 1. Crystal violet-stained HeLa cells incubated for 48 h with concentrated *H. pylori* culture supernatants from the Tox$^-$ strain Tx30a (A) and the Tox$^+$ strain 60190 (B), as described in the text. Prominent intracellular vacuolization occurred in response to *H. pylori* cytotoxin. Magnification, ×250.

1:1,000 (Dako, Copenhagen, Denmark) were used in initial studies. The use of Tris-saline blotting buffer and an alkaline phosphatase conjugate of goat anti-human IgG diluted 1:3,000 (Miles-Yeda Ltd., Rehovot, Israel) produced equivalent results with clearer resolution of bands and was used subsequently (2). All serum samples studied were tested in duplicate.

Pronase treatment of concentrated culture supernatants. Pronase (Calbiochem-Behring, La Jolla, Calif.) was diluted to 0.25 mg/ml in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2) containing 5 mM $CaCl_2$. Equal volumes of CCS from strain 60190 and pronase in buffer were rotated at 37°C for 3 h. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was then added in a final concentration of 33 mg/ml to stop the reaction. CCS was incubated in buffer without pronase as a control.

Statistical methods. Proportions were compared by using a one-tailed Fisher exact test or chi-squared analysis. Distributions of optical density values were compared by using a two-tailed Student's *t* test for independent variables.

RESULTS

Cytotoxin production by *H. pylori*. We tested 28 clinical *H. pylori* isolates and the two control strains for the production of vacuolizing cytotoxins. As previously reported (16), CCS from the Tox$^+$ control strain 60190 produced vacuolization of HeLa cells in a titer of 1:320, whereas CCS from the Tox$^-$ strain Tx30a did not cause vacuolization in any dilution tested (Fig. 1). Uninoculated brucella broth containing 5% fetal bovine serum, which was processed in the same manner as culture supernatants, also failed to induce vacuolization in HeLa cells. Unconcentrated culture supernatant from strain 60190 produced vacuolization in a titer of 1:20. After the concentration of CCS from strain 60190 by ultrafiltration, the retentate induced vacuolization in HeLa cells, but the filtrate did not. Concentrated culture supernatants from 15 of the 28 (53.6%) clinical *H. pylori* isolates tested produced vacuolization in titers ranging from 1:10 to 1:160. The median titer of vacuolization produced by CCS from these 15 cytotoxigenic strains was 1:40. Thus, using a new group of *H. pylori* strains, we found vacuolizing cytotoxin activity in a proportion of supernatants similar to that reported by Leunk et al. (16).

Relationship between gastric histology and cytotoxin production by *H. pylori* isolates. To determine whether cytotoxin production by *H. pylori* was associated with specific gastric histology, we correlated *H. pylori* cytotoxin production with the type of gastric inflammation present in 19 *H. pylori*-infected patients from Denver. Thirteen patients had active or active chronic histologic gastritis, five patients had chronic gastritis only, and one patient had no detectable gastric inflammation. Tox$^+$ *H. pylori* isolates were cultured from five of the patients with active gastritis, two of the patients with chronic gastritis only, and the one patient without detectable gastric inflammation. The gastritis scores of patients infected with Tox$^+$ and Tox$^-$ *H. pylori* isolates were not significantly different. Therefore, there was no obvious association between the cytotoxin activity of the *H. pylori* isolate and the histologic characteristics of the involved tissues.

Detection of an 82-kDa band in concentrated culture supernatants from Tox$^+$ strains by SDS-PAGE. To identify specific bacterial elements associated with vacuolizing activity, we compared the protein profiles of Tox$^+$ and Tox$^-$ *H. pylori* supernatants by SDS-PAGE and silver staining. We studied

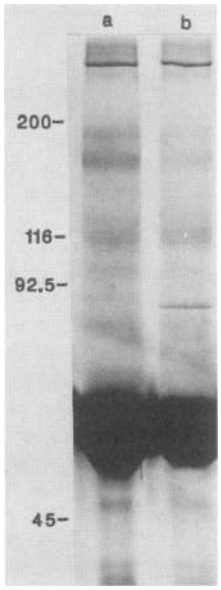

FIG. 2. Silver stain following SDS-PAGE on a 7.0% polyacrylamide gel of Tox$^-$ CCS from *H. pylori* Tx30a (lane a) and Tox$^+$ CCS from strain 60190 (lane b). An 82-kDa band was present in CCS of the Tox$^+$ strain but not the Tox$^-$ strain. There was no visualization of a 128-kDa band in either preparation. The darkly staining band corresponds to albumin in the culture medium. Numbers to the left of the gel are in kilodaltons.

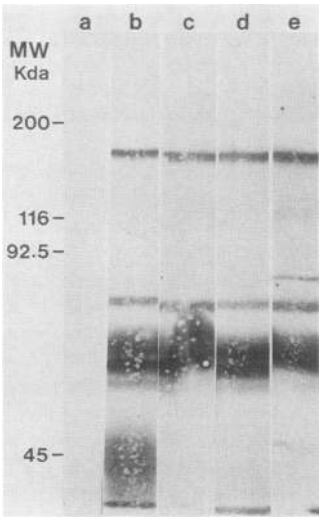

FIG. 3. Immunoblots of concentrated culture supernatants from *H. pylori* strains 60190 (lanes a, c, and e) and Tx30a (lanes b and d), Tox$^+$ and Tox$^-$, respectively with preimmune and immune rabbit serum. Preimmune serum (lane a) failed to recognize any CCS proteins. Serum from a rabbit immunized with Tox$^-$ CCS from strain Tx30a (lanes b and c) recognized several bands that were present in both Tox$^-$ and Tox$^+$ CCSs. Serum from a rabbit immunized with Tox$^+$ CCS from strain 60190 recognized an 82-kDa band in Tox$^+$ CCS (lane e) that was absent from Tox$^-$ CCS (lane d). The 82-kDa band is thus a potential cytotoxin-associated protein.

supernatants from 30 *H. pylori* strains (Table 1), including the two control strains. Of the 16 strains that produced vacuolizing cytotoxins, CCS from the control strain 60190 and CCS from two other strains contained an 82-kDa band (Fig. 2). In contrast, the 82-kDa band was not visualized in any of the 14 CCSs without vacuolizing activity ($P = 0.138$) and was not visualized in a concentrated preparation of uninoculated brucella broth containing 5% fetal bovine serum. Thus, there was not a strong association between the presence of the 82-kDa band and cytotoxin activity.

Recognition of an 82-kDa band by immune rabbit sera. To examine further the antigenic differences between Tox$^+$ and Tox$^-$ strains, we immunized rabbits with supernatant from the control *H. pylori* strain 60190 (Tox$^+$) or strain Tx30a (Tox$^-$). CCS from each of these strains was then immunoblotted with preimmune and immune rabbit sera. As shown in Fig. 3, serum from the rabbit immunized with Tox$^+$ CCS recognized an 82-kDa band in CCS from the Tox$^+$ strain 60190 but not in CCS from the Tox$^-$ strain Tx30a. Thus, CCS from the Tox$^+$ strain (60190) contained an 82-kDa protein that was absent from Tox$^-$ CCS (Tx30a) and that was antigenic to rabbits. The 82-kDa band was also recognized in CCS from strain 60190 by serum from a rabbit immunized with a French press preparation of strain 60190, but not by serum from a rabbit immunized with the corresponding preparation of strain Tx30a. Serum from a rabbit that was immunized with a formalinized whole-cell preparation of strain 60190 weakly recognized the 82-kDa band in CCS from strain 60190.

Recognition of 128- and 82-kDa bands by human sera. To identify additional bacterial elements associated with vacuolizing activity, CCS from Tox$^+$ strain 60190 or Tox$^-$ strain Tx30a was immunoblotted with serum from each of the 28 *H. pylori*-infected patients for whom *H. pylori* isolates were available. Of the 28 serum samples, 18 (64.3%) contained IgG antibodies that recognized a 128-kDa band in CCS from the Tox$^+$ strain. No 128-kDa band was recognized in CCS from the Tox$^-$ strain by any of the 28 serum samples, suggesting that this band was not present (Fig. 4). In addition, no band migrating at 128 kDa was recognized in a concentrated preparation of uninoculated brucella broth containing 5% fetal bovine serum. Of the 18 serum samples described above that recognized the 128-kDa band, 4 serum samples also recognized the 82-kDa band in CCS from the Tox$^+$ strain. Several bands of lower molecular weight were recognized in CCSs from both the Tox$^+$ and Tox$^-$ strains. Therefore, human sera recognized a 128-kDa band in CCS from a Tox$^+$ strain but not in CCS from a Tox$^-$ strain. To determine which class of antibodies best recognized the 128-kDa band, we compared IgG, IgA, and IgM recognition of this band by three sera. The 128-kDa band in CCS from strain 60190 was strongly recognized by IgG antibodies in all three serum samples, was weakly recognized by IgA antibodies in one serum sample, and was not recognized by IgM antibodies in any of the serum samples. Subsequent studies therefore investigated only serum IgG recognition of the 128-kDa band.

Presence of immunoreactive 128- and 82-kDa bands in CCSs from other strains. To determine whether 128- and 82-kDa bands were present in CCSs from other Tox$^+$ strains as well, CCSs from an additional two Tox$^+$ and two Tox$^-$ *H. pylori* strains were each immunoblotted with human sera. The 128- and 82-kDa bands were recognized in both of the Tox$^+$ CCSs but in neither of the Tox$^-$ CCSs by sera from persons infected with Tox$^+$ *H. pylori* strains (Table 2). These findings provided evidence that 128- and 82-kDa bands were commonly present in *H. pylori* culture supernatants with vacuolizing activity.

Association between vacuolizing cytotoxin activity and the presence of 128-kDa bands in *H. pylori* culture supernatants. To clarify further the relationship between vacuolizing cyto-

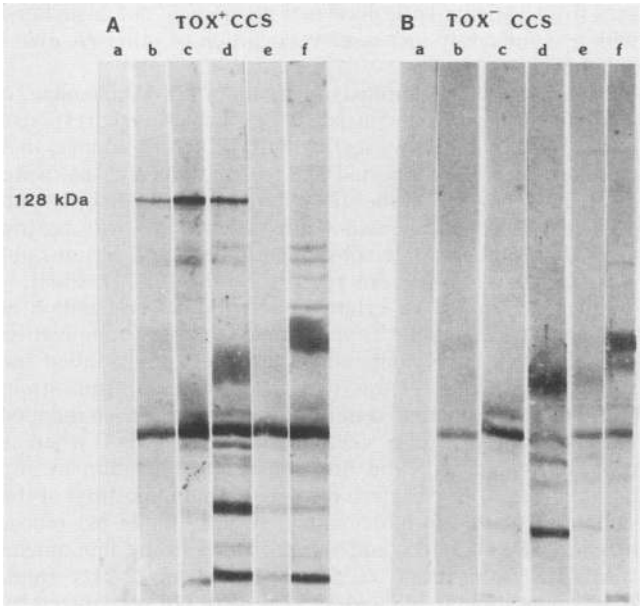

FIG. 4. Immunoblots of concentrated culture supernatants from Tox+ *H. pylori* 60190 (A) and Tox- *H. pylori* Tx30a (B) with 1:100 dilutions of human sera. The second antibody used was peroxidase-conjugated goat anti-human IgG diluted 1:2,500. The sera tested were from persons who were not infected with *H. pylori* (lanes a), those who were infected with Tox+ *H. pylori* (lanes b, c, and d), or those who were infected with Tox- *H. pylori* (lanes e and f). A 128-kDa band was recognized in Tox+ CCS by sera from the persons infected with Tox+ strains. The 82-kDa band was not recognized by any of the sera tested in this immunoblot.

toxin activity in *H. pylori* culture supernatants and the presence of 128-kDa bands, we immunoblotted CCSs from each of the 28 *H. pylori* clinical isolates and the two control strains with serum sample M1. This serum sample from a *H. pylori*-infected person was chosen because of its strong reactivity with the 128-kDa band in CCS from the control Tox+ strain. Serum sample M1 recognized a 128-kDa band in all 16 of the CCSs with vacuolizing cytotoxin activity and in 9 (64%) of the 14 CCSs without vacuolizing cytotoxin activity ($P = 0.014$). Thus, culture supernatants from Tox+ strains contained the 128-kDa band more frequently than did supernatants from Tox- strains. The 128-kDa protein was therefore a potential marker for vacuolizing cytotoxin activity.

Relationship between serologic recognition of 128- and 82-kDa bands and cytotoxicity of patient isolates. We next studied the relationship between serologic recognition of 128- and 82-kDa bands and the cytotoxin phenotype of the *H. pylori* strains with which persons were infected. Of the 28 patients for whom both sera and *H. pylori* isolates were available, 2 patients known to be infected with the human immunodeficiency virus were excluded from this analysis. Sera from 12 of 15 (80%) persons infected with Tox+ strains recognized 128-kDa bands in CCS from the control Tox+ strain, as did 5 of the 11 (45%) serum samples from persons infected with apparently Tox- strains ($P = 0.079$). Therefore, serologic recognition of the 128-kDa band was more common among patients infected with Tox+ strains than among those infected with Tox- strains, but the difference was not statistically significant. Two patients infected with Tox+ *H. pylori* whose sera did not recognize the 128-kDa band were from Thailand; the third such patient had renal insufficiency and had undergone recent surgery for an abdominal aortic aneurysm. Sera from four patients recognized the 82-kDa band, in addition to the 128-kDa band; three of these persons were infected with Tox+ strains and one was infected with a Tox- strain. Subsequent analysis was limited to the 128-kDa band, since its recognition by human sera was stronger and more consistently reproducible than was recognition of the 82-kDa band.

Relationship between gastric histology and serologic recognition of 128-kDa bands. To determine whether serologic recognition of the 128-kDa band was associated with specific gastric histology, we correlated such recognition with the type of gastric inflammation present in 49 asymptomatic *H. pylori*-infected persons from Los Angeles. Of the 49 persons, 30 had active chronic gastritis, 17 had chronic gastritis, and 2 had normal gastric histology. Sera from 20 of the 30 (66.7%) persons with active chronic gastritis and 8 of the 17 (47.1%) persons with chronic gastritis recognized the 128-kDa band in CCS from strain 60190 ($P = 0.31$). Thus, gastric histology and recognition of the 128-kDa band were not closely related.

TABLE 2. Recognition of 128- and 82-kDa bands in Tox+ and Tox- *H. pylori* culture supernatants by sera from *H. pylori*-infected persons[a]

Serum sample	Bands (kDa) recognized by the indicated sera in concentrated culture supernatants from the specified strains:					
	60190 (Tox+)	87-199 (Tox+)	87-29 (Tox+)	86-313 (Tox-)	86-385 (Tox-)	Tx30a (Tox-)
87-342 (patient infected with Tox+ strain 87-199)	128, 82	128, 82	82	None	None	None
87-262 (patient infected with Tox+ strain 87-29)	128	128, 82	82	None	None	None
Immune serum from rabbit immunized with CCS from strain 60190	82	82	82	None	None	None
Immune serum from rabbit immunized with CCS from strain Tx30a	None	None	None	None	None	None
87-56 (patient infected with Tox- strain 86-313)	None	None	None	None	None	None
87-75 (patient infected with Tox- strain 86-385)	None	None	None	None	None	None
87-241 (uninfected patient)	None	None	None	None	None	None

[a] Human sera were used as probes to detect cytotoxin-associated proteins in concentrated culture supernatants of six *H. pylori* strains. Bands migrating at 128 and 82 kDa were recognized in concentrated supernatants of Tox+ strains by sera from patients infected with Tox+ strains.

TABLE 3. Serum IgG recognition of a 128-kDa band in culture supernatant from *H. pylori* 60190 by 166 persons with defined gastroduodenal pathology[a]

Lesion	Gastrointestinal symptoms	*H. pylori* culture	*H. pylori* serology[b]	No.	No. (%) of serum samples that recognized 128-kDa band
None	No	−	−	49	3 (6.1)
Gastritis	No	+	+	49	30 (61.2)
Gastritis	Yes	+	+	25	15 (60)
Duodenal ulcer	Yes	+	+	31	31 (100)
Gastric ulcer	Yes	+	+	12	10 (83.3)

[a] Concentrated supernatant from *H. pylori* 60190 was immunoblotted with sera from persons with the indicated lesions and symptoms. A 128-kDa band was recognized by sera from patients with duodenal ulcers significantly more frequently than by sera from infected persons without ulcer disease (100 versus 60.8%; $P = 0.0001$).

[b] Sera were tested for reactivity with a pool of sonicated *H. pylori* strains in an ELISA. The optical density threshold for IgG positivity was 0.91, as described previously (23).

IgG antibody in serum to 128-kDa bands in specific gastroduodenal disease states. We next studied the prevalence of serologic recognition of the 128-kDa band among patients with defined gastroduodenal disease states. CCS from the control Tox^+ strain 60190 was immunoblotted with sera from 117 *H. pylori*-infected persons and 49 uninfected volunteers from Los Angeles (Table 1). The prevalence of reactivity with the 128-kDa band was then determined for sera from uninfected persons; sera from asymptomatic infected volunteers; and sera from infected patients with duodenal ulceration, gastric ulceration, and non-ulcer dyspepsia (Table 3). The 128-kDa band was recognized by sera from infected patients significantly more frequently than by sera from uninfected persons (73.5 versus 6.1%; $P < 0.0001$). Sera from *H. pylori*-infected patients with duodenal ulcer disease recognized the 128-kDa band significantly more frequently than did sera from infected persons without peptic ulceration, regardless of their symptomatic status (100 versus 60.8%; $P = 0.0001$). In contrast to the universal recognition of the 128-kDa band by sera from patients with duodenal ulcer disease, sera from 2 of the 12 *H. pylori*-infected patients with gastric ulcers failed to recognize the 128-kDa band. These data indicated that serologic recognition of the 128-kDa band was more common in *H. pylori*-infected persons than in uninfected persons and that sera from *H. pylori*-infected patients with duodenal ulcer disease recognized this band more frequently than did sera from infected persons without peptic ulcer disease.

Reactivity of sera with *H. pylori* antigens in an ELISA. We next examined whether the high prevalence of serologic recognition of the 128-kDa band among patients with duodenal ulcers was related to an overall hyperresponsiveness to *H. pylori* antigens. For each of the patient groups listed in Table 3, we determined the mean IgG reactivity in serum with a pool of sonicates from five *H. pylori* strains; these data were previously determined by ELISA (23, 24). The mean optical densities (optical densities ± standard errors of the mean) produced by each group of sera were as follows: duodenal ulcer patients, 1.158 ± 0.058; gastric ulcer patients, 1.305 ± 0.144; asymptomatic infected persons, 1.339 ± 0.084; and uninfected volunteers, 0.177 ± 0.024. The mean optical densities of sera from *H. pylori*-infected patients without ulcers and sera from infected patients with duodenal ulcers were not significantly different ($P = 0.072$). Thus, the more frequent recognition of the 128-kDa band by sera from patients with duodenal ulcers was not associated with a significantly increased recognition of other *H. pylori* antigens.

Persistence of IgG antibody in serum to 128-kDa bands. To study the duration and consistency of the serologic response to the 128-kDa band, we tested multiple serum samples that were collected over a period of 2 to 5 months and that were available from 12 of the *H. pylori*-infected persons with duodenal ulcer disease and 4 infected persons with gastric ulcers. All initial and 20 subsequently collected serum samples from these 16 persons recognized the 128-kDa band.

Effect of pronase on cytotoxin activity and recognition of 128- and 82-kDa bands. To determine whether vacuolization of HeLa cells was mediated by a protein, we studied the effects of pronase on this phenomenon. CCS from strain 60190 that was incubated in buffer without pronase induced vacuolization in HeLa cells in a titer of 1:80, whereas pronase-treated CCS did not induce vacuolization in any dilution tested. By Western blotting (immunoblotting) of the sample incubated in buffer alone, serum sample M1 recognized a 128-kDa band, and serum from a rabbit immunized with CCS from strain 60190 recognized an 82-kDa band. Bands migrating at 128 and 82 kDa were not recognized by the respective sera in the pronase-treated preparation. These results indicate that a protease could abolish the vacuolizing activity in CCS and abolish the antigenicity of the 128- and 82-kDa bands.

DISCUSSION

The studies presented here have confirmed the induction of vacuolization in HeLa cells by *H. pylori* culture supernatants. The proportion of strains with vacuolizing activity in our study was similar to the 55% and 30 to 67% frequencies reported in the original and subsequent reports (9, 16). The mechanism whereby *H. pylori* supernatants induce vacuolization in cultured cells is not known, but Leunk et al. (16) have suggested that a cytotoxin is present. The relationship between the vacuolizing cytotoxin activity in *H. pylori* supernatants and other reported cytolethal toxins of *H. pylori* has not been defined (14).

Neutralization of vacuolizing cytotoxin activity in vitro by sera from patients infected with *H. pylori* suggests that the vacuolizing cytotoxin is active in vivo (D. Morgan, S. Krakowka, K. Eaton, and R. Leunk, Abstr. Int. Workshop on *Campylobacter pylori* infections, Bordeaux, France, 1988; A. Simor, R. Leunk, D. Low, D. Gregson, and D. Morgan, Abstr. Vth Int. Workshop on *Campylobacter* infections, Puerto Vallarta, Mexico, 1989). A functional role for the vacuolizing cytotoxin of *H. pylori* has also been suggested by the experimental infection of gnotobiotic piglets; toxigenic *H. pylori* colonized and induced gastric inflammation in gnotobiotic piglets, whereas a nontoxigenic strain failed to colonize or induce inflammation (8). Vacuolization has been observed in gastric epithelial cells of gnotobiotic piglets that were experimentally infected with toxigenic *H. pylori* (8), as well as in the gastric epithelial cells of humans infected with *H. pylori* (27).

To identify proteins associated with vacuolizing cytotoxin activity, we compared the proteins present in culture supernatants from Tox^+ and Tox^- strains. Supernatants from Tox^+ and Tox^- strains shared multiple bands, but the presence of 128- and 82-kDa bands was associated with vacuolizing activity. Treatment with a protease abolished the vacuolizing activity in supernatants and also eliminated the antigenicity of the 128- and 82-kDa bands. Although

these proteins may be useful markers for the presence of cytotoxin activity, the purification of the vacuolizing cytotoxin will be required to ascribe specific activity to these proteins or other elements synthesized by *H. pylori*.

The relationship, if any, between the 128- and 82-kDa bands is not yet defined. The 82-kDa band in Tox^+ CCS was visualized by both silver staining and immunoblotting, whereas the 128-kDa band was recognized only by immunoblotting. The 128-kDa protein may thus be present in concentrations that are too low to be detected by silver staining. The 128-kDa band was strongly recognized by sera from the majority of humans infected with *H. pylori*, whereas the 82-kDa band was recognized less frequently. Conversely, serum from a rabbit immunized with supernatant from a toxigenic *H. pylori* strain recognized the 82-kDa band but not the 128-kDa band. These differences between human and rabbit serologic responses may have resulted from the different routes of antigenic presentation. Alternatively, protein synthesis and processing by *H. pylori* in culture media may be different from that which occurs in vivo. Further investigation may demonstrate that the 82-kDa band is a degradation product or a subunit of the 128-kDa band, or that both bands are degradation products of a larger protein.

We studied recognition of the 128-kDa protein by sera collected from persons with defined gastroduodenal pathology. Sera from persons with *H. pylori* infection recognized the 128-kDa band significantly more frequently than did sera from uninfected persons. Sera from *H. pylori*-infected patients with duodenal ulcer disease universally recognized the 128-kDa band, whereas recognition was significantly less frequent by sera from infected persons with gastritis only. The latter observation may be related either to differences in the *H. pylori* strains with which these patients were infected or to differences in the host responses to *H. pylori* infection. There was not a significant difference in the reactivities of the sera from these two groups of patients with a pool of *H. pylori* antigens, suggesting that the host responses of these patients were similar.

A recent Italian study comparing vacuolizing cytotoxin production by *H. pylori* isolates from patients with peptic ulcer disease and isolates from patients with chronic gastritis only showed that isolates from the ulcer patients produced vacuolizing cytotoxins more frequently than did isolates from the patients without ulcers (66.6 versus 30.1%) (9). The serologic data presented in our study, based on the use of an antigenic 128-kDa protein as a marker for vacuolizing cytotoxin activity, also suggest that duodenal ulcer disease may be associated with cytotoxigenic *H. pylori* infection. Because the *H. pylori* isolates were no longer available from most patients presented in our study, we were unable to test this hypothesis directly. Further studies are required to clarify the relationship between vacuolizing cytotoxin production by *H. pylori* and the occurrence of peptic ulcer disease.

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