JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1203–1205 Vol. 33, No. 5
0095-1137/95/$04.00+0

Cytotoxin Production by *Helicobacter pylori* from Patients with Upper Gastrointestinal Tract Diseases

WEE TEE,[1]* JOHN R. LAMBERT,[2] AND BRIAN DWYER[1]

Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital, Fairfield,[1] and Gastroenterology Research Group, Mornington Peninsula Hospital, Frankston,[2] Victoria, Australia

Received 17 October 1994/Returned for modification 7 December 1994/Accepted 31 January 1995

A cytotoxin produced by some *Helicobacter pylori* strains has recently been identified. The cytotoxin induces intracellular vacuolization of cultured cells. The aim of the present study was to examine the frequency of occurrence of cytotoxin-producing strains of *H. pylori* from subjects with upper gastrointestinal disease including nonulcer dyspepsia, gastric and duodenal ulcer disease, gastroesophageal reflux disease, and gastric cancer. Broth culture filtrates of clinical isolates of *H. pylori* recovered from 175 patients were used to inoculate Vero and HeLa cell monolayers for the detection of vacuolating cytotoxin activity. The results obtained demonstrated that the highest percentage of strains producing cytotoxin were found in subjects with peptic ulcer disease (gastric ulcer, 65%; duodenal ulcer, 66%; $P < 0.01$ compared with nonulcer dyspepsia, 38%). Of the 11 patients with gastroesophageal reflux disease, 4 of 5 patients in this group who had esophageal ulcers, were found to be infected with strains that produced cytotoxin. Three of the four patients with carcinoma of the stomach were also found to be infected with cytotoxic strains of *H. pylori*. With increasing severity of mucosal damage in subjects with a normal upper gastrointestinal tract, macroscopic gastritis, duodenitis, and peptic ulceration, there were corresponding increase in the proportion of strains producing cytotoxin; these increases were 32, 46, 50, and 66%, respectively. *H. pylori* strains from subjects with ulcer disease commonly produced vacuolating cytotoxin, suggesting that it may be a virulence factor in the pathogenesis of peptic ulcer disease.

Considerable amounts of data in the literature demonstrate the strong association of *Helicobacter pylori* infection with histologic gastritis and peptic ulcer disease, but the exact nature or mechanism which *H. pylori* may cause inflammation or tissue injury is not well understood. *H. pylori* resides in gastric-type epithelium within the overlying mucous gel and in gastric glands, with invasion of the gastric mucosa by this organism rarely being demonstrated (1, 2). It has been suggested that extracellular materials or metabolic products elaborated by the organism, such as urease, protease, adhesins, cytotoxins, and mediators of inflammation, may play a role in inciting mucosal damage.

Leunk et al. (19) have described a heat-labile, trypsin-sensitive cytotoxin that induced vacuolization in cultured cells, and in the initial study cytotoxin was found in 55% of *H. pylori* strains tested, including isolates obtained from four geographically different regions worldwide. The clinical data were not provided in that study. Figura and colleagues (13) have shown that *H. pylori* strains isolated from patients with duodenal ulcers were more likely to produce vacuolating cytotoxin than were those from patients with gastritis. In that study (13), only isolates from subjects with a restricted range of gastrointestinal diseases were selected for examination for the production of cytotoxin. Few documented studies (5, 13, 19) have shown a relationship between the production of cytotoxin and the severity of clinical disease associated with *H. pylori* infection. *H. pylori* infection is extremely common, yet not all of those who are infected develop gastric or duodenal ulcer disease. It is reasonable to postulate that more severe clinical presentations such as ulceration may be due in part to the activities of toxins. In the study described here, we prospectively evaluated 175 culture-positive subjects with various gastrointestinal diseases who presented for elective gastroscopy in a public hospital. We aimed to characterize the *H. pylori* strains according to their abilities to produce cytotoxin and to examine the frequency of cytotoxin production in relation to clinical diseases.

* Corresponding author. Mailing address: Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital, Yarra Bend Road, Fairfield, Victoria 3078, Australia. Phone: 61-3-2802523. Fax: 61-3-4816784.

MATERIALS AND METHODS

Subjects. A total of 175 patients infected with *H. pylori* were enrolled in the study. Patients underwent routine upper endoscopy, and the clinical diseases in these patients are presented in Table 1. The endoscopic appearance was recorded by the endoscopist at the completion of each procedure. Gastritis is defined as erythema, edema, and/or erosions. Erosions are defined as small mucosal defects without depth. Gastric and duodenal ulcers are defined as mucosal defects of >4 mm in diameter, with surface indentation to the lesion. Nonulcer dyspepsia describes disease among subjects with upper gastrointestinal symptoms without ulcer disease, esophageal disease, or some other identified pathology.

Isolation and identification of *H. pylori*. Gastric mucosal biopsy specimens were obtained from a 5-cm radius around the pylorus during routine endoscopy. The two biopsy specimens were finely minced and were inoculated onto each of two plates containing selective media (Skirrow's medium and Dent's CP medium [Oxoid, Basingstoke, England]) and a chocolate agar plate. After incubation at 37°C under microaerobic conditions (10% CO_2 and 6% O_2 in 84% N_2) for 5 to 7 days, colonies that exhibited characteristic colonial morphologies were identified as *H. pylori* if they rapidly hydrolyzed urea, produced catalase and oxidase, did not reduce nitrate, and were gram-negative spiral rods on Gram smears. For preservation, sloppy blood agar consisting of 6% sheep blood and 0.4% agar in nutrient broth (Nutrient broth no. 2; CM67; Oxoid) was inoculated with *H. pylori*, and the plates were incubated under microaerophilic conditions for 4 to 5 days. The liquid-phase culture at the base of the slope was then removed and was placed in a sterile vial, and the vial was immediately stored frozen at −70°C. Multiple slopes were inoculated for each strain to maintain the maximum number of viable strains.

Cytotoxin assay. Stored *H. pylori* strains were thawed and cultured onto 6% sheep blood agar, and the plates were incubated at 37°C in a microaerophilic atmosphere for up to 7 days. A packed loop (1/200 ml; MW 191; Medical Wire and Equipment Co. Ltd., Corsham, England) of viable organisms from these cultures was inoculated into 10 ml of sterile brucella broth with 10% fetal calf serum (FCS), and the broth culture was incubated at 37°C (microaerophilic atmosphere) for 2 to 3 days with continuous agitation (100 oscillations per min). After centrifugation at 2,500 × *g* for 15 min the supernatant was passed through a 0.2-μm-pore-size filter and was used to inoculate Vero (African Green Monkey

TABLE 1. Production of cytotoxin by *H. pylori* isolates recovered from 175 patients with various gastroduodenal diseases

Clinical disease		No. of patients	No. (%) of cytotoxic strains
Nonulcer dyspepsia ($n = 63$)	Normal mucosa	40	13 (32)
	Gastritis	13	6 (46)
	Duodenitis	10	5 (50)
Peptic ulcer ($n = 93$)	Gastric ulcer	20	13 (65)
	Duodenal ulcer	73	48 (66)
Gastroesophageal reflux diseases ($n = 11$)	Ulcer	5	4 (80)
	Nonulcer	6	1 (17)
Other ($n = 8$)	Esophageal varices	4	1 (25)
	Gastric cancer	4	3 (75)

kidney) and HeLa (human cervical carcinoma) cell monolayers. These cells were supplied by Commonwealth Serum Laboratories, Melbourne, Australia. Vero cells were grown in a 75-cm^2 flask in Earle's minimum essential medium (Gibco) supplemented with nonessential amino acids (Gibco), 5% fetal calf FCS, and 1 ml of antibiotic stock solution to a final concentration of 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 80 μg of neomycin per ml. The culture medium used for HeLa cells was basal minimum eagle (Flow, North Ryde, N.S.W., Australia) containing 5% FCS and 1 ml of the antibiotic stock solution described above. Confluent cell cultures were trypsinized and adjusted to a concentration of 10^5 cells per ml. A total of 0.1 ml of the cell suspension from each cell culture was seeded into each well of a 96-well microtiter plate to produce a confluent monolayer after 24 h of incubation. The culture medium was removed from each well and was replaced with 150 μl of fresh medium prior to inoculation of test samples. A total of 50 or 100 μl of the broth culture filtrate of each test strain of *H. pylori* was then added to the wells, yielding dilutions of 1:4 or 1:2.5, respectively. Uninoculated brucella broth was used as a negative control, and a culture filtrate of *H. pylori* type strain NCTC 11637 was used as a positive cytotoxin control. Intracellular vacuolization was read after 24 and 48 h of incubation at 35°C in the presence of CO_2.

Statistical analyses of 2-by-2 tables were performed by Yates' corrected chi-square and Fisher's exact tests.

RESULTS

The cytopathic effects seen on the HeLa and the Vero cell monolayers were intracellular vacuolization similar to that described by Leunk et al. (19) and Figura et al. (13). Of 175 strains of *H. pylori* tested, 94 (53%) strains produced a vacuolating cytotoxin. Forty-three strains were positive for cytotoxin production in HeLa cells alone, whereas 6 strains were positive in Vero cells alone. Forty-five strains produced cell vacuolization in both HeLa cell and Vero cell monolayers. HeLa cells appear to be the most sensitive cell culture for use in the detection of *H. pylori* cytotoxin production.

Most of the vacuolization was detected 18 to 24 h after the addition of the soluble broth filtrate of *H. pylori* to the tissue culture, and the vacuolization remained positive at 48 h. Only two strains of *H. pylori* were found to be positive for intracellular vacuolization at 48 h. Intracellular vacuolization was detected at the 1:2.5 dilution for all 87 positive samples, and only 7 samples positive for intracellular vacuolization were not detected at the 1:4 dilution.

Table 1 shows the results of the cytotoxin assay for samples from the 175 patients. The highest percentage of strains producing cytotoxin was found among strains from subjects with peptic ulcer disease (gastric ulcer, 65%; duodenal ulcer, 66%). Of the 11 patients with gastroesophageal reflux disease, cytotoxic strains were isolated from 4 of 5 patients who had esophageal ulcers. Three of four patients with carcinoma of the stomach also yielded cytotoxic strains of *H. pylori*. In contrast, only 13 of 40 subjects with a normal upper gastrointestinal tract were found to produce cytotoxic strains. Subjects who had endoscopic findings of gastritis ($n = 13$) or duodenitis ($n = 10$) yielded 46 and 50% of cytotoxic *H. pylori* strains, respectively. The difference between cytotoxin production in patients with peptic ulcers (65.5%) and cytotoxin production in patients with an endoscopically normal stomach (32%) was statistically significant ($P < 0.001$). With increasing severity of mucosal damage in subjects with a normal upper gastrointestinal tract, gastritis, duodenitis, or peptic ulceration, a corresponding increase in the percentage of strains producing cytotoxin occurred (32, 46, 50, and 66%, respectively; chi-square per linear trend, 12:740; $P = 0.0004$), indicating the significant association between cytotoxin production by *H. pylori* and the severity of the disease. This association between cytotoxin production of *H. pylori* and clinical diseases is shown in Fig. 1.

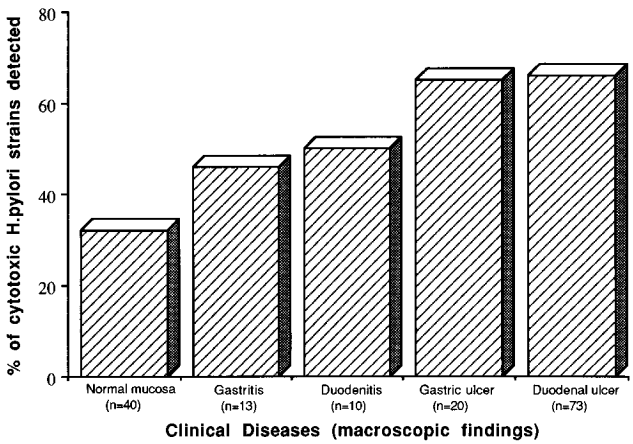

FIG. 1. Association between cytotoxin production by *H. pylori* isolates and clinical disease. Gastritis and duodenitis were defined as macroscopic erythema, edema, and/or erosions.

DISCUSSION

Vacuolating cytotoxin was detected in 54% of the *H. pylori* isolates examined in the present study by using HeLa and Vero cell monolayers. This finding is consistent with those obtained in previous investigations (5, 13, 18) that the toxin is produced in vitro by 50 to 60% of *H. pylori* strains. Of the two cell lines used, HeLa cells were the most susceptible.

Early reports have described at least two factors that appear to cause vacuolization of cultured epithelial cells in vitro. Xu et al. (22) have found that all *H. pylori* strains tested caused intracellular vacuolization in the presence of physiological concentrations of urea and that this effect can be blocked by the urea inhibitor acetohydroxamic acid. A later study by Konishi et al. (16) has shown that a urease-negative mutant of *H. pylori* produces similar vacuolization on cell culture in the absence of urea, suggesting that *H. pylori* produces a cytotoxic substance other than urease. In contrast, others showed that a broth culture filtrate of *H. pylori* did not contain urease activity (6, 16, 17). Cover and colleagues (3, 5), have identified an 82-kDa protein (later changed to an 87-kDa protein) and a 128-kDa protein in an *H. pylori* broth culture filtrate with vacuolating activity. The 87-kDa protein is distinct from *H. pylori* urease (6, 12, 20). Recent studies (2, 3, 8) have indicated that the predominant *H. pylori* product causing cell vacoulization is a cytotoxin with a molecular mass of 87 kDa. This 87-kDa protein has been purified and characterized (3). Inhibitors of eukaryotic ion-transporting ATPase have been shown to significantly

alter the interaction between *H. pylori* toxin and HeLa cells, suggesting that vacuole formation in HeLa cells by the cytotoxin requires the activity of vacuolar H^+-ATPase and is associated with altered cation transport involving Na^+ K^+ ATPase within eukaryotic cells (7).

Bacterial lysates from *H. pylori* strains also express a protein which appears to be distinct from the vacuolating cytotoxin and that has a molecular mass estimated to be 120, 128 or 130 kDa (2, 5, 9, 21). This 128-kDa protein has been shown to be immunogenic in humans infected with *H. pylori* (15) and closely related to the production of cytotoxin, but it does not directly mediate toxin activity (2, 21). This protein is known as CagA (cytotoxin-associated gene A) protein and has been purified and characterized (15), and its gene (*cagA* gene) has also been cloned and sequenced (2, 21). Recent studies (2, 21) indicate that although the CagA protein is associated with cytotoxin production, it is not required for the expression of vacuolating toxin activity and is not immunologically cross-reactive with the 87-kDa (VacA) protein. CagA is probably needed as an assessory protein for folding and export of the 87-kDa (VacA) protein. Previous studies (2, 4, 10) have shown that the presence of antibodies to the 120- to 128-kDa protein in either sera or mucosal secretions is associated with the presence of peptic ulceration in the subjects studied. In our study, 54% of *H. pylori* strains recovered from *H. pylori*-infected subjects produced vacuolating cytotoxin, and this cytotoxin was detectable in culture supernatants of *H. pylori* isolates recovered from 66% of the patients with peptic ulceration.

The correlation between the production of cytotoxin and the severity of clinical disease associated with *H. pylori* infection was provided by two recent studies. Figura et al. (13) have shown that cytotoxin-producing *H. pylori* strains were isolated more frequently from patients with peptic ulcer disease than from patients with chronic gastritis only (67 versus 30%; $P < 0.01$). Similarly, by immunoblotting with human sera, Cover et al. (5) observed a specific serologic response to proteins isolated from *H. pylori* culture supernatants, with vacuolating cytotoxin activity more frequently among *H. pylori* isolates from infected individuals with duodenal ulcers than among those from individuals without peptic ulceration (100 versus 61%, respectively). Our data, showing that 32% of cytotoxic strains were found within subjects with endoscopically normal stomachs infected with *H. pylori* compared with 66% of patients with peptic ulceration, support their findings. Our data also showed that 80% of *H. pylori* strains isolated from patients with esophageal ulcers produce cytotoxin and that 75% of patients with carcinoma of the stomach were infected with cytotoxin-producing strains. However, the sample population of these two groups of patients was small and the results should be interpreted with caution. Nevertheless, a recent report (11) indicates that patients with gastric cancer also showed increased serological recognition of the CagA protein relative to that among subjects with nonulcer dyspepsia. It appears that *H. pylori* isolates associated with ulcer disease commonly produce a vacuolating cytotoxin, indicating that this factor may play a role in the pathogenesis of mucosal ulceration in *H. pylori*-infected subjects.

The severity of the clinical disease correlates with the virulence (cytotoxicity) of *H. pylori*. The study reported here shows that an increase in the percentage of cytotoxic strains of *H. pylori* corresponds significantly with the progressive increase in the severity of mucosal damage in subjects with normal upper gastrointestinal tracts, gastritis, duodenitis, and peptic ulceration. This association suggests that vacuolating cytotoxin may play an important role in the pathogenesis of peptic ulcer disease.

REFERENCES

1. **Chen, X. G., P. Correa, J. Offerhaus, E. Rodriguez, F. Janney, E. Hoffman, J. Fox, F. Hunter, and S. Diavolitsis.** 1986. Ultrastructure of the gastric mucosa harboring Campylobacter-like organisms. Am. J. Clin. Pathol. **86:** 575–582.
2. **Covaccin, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, S. Xiang, N. Figura, and R. Rappuoli.** 1993. Molecular characterization of the 128 kDa immunodominant antigen of *Helicobacter* pylori associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA **90:**5791–5795.
3. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterisation of the vacuolating toxin for *Helicobacter pylori*. J. Biol. Chem. **267:**10570–10575.
4. **Cover, T. L., P. Cao, C. D. Lind, K. T. Tham, and M. J. Blaser.** 1993. Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates in vitro and in vivo. Infect. Immun. **61:**5008–5012.
5. **Cover, T. L., C. P. Dooley, and M. J. Blaser.** 1990. Characterization of and human serologic responses to proteins in *Helicobacter pylori* broth culture supernatant with vacuolizing cytotoxin activity. Infect. Immun. **58:**603–610.
6. **Cover, T. L., W. Puryear, G. I. Perez-Perez, and M. J. Blaser.** 1991. Effect of urease in HeLa cell vaculation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59:**1264–1270.
7. **Cover, T. L., L. Y. Reddy, and M. J. Blaser.** 1993. Effects of ATPase inhibitors on the response of HeLa cells to *Helicobacter pylori* vacuolating toxin. Infect. Immun. **61:**1427–1431.
8. **Cover, T. L., M. K. K. Tummuru, and M. J. Blaser.** 1993. Conservation of genetic sequence for the vacuolating cytotoxin among all *Helicobacter pylori* strains. Acta Gastroenterol. Belg. **56**(Suppl.)**:**47. (Abstract.)
9. **Crabtree, J. E., N. Figura, J. D. Taylor, M. Bugnoli, D. Armellini, and D. S. Tompkins.** 1992. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. J. Clin. Pathol. **45:**733–734.
10. **Crabtree, J. E., J. D. Taylor, J. I. Wyatt, R. V. Heatley, T. M. Shallcross, D. S. Tompkins, and B. J. Rathbone.** 1991. Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration and gastric pathology. Lancet **338:**332–334.
11. **Crabtree, J. E., J. I. Wyatt, G. M. Sobalo, G. Miller, D. S. Tompkins, J. N. Primrose, and A. G. Morgan.** 1993. Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer. Gut **34:**1339–1343.
12. **Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser.** 1990, Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265:**9464–9469.
13. **Figura, N., P. Guglielmetti, A. Rossolini, A. Barberi, G. Cusi, R. A. Musmanno, M. Russi, and S. Quaranta.** 1989. Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. J. Clin. Microbiol. **27:**225–226.
14. **Fox, J. G., P. Correa, N. S. Taylor, N. Thompson, E. Fontham, F. Janney, M. Sobhan, B. Riuz, and F. Hunter.** 1992. High prevalence and persistence of cytotoxin-positive *Helicobacter pylori* strains in a population with high prevalence of atrophic gastritis. Am. J. Gastroenterol. **87:**1554–1560.
15. **Gertenecker, B. B., H. Eschweiler, H. K. Vogele, U. Koch, C. H. Helleri, and M. Kist.** 1992. Serodiagnosis of Helicobacter pylori infections with an enzyme immunoassay using the chromatographically purified 120 kilodalton protein. Eur. J. Clin. Microbiol. Infect. Dis. **11:**595–560.
16. **Konishi, H., M. Ishibashi, M. G. Morshed, and T. Makazawa.** 1992. Cytopathic effect of *Helicbacter pylori* on cultured mammalian cells. J. Med. Microbiol. **37:**118–122.
17. **Leunk, R. D.** 1991. Production of a cytotoxin by *Helicobacter pylori*. Rev. Infect. Dis. **3**(Suppl. 8)**:**S686–S689.
18. **Leunk, R. D., M. A. Ferguson, D. R. Morgan, D. E. Low, and A. E. Simor.** 1990. Antibody to cytotoxin in infection by *Helicobacter pylori*. J. Clin. Microbiol. **28:**1181–1184.
19. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.
20. **Mobley, H. L. T., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol. **26:**831–836.
21. **Tummuru, M. K. R., M. L. Cover, and M. J. Blaser.** 1993. Cloning and expression of a high-molecular-mass major antigen if *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect. Immun. **61:**1799–1809.
22. **Xu, J. K., C. S. Goodwin, M. Cooper, and J. Robinson.** 1990. Intracellular vacuolization caused by the urease of *Helicobacter pylori*. J. Infect. Dis. **161:**1302–1304.