Cytotoxin Production by *Campylobacter pylori*

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C. pylori-associated gastritis may sometimes be associated with severe acid-peptic disease. We have proposed that certain *C. pylori* (CP) might produce a cytotoxic product analogous to that produced by *C jejuni, Shigella, C. difficile,* or enterohemorrhagic *E. coli.* Sixty-six consecutive clinical *C. pylori* isolates were cultured on horse blood agar and assessed for cytotoxic activity in polymyxin B lysates in Chinese hamster ovary (CHO) cells *in vitro.* Significant cytotoxicity with rounded, damaged cells was found in 32 of 66 (48%) unconcentrated polymyxin B lysates. To date, prospectively recorded major symptoms, total symptom score, endoscopic appearance, severity of gastritis and density of *C. pylori* colonization has not correlated with cytotoxic titer. In addition, a qualitatively different vacuolating cytotoxin, analogous to that reported by Leunk *et al.* (ASM 1987), was found in 1 of 10-14 of these isolates, with broth cultures tested on human intestine 407 cells or CHO cells, respectively. Neither effect was neutralized by antisera against Shiga toxins 1 or 2, C. difficile toxins A and B or *C jejuni* cytotoxin. We conclude that CP often produce one or more unique cytotoxins. Their further characterization and role in causing disease remains to be defined.

Inflammatory or enterohemorrhagic enteric pathogens characteristically produce cytotoxins (sometimes requering polymyxin-release). Examples include Shiga-toxin produced by *Shigella* organisms, Verocytotoxins I and II (SLT-I, SLT-II) produced by enterohemorrhagic *E. coli* (EHEC) 0157:H7,026 et al, *C. difficile* toxins A and B; and a cytotoxin produced by *C. jejuni.* Because *C. pylori* is characteristically associated with gastritis and occasionally severe acid-peptic disease, we propose that certain *C. pylori* strains might produce a cytotoxic product analogous to those mentioned above.

Our purposes were to examine fresh, characterized clinical isolates of *C. pylori,* for cytotoxic product (s) *in vitro;* to characterize any cytotoxin with respect to need for polymyxin release, dialyzability and heat and trypsin lability; and to determine the relationship of any *C. pylori* cytotoxin(s) found to recognized cytotoxins of *Shigella,* EHEC, *C. difficile* and *C. jejuni.*

Methods

Methods included preparation of polymyxin lysates by growing 66 clinical isolates from gastric biopsies on horse blood agar slants with GCHI (Remel Laboratories, Lenexa, KA), in 10% CO_2 for 3 days. Organisms were then suspended in PBS, with 2 mg/ml polymyxin B, to a McFarland 6-8 cloudy suspension and incubated for 1 hour for 37 °C. After centrifugation at 3000 rpm x 20 min., supernatants were filtered (0.2μm) and tested immediately in serial dilutions in PBS on CHO cells or stored at -70 °C. Cytotoxicity was observed in Giemsa-stained cells after 24 hours.

In testing I-407 and CHO cells, brucella broth culture filtrates were prepared in shaking culture (150 oscillations per minute) in 85% into 10% CO_2 and 5% 0_2 at 37 °C for 48 hours, centrifuged at 3000 rpm for 20 minutes and filtered through 0.45μm for dilution in PBS in order to place on CHO or I-407 cells to observe for vacuolization or other cytotoxicity after 24 hours in Giemsa- stained cells. Polymyxin lysate filtrates were prepared from three different *C. pylori* isolates for incubation with 0.25% trypsin, 3.3 mg/ml trypsin inhibitor (control) or antisera, for 30 minutes at 37 °C before placing the mixture in two-fold dilution on CHO cells, to be read at 24 hours as noted above.

Results

Of 66 clinical *C. pylori* isolates examined for polymyxin-lysate cytotoxicity, 32 (48%) produced cytotoxin titers 1:32. In studies of antiserum neutralization, titers of *C. pylori* polymyxin-lysate cytotoxicity were not altered by antisera against *C. difficile* toxins A + B (1:25), SLT I, SLT II (both at 1:25), cholera toxin (SSVI, 1:100) or *C. jejuni* cytotoxin (1:5) prepared by Dr. Donna Morgan. While antiserum against the vacuolating cytotoxin failed to neutralize the polymyxin-released cytotoxin, specific neutralization with rabbit antiserum, kindly provided by Drs. Leunk and Morgan, provided variable neutralization of their homologous vacuolating cytotoxin.

Most polymyxin-released *C. pylori* cytotoxicity from 2 strains was destroyed by heating to 70°C for 30 minutes. The protein nature of this cytotoxin was suggested by its complete inactivation by trypsin 0.25% for 30 minutes, an effect that was specifically inhibitable by prior incubation of the trypsin with trypsin inhibitor.

Conclusions

In summary, *C. pylori* isolates from gastric biopsies from patients with gastritis may produce 2 distinct cytotoxins. Of 66 strains tested, 48% produced a CHO cell-cytotoxin, detected in polymyxin-lysate at titers 1:32. This cytotoxin is nondialyzable, moderately heat-labile and trypsin-labile. Out of 14 strains tested in unconcentrated broth filtrates, one produced a vacuolating cytotoxin detected in I-407 cells at a titer of 1:4. Neither cytotoxin was neutralized by cytotoxin sera prepared against *C. jejuni* cytotoxin, SLT I, SLT II or *C. difficile* toxin A + B. The antiserum prepared against the vacuolating cytotoxin by Drs. Leunk and Morgan, did not neutralize the polymyxin-released *C. pylori* cytotoxin. The severity of symptoms, endoscopy or histopathology scores did not show an obvious correlation with cytotoxin titer. The role of polymyxin-releasable and vacuolating cytotoxins in the pathogenesis of gastritis or ulcer disease requires further study.

Characterization of the Cytotoxin from *Campylobacter pylori*

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Leunk *et al.* recently described a cytotoxin present in broth culture supernatants of *C. pylori,* which caused vacuolization of cultured cells. We sought to confirm toxigenesis, characterize the toxin further, and determine conditions which maximized toxigenesis *in vitro.* A known tox+ (60190) and tox- (Tx30a) strains were grown in brucella broth with 5% FBS. Concentrated supernatants were incubated in a final protein concentration of 1.5 mg/ml for 48h with cultured HeLa cells. Toxigenesis was maximal, causing 50% vacuolization in titers of 1:320 in the plateau phase of growth, corresponding to approx. 10^6 CFU/ml, OD_{450} values of 1:100 to 1.300, and 7 to 11 days of incubation. Concentrated culture supernatants (CCS) of the tox+ and tox- strains were Western-blotted with sera from immunized rabbits and human sera. Sera from a rabbit immunized with tox+ CCS recognized an 82kDa band in tox+ CCS but not in tox- CCS; this band was not recognized by sera from a rabbit immunized with the tox-CCS. Sera from 11 of 17 *C. pylori*-infected humans recognized 95 or 132kDa bands in tox+ CCS but not in tox-CCS; 4 of the 17 sera recognized an 82kDa band. None of these bands were recognized by sera from 11 uninfected persons with *C. pylori.* These results indicate that *C. pylori* may produce a vacuolizing cytotoxin associated with CCS proteins of 82, 95, or 132kDa.