Bacillus pumilus in the Induction of Clindamycin-Associated Enterocolitis in Guinea Pigs

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Antibiotic-associated enterocolitis was induced in guinea pigs by the intraperitoneal injection of clindamycin. The colonial and cecal mucosa and feces of acutely ill animals were cultured under aerobic and anaerobic conditions on 5% sheep blood agar plates and on a selective and differential medium for Clostridium difficile. All morphologically distinct colony types were isolated in pure culture and identified. A sterile cell-free filtrate of each isolate was tested for ability to induce morphological changes in cultured monolayers of mouse adrenal cells. The filtrate of a predominant isolate, Bacillus pumilus, induced an alteration of cellular morphology; the sterile filtrates of other isolates were unreactive. Toxin contained in cell-free filtrates of B. pumilus caused a syndrome identical to clindamycin-associated enterocolitis when injected intracecellularly into guinea pigs. The toxin had a molecular weight of 6,500 daltons as determined by molecular sieve chromatography and was inactivated with pronase, lipase, and trypsin. The minimal inhibitory concentrations of clindamycin and vancomycin for B. pumilus were 50 μg/ml and =0.4 μg/ml, respectively.

Enterocolitis is a well-recognized complication of antibiotic therapy (2, 4). Although this complication has been observed with the administration of various antibiotics, it is more often associated with clindamycin, lincomycin, and ampicillin (2).

Studies to define the etiology of enterocolitis followed the demonstration of a bacterial toxin in the feces of patients treated with antibiotics. The clinical significance of Clostridium difficile toxin in the etiology of enterocolitis was demonstrated previously (4-6, 22-24, 26, 29, 32). The toxin can be neutralized both in vitro and in vivo by Clostridium sordellii or by polyvalent gas gangrene antitoxin (3, 6, 7, 23, 29).

Recent observations by Knoop (20), Lamont et al. (21), Rehg (28), and Lowe et al. (24) have reported, using animal systems, the presence of a bacterial toxin(s) that can be neutralized by antiserum specific for Clostridium histolyticum, Clostridium perfringens (type E), C. sordellii, and C. difficile, respectively. Further, a number of case reports have implicated toxin-producing C. perfringens (type C) (32), Clostridium sporogenes (26), and Staphylococcus aureus (D. H. Batts, J. Silva, and R. Fekety, Abstr. 11th Internat. Congr. Chemother.; 19th Intersci. Conf. Antimicrob. Agents Chemother. 1979, p. 181) in the etiology of colitis in humans.

This study was designed to define the etiology of clindamycin-associated enterocolitis in guinea pigs. The microbial flora of cecal and colonic mucosa and feces of guinea pigs, in which enterocolitis had been induced with clindamycin, was studied. The production of extracellular microbial products that may relate to pathogenesis and clinical disease was determined.

MATERIALS AND METHODS

Drug challenge. Albino guinea pigs (200 g) were purchased from Daubert Rabbity, Omaha, Nebr. Experimental groups of six guinea pigs were challenged intraperitoneally with clindamycin phosphate (75 mg/kg of body weight; The Upjohn Co., Kalamazoo, Mich.) in physiological 0.067 M potassium phosphate buffer (pH 7.25) by previous methods (20). Control groups received an equal volume of buffer without antibiotic.

Culture procedures. Samples for culture before antibiotic challenge (day 0) were obtained by rectal swabs and collection of fresh feces. All experimental animals were symptomatic on day 2 after antibiotic challenge and were sacrificed for postmortem examination. Samples from the distal colonic, mid-colonic, and cecal mucosa and feces were obtained for culture; the feces were heated at 80°C for 10 min before culture. All samples were plated on tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood or on a selective and differential medium for C. difficile as described elsewhere (15). To determine quantitative changes in fecal microflora, feces were serially diluted 10-fold before culture. The plates were incubated at 37°C for 48 h in GasPak anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.), or at 37°C in air for 24 h. After incubation, all morphologically distinct colonies were isolated on 5% sheep blood agar plates. The isolates were then identified by the Enterotube II (Roche Diagnostics, Nutley, N.J.) or API 20A (Analytab Products, Plain
view, N.Y.) identification system. Identification of Bacillus isolates was performed by Ruth Gordon (Waksman Institute of Microbiology, New Brunswick, N.J.).

Toxin production and assay. The ability to produce toxin was determined for all isolates. Aerobic cultures were grown for 24 h at 37°C in screw-cap tubes containing 10 ml of brain heart infusion broth (BHIB; Difco); anaerobic cultures were grown for 48 h at 37°C in 50-ml tubes containing 30 ml of BHIB in an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. After incubation, the cultures were centrifuged (12,100 × g for 30 min), and the supernatant was sterilized by membrane filtration (0.45 μm; Gelman Sciences Inc., Ann Arbor, Mich.).

To detect the presence of toxin contained in cell-free filtrate (CFF), mouse Y1 adrenal cells were cultured in 96-well microtiter plates and maintained on F15 minimal essential medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 200 U of penicillin G, 20 μg of gentamicin, and 1.25 μg of amphotericin B per ml (20). The microtiter wells were challenged (20 μl per well) with serial twofold dilutions of CFF made in Dulbecco phosphate-buffered saline (D-PBS; pH 7.25). After incubation for 18 h, the cells were observed by light microscopy for morphological changes. The highest dilution showing in excess of 90% cell rounding was considered an endpoint; the reciprocal of the highest dilution in which duplicate wells were positive was multiplied by 10 to correct for final dilution and expressed as toxic units per milliliter. The toxin of C. difficile (ATCC 9689) was purified by the method of Rolfe and Finegold (30) and used as a positive control.

Culture isolates that induced a toxic response were grown in 2-liter Erlenmeyer flasks containing 1 liter of BHIB supplemented with 5% fetal calf serum (GIBCO). Each flask was inoculated from an overnight BHIB broth culture, resulting in an initial optical density (at 540 nm) of 0.05, and cultured at 37°C for 5 days without shaking, as indicated by preliminary studies for peak toxin titer. After incubation, the cells were removed by centrifugation (16,300 × g for 2 h) at 4°C, and the supernatant was sterilized by membrane filtration (0.45 μm; Gelman). The CFF was concentrated and twice washed with an equal volume (10 ml) of D-PBS (pH 7.25) on a Diaflo PM-10 ultrafiltration membrane (terminal molecular weight cutoff = 10,000; Amicon Corp., Lexington, Mass.) and stored at −70°C until required.

Animal challenge with Bacillus pumilus toxin. Three groups of two guinea pigs were challenged intracereally with 1.0 ml of either concentrated CFF from B. pumilus (1,280 toxic units) or highly purified toxin from C. difficile (1,280 toxic units); control animals were injected with an equal volume of D-PBS (pH 7.25). After surgery, the animals were placed in separate cages and observed for signs of enterocolitis (20). All animals were sacrificed 4 days after surgery to observe cecal and intestinal pathology.

Drug sensitivity. The sensitivity of B. pumilus to clindamycin and vancomycin was performed as described elsewhere (17).

Molecular sieve chromatography. B. pumilus toxin was produced as previously described. The PM-10 concentrate was chromatographed on a column (2.5 by 100 cm) of Bio-Gel A5m (BioRad Laboratories, Rich-
plates for *C. difficile* were consistently negative for bacterial growth.

Each isolate was grown in BHIB and the sterile cell-free culture filtrate assayed for the presence of toxin by using cultured monolayers of mouse adrenal cells. Culture filtrates of six isolates of *B. pumilus* caused a cytotoxic response; culture filtrates from all other isolates were negative. There was no difference in the ability of pre- and postchallenge isolates to produce toxin (Table 1).

Feces from drug-treated and control animals were serially diluted 10-fold to determine quantitative changes in the intestinal flora. A decrease in the number of anaerobic organisms was observed with the exception of anaerobic gram-positive spore-forming rods, which remained essentially the same. An increase of 100- to 1,000-fold was observed for facultative gram-negative rods. Facultative and aerobic gram-positive spore-forming rods increased by 200- to 500-fold over parallel controls (data not shown).

**Effect of *B. pumilus* toxin on cultured cells.** The toxin of *B. pumilus* produced morphological changes similar, but not identical, to those caused by the highly purified toxin of *C. difficile* (Fig. 1). Cultured cells treated with *C. difficile* toxin showed cytoplasmic extensions in contrast to the aggregates or clusters of cells observed after treatment with *B. pumilus* toxin. Further, in samples containing a high titer of *B. pumilus* toxin (>80 toxic units per ml), cytopathic effects could be observed at 4 h postchallenge, and essentially complete cell lysis was observed at 18 h. Cells exposed to *B. pumilus* toxin were not viable after 18 h of exposure as determined by trypan blue exclusion. No effect on the toxin titer was observed in the absence of antibiotics.

**Animal challenge.** The role of *B. pumilus* toxin in the induction of enterocolitis in guinea pigs was determined. One milliliter of concentrated CFF was used to intraceally challenge guinea pigs; control animals were administered an equal volume of *C. difficile* toxin or D-PBS (surgery control). An untreated group of animals without surgical intervention was used for direct comparison. Experimental animals receiving either *B. pumilus* or *C. difficile* toxin showed reduced water and food intake; fecal pellets were abnormal in size. Postmortem examination of experimental animals showed an enlarged small bowel and massive cecal dilation (20). The ceca of experimental animals were filled with watery fecal material; the intestine proximal to the cecum in animals treated with *B. pumilus* toxin showed macroscopic areas of hemorrhage. No intestinal pathology was noted at postmortem examination of control animals.

**Drug sensitivity.** The sensitivity of *B. pumilus* to clindamycin and vancomycin was obtained by using a standard broth dilution method (17). The minimum inhibitory concentrations of clindamycin and vancomycin for *B. pumilus* were 50 μg/ml and ≤0.4 μg/ml, respectively. These observations indicate that *B. pumilus* was resistant to clindamycin and sensitive to vancomycin.

**Molecular sieve chromatography.** Although the fractionation of concentrated cell-free filtrate from *B. pumilus* on a Bio-Gel A5m column resulted in the separation of several components, both toxic and proteolytic activities were associated with a major protein peak (data not shown). Therefore, these fractions were pooled, concentrated by ultrafiltration, and applied to a column of Sephadex G-75 in an effort to separate toxin from proteolytic activity. These results are

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**TABLE 1. Culture isolates from guinea pigs before and after the induction of clindamycin-associated enterocolitis**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Preculture</th>
<th>Feces</th>
<th>Cecum</th>
<th>Mid-colon</th>
<th>Distal colon</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><em>Clostridium clostridioforme</em></td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacteroides ovatus</em></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Peptococcus magnus</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Propionibacter sp.</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND* means no detection.

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*a* All cultures were plated on 5% sheep blood agar; +, isolation; ND, not detected.

*b* Samples obtained by rectal swabs and collection of fresh feces.

*c* Feces were heated to 80°C for 10 min before culture.

*d* -, Negative; +, positive; see text for details.
The specific activity of *B. pumilus* toxin increased from 1.5 toxic units per mg of protein in the cell-free filtrate to 2.4 toxic units per mg of protein in pooled fractions from the Bio-Gel A5m column; a specific activity of 8.2 toxic units per mg of protein was observed after Sephadex G-75 molecular sieve chromatography.

**Enzymatic studies.** The action of proteolytic, lipolytic, and nucleolytic enzymes on *B. pumilus* toxin was determined. The treatment of CFF with DNase or RNase for 1 h at 37°C did not cause a decrease in toxic activity (data not shown). However, the treatment of CFF with lipase, trypsin, or pronase resulted in a significant inactivation (≥88%) of toxic activity. These observations suggest that the toxin of *B. pumilus* may be a lipoprotein.

**pH and heat effect on toxin.** The effect of pH on *B. pumilus* toxin was determined. Although a decrease in toxic activity was not observed in the pH range of 6 to 8 (data not shown), an inhibition (>50%) over the pH ranges of 4 to 6 and 8 to 10 was observed.

To assess the effect of temperature on toxin stability, samples of CFF were heated at 56, 80, and 100°C for 1 h before assay. Toxic activity was found to be stable at 56°C but labile at 80 and 100°C.

**DISCUSSION**

The results of this study implicate *B. pumilus* in the etiology of clindamycin-associated enterocolitis in guinea pigs. This organism was a predominant member of the cecal flora of all symptomatic animals and could be easily isolated under aerobic conditions on 5% sheep blood agar plates. Broth culture filtrates of *B. pumilus* contained a toxin that caused a pathology similar to, with the exception of intestinal hemorrhage, that induced by drug treatment or *C. difficile* toxin. In all cases, the pathology was marked by small bowel and cecal dilatation; the ceca were distended and filled with watery fecal material. The toxin of *B. pumilus* was found to be pH sensitive and heat labile.

Previous studies have demonstrated that toxic activity contained in feces could be neutralized by *C. histolyticum* (20), *C. sordellii* (24, 28), or *C. difficile* (24) antitoxin. The toxin produced by *B. pumilus* was not neutralized by these or other (*C. septicum*, *C. novyi*, or *C. perfringens* type A) clostridial antisera (unpublished data). Although not determined, the presence of a potent protease in broth filtrates of *B. pumilus* may inhibit specific serological neutralization. Other reports on toxin-associated enterocolitis in animals and humans have demonstrated the neutralization of toxic activity in feces with clostridial antisera (7, 21, 29). Recently, some concern

isolated from ticks produces cataracts and neutrophils as shown in Fig. 2. As indicated, the major portion of proteolytic activity was found to be associated with the first protein peak; toxic activity was associated with the second protein peak. The molecular weight of toxin when compared with protein standards eluted from the same column was 6,500 (Fig. 2). These observations indicate that the action of *B. pumilus* toxin is not associated with proteolytic activity, since those fractions containing enzymatic activity did not induce alterations in the cultured cell system.
over the specificity of these antisera has been reported (1).

In the present study, C. histolyticum, C. sor- delli, and C. difficile could not be isolated from healthy or symptomatic guinea pigs; this may be due to the small number of organisms present and therefore undetectable by the culture procedures used. This seems unlikely, however, since other investigators have demonstrated a quantitative increase of C. difficile in symptomatic animals (4, 5). Quantitative analysis of cecal contents revealed an increase in aerobic gram-positive spore-forming rods (Bacillus sp.) and facultative gram-negative rods, with a decrease in anaerobic organisms. Further analysis of all isolates for toxin production indicated that an aerobic gram-positive spore-forming rod, B. pu- nilus, was toxigenic. Since no difference could be found in the production of toxin between pre- and posttreatment isolates, disease production by this organism may be the result of an increase in cell numbers.

B. pumilus is commonly isolated from a variety of environmental sources. The organism grows as a smooth colony that becomes yellow with increased incubation. Gordon et al. (18) reported the characteristics of the species. These include a requirement for biotin, with a further requirement for amino acids in some strains. Typically, the organism is motile, cata- lase positive, and salt tolerant, and it will not grow under strict anaerobic conditions.

The toxin of B. pumilus had a molecular weight of 6,500 as determined by molecular sieve chromatography. The relation of this toxin to toxic factors produced by other Bacillus species is not known. However, the production of a low-molecular-weight peptide (<5,000), toxic to silkworm larvae, has been reported for Bacillus thuringiensis (9). The CFF of B. pumilus con- tained a dermatonecrotic factor which produced a lesion similar to that produced by Bacillus cereus when injected into the back of a guinea pig (16); this observation may relate to the production of proteolytic enzymes by these or- ganisms. Studies should be directed at the relationship of B. pumilus toxin to other microbial toxins, including the enterotoxin of B. cereus (33).

Several studies have shown that between 47 and 92% of normal healthy people contain members of the Bacillus genus, ranging from $10^{3}$ to $10^{6}$ colony-forming units per g of feces (11, 12, 13). B. pumilus, a nonpathogenic enteric organ- ism, possesses an antigen which cross-reacts with meningococcal group A polysaccharide (36). Thus, the organism has been implicated as a source of natural meningococcal immunity (27). The presence of B. pumilus in the intestine during antibiotic therapy may allow the organ- ism to multiply and produce disease; the present- ation of disease may depend on the action of the toxin and sensitivity of the host.

Whether the animal species determines which
microorganism will overgrow in the intestine as a result of antibiotic exposure is not known. It is becoming apparent that a number of different organisms are capable of inducing similar enteric diseases in animals (6, 10, 20, 21, 24, 28) and humans (22, 23, 26, 29, 32). The induction of enterocolitis may depend on the antibiotic or composition of the normal microflora, as well as the presence of other enteric pathogens (8).

Because of the resistance of Bacillus spores to heat and other bactericidal agents, they are of economic importance in the production of canned foods and the preparation of sterile products. Evidence is rapidly accumulating to indicate that opportunistic human infections may result from a number of Bacillus species that are generally believed to be nonpathogenic (14, 19, 34, 35).

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LITERATURE CITED


32. Schwartz, J. N., J. P. Hamilton, R. Fekety, E. G. Green, L.


