Cytotoxicity of *Bacillus piliformis*

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Summary. Seven isolates of *B. piliformis*, the agent of Tyzzer's disease, obtained from various host species, were examined for cytotoxic activity by incubating culture filtrates on BRL 3A rat-hepatocyte and 3T3 mouse-fibroblast cell lines. One isolate exhibited cytopathic effects on BRL 3A cells, but not on 3T3 cells. Three other isolates were strongly cytotoxic for 3T3 cells but only slightly so for BRL 3A cells. The remaining three isolates showed no cytotoxicity for either cell line. The cytotoxic products were > 100 kDa in mol. wt, thermolabile, and partly destroyed by trypsin treatment. The data show that some *B. piliformis* isolates produce cytotoxic proteins, which may contribute to the pathogenesis of Tyzzer's disease.

Introduction

Bacillus piliformis is a long (10–40 µm), rod-shaped, gram-negative, spore-forming, motile, obligately intracellular bacterium, originally described and named by Tyzzer.¹ It causes an often fatal enterohepatic syndrome in a wide range of domestic, laboratory, and wild animal species,^{2–4} in primates,⁵ and possibly in man.⁶ Infection may produce a watery to pasty diarrhoea^{2,3} and oedema, haemorrhage, inflammation, and focal ulceration in intestinal and hepatic tissue. The intestinal lamina propria is distended and contains a polymorphonuclear cell infiltrate. *B. piliformis* organisms are visible within the absorptive and crypt epithelial cells of the terminal ileum and colon.

Diarrhoea with histopathological changes such as those described is characteristic of cytotoxin-producing bacteria, such as *Shigella* spp.,⁷ *Escherichia coli* strains that produce Shiga-like toxins,⁸ and *Campylobacter jejuni*.^{9,10} The present study was undertaken to examine the possibility that *B. piliformis* may produce cytotoxins.

Materials and methods

Preparation of culture filtrates

Seven isolates (B, G, H, GP, M, E, R1) of *B. piliformis* obtained from naturally infected animals in diverse geographical locations¹¹ were used. Isolate B was grown on 3T3 mouse fibroblasts (MA Bio-products, Walkersville, MD, USA); all other isolates were grown on buffalo rat liver cells (BRL 3A; ATCC CRL 1442) as previously described,¹¹ unless otherwise

noted. Briefly, mammalian cell monolayers were grown at 37°C in Dulbecco Modified Eagle Medium (DMEM) supplemented with Serum Plus (Hazelton, Lenexa, KS, USA) 10% and 2 mM L-glutamine (Sigma Chemical Co., St Louis, MO, USA) until 70–80% confluent. The monolayers were inoculated with *B. piliformis* and incubated at 37°C until the bacterial concentrations reached 10⁵–10⁶/ml. Culture medium was harvested and filtered through a 0.22-µm membrane filter. Except where specifically indicated, filtrates were either used immediately or after storage at 4°C. For trypsin sensitivity experiments, bacterial cultures were grown as described above, except that the Serum Plus was decreased to 1%.

As controls, culture filtrates and mammalian cell extracts were prepared from analogous monolayers not infected with *B. piliformis*. The extracts were prepared from monolayers that were harvested by scraping and disrupted ultrasonically at 105 W (Branson Sonic Power Company, Danbury, CT, USA) on ice for three 30-s bursts.

Cytotoxin assays

A modification of the spectrophotometric method of Gentry and Dalrymple¹² was used to quantitate cytotoxic activity in culture filtrates. Mammalian cell lines used as target cells in cytotoxicity assays included 3T3 mouse fibroblast, BRL 3A rat liver, monkey kidney (Vero; ATCC CCL 81), human embryonic intestine (Henle 407; ATCC CCL 6), and human cervical epithelioid carcinoma (HeLa; ATCC CCL2). Plastic plates containing 96 wells (Becton Dickinson Labware, Lincoln Park, NJ, USA) were seeded with target cells (16000/well) and incubated overnight at 37°C; the growth medium (0.2 ml/well) was then removed. To each well was next added 0.2 ml of culture filtrate diluted 1 in 2 with fresh medium, unless

otherwise stated; for control purposes fresh medium (0.2 ml) was added to each well. After incubation for 48 h at 37°C, plates were washed with phosphate-buffered saline, pH 7.2 (PBS), to remove detached cells. Remaining cells were fixed in formalin and stained with crystal violet. Excess stain was removed by rinsing with water, and the dye was eluted with ethanol 50%. The percentage of surviving cells was quantitated by comparing the absorbance of the eluted dye at 570 nm with that of the appropriate control. All experiments were performed on 3–6 culture-filtrate samples collected and assayed on separate occasions, each sample being tested in triplicate. Percentage of cytotoxicity was calculated as $100 - [(A_{570} \text{ of experimental sample} / A_{570} \text{ of control samples}) \times 100]$.

Characterisation of cytotoxic activity

To assess the effect of storage on cytotoxicity, samples of culture filtrates were stored at 0 and –20°C. At intervals, samples were removed and tested for cytotoxic activity on HeLa cells. The cytotoxicity values were compared with those determined immediately after harvest of culture filtrate.

To define the heat stability of cytotoxin, culture filtrates were heated in a water bath at 50, 60 or 80°C for 30 min. Cytotoxicity for HeLa cells was compared with that of unheated culture filtrate.

Mol.-wt ranges of cytotoxic factors were determined by ultrafiltration of culture filtrates on Amicon membranes (Amicon Corp., Danvers, MA, USA). Membranes were presoaked for 30 min in NaCl 5% and then rinsed for 1 h in three changes in deionised water before use. Fractions of defined size ranges (< 10, 10–30, 30–100 and > 100 kDa) were prepared from each culture filtrate. The cytotoxicity of each fraction, as compared with that of unfractionated culture filtrate, was then determined on HeLa cells.

To determine whether cytotoxins were composed of protein, culture filtrates were treated with trypsin (2 mg/ml) (Sigma) as previously described.¹³ After incubation for 30 min at 37°C, the trypsin was inactivated by the addition of soy bean trypsin inhibitor at a ratio of 2.7 mg of inhibitor to 1.0 mg of trypsin, and the percent cytotoxicity was determined. Controls consisted of untreated culture filtrates and culture filtrates treated with trypsin which had been pre-incubated with trypsin inhibitor.

Statistical analyses

Statistical analyses were performed by Student's *t* test.

Results

Cytotoxicity of culture filtrates

To investigate cytotoxicity, filtrates from cultures of mammalian cells infected with various *B. piliformis*

Fig. 1. Effect of culture filtrates on mammalian cell monolayers. BRL 3A cells incubated with filtrate from *B. piliformis*-infected (A) and uninfected (B) cell cultures. Bar = 20 µm.

isolates were tested initially on BRL 3A rat-hepatocyte and 3T3 mouse-fibroblast cell lines. After incubation of filtrates from isolate B with BRL 3A cells at 37°C for 48 h, phase-contrast light microscopy revealed rounding and detachment of the cells; identical monolayers incubated with filtrates from uninfected mammalian cell cultures remained attached and almost confluent (fig. 1). Similar cytopathic effects were seen with filtrates from isolates G, H and GP on 3T3 cells.

Cytotoxic activity was assayed in culture filtrates prepared from seven *B. piliformis* isolates on BRL 3A and 3T3 cells (table I). Filtrate from isolate B was cytotoxic for BRL 3A cells but not for 3T3 cells. In contrast, filtrates from isolates G, H and GP showed

Table I. Cytotoxicity of *B. piliformis* culture filtrates on 3T3 and BRL 3A mammalian cells

Isolate	Number of samples tested	Mean (SEM) percentage cytotoxicity on mammalian cells	
		3T3	BRL 3A
B	6	0.2 (0.6)	92.8 (1.0)
G	3	62.6 (2.9)	9.3 (2.9)
H	3	56.0 (3.2)	1.5 (1.2)
GP	3	67.0 (3.3)	18.3 (2.1)
M	3	0.0 (2.1)	0.0 (1.9)
E	3	0.0 (3.2)	0.0 (2.3)
R1	3	0.0 (2.2)	0.0 (3.6)

Table II. Cytotoxicity of *B. piliformis* filtrates on various mammalian cell lines

Isolate	Number of samples tested	Mean (SEM) percentage cytotoxicity on mammalian cells		
		Vero	Henle	HeLa
B	6	86.5 (1.5)	31.3 (2.6)	87.7 (1.8)
G	3	96.3 (2.3)	90.0 (1.3)	89.3 (0.7)
H	3	46.0 (2.8)	72.5 (1.6)	77.0 (1.0)
GP	3	89.3 (2.6)	88.8 (1.4)	90.0 (1.3)

much greater cytotoxicity for 3T3 cells than for BRL 3A cells. No significant difference in cytotoxicity for 3T3 cells was noted between filtrates from isolates G, H and GP. Culture filtrates from isolates M, E and R1 showed no cytotoxic activity against either cell line. Similar results were obtained when isolates G, H, GP, M, E and R1 were grown in 3T3 cells rather than BRL 3A cells, although the cytotoxic activities of isolates G, H and GP were reduced (data not shown). Analogous experiments in which isolate B was grown on BRL 3A cells were not performed as the organism could not be propagated in this cell line despite repeated attempts; the cells in BRL 3A monolayers inoculated with isolate B invariably became rounded and detached, preventing bacterial growth. Because no cytotoxicity was observed with isolates M, E and R1, the remainder of the studies were performed only on isolates B, G, H and GP.

To confirm that degradative products from lysed mammalian cells in which bacteria were grown were not responsible for the observed cytopathic effects, extracts of uninfected 3T3 and BRL 3A cells were prepared and applied to BRL 3A and 3T3 target cell monolayers, respectively. Monolayers were examined for cytopathic effects after incubation at 37°C for 24, 48 and 72 h. The results were negative, indicating that mammalian cell components were not cytotoxic.

To prove that cytotoxicity was not due to the depletion of medium nutrients during the incubation of cytotoxicity assays, culture filtrates of isolate B were diluted 1 in 50 in fresh medium and the toxicity was assayed. The diluted samples showed 93% of the cytotoxicity of undiluted culture filtrates. Similar effects were seen with culture filtrates from other cytotoxinogenic isolates. These data, coupled with the lack of cytotoxicity in assays of filtrates from isolates M, E and R1, indicated that nutrient depletion was not responsible for the cytopathic effects.

To assess whether other cell lines previously documented as sensitive to cytotoxins of invasive bacteria were sensitive to *B. piliformis* cytotoxin, culture filtrates from the four cytotoxinogenic isolates shown were incubated with Vero, Henle 407 and HeLa cells (table II). All four had significantly increased cytotoxic activity against these cell lines as compared with filtrates from uninfected control cultures ($p \leq 0.01$). Control wells in which extracts of uninfected BRL 3A

Fig. 2. Effect of heat treatment (▨, 50°C; ■, 60°C; □, 80°C) on cytotoxic activity of isolates B, G, H and GP. Values represent the mean percentage of cytotoxicity of the appropriate untreated control (□).

and 3T3 cells were applied to Vero, Henle 407 and HeLa cells exhibited no cytotoxicity, indicating that toxicity on these cell lines, like that described in BRL 3A and 3T3 cells, was not due to a mammalian cell product.

Characterisation of cytotoxic products

To facilitate characterisation and comparison of cytotoxins produced by the various isolates of *B. piliformis*, all subsequent assays were performed on HeLa cell monolayers.

The stability of cytotoxin was evaluated at various temperatures. Filtrates from infected cultures stored at 4°C showed < 10% loss of activity in 60 days. Samples stored at -20°C for 2 weeks had lost > 60% of cytotoxic activity. Thermal stability of culture filtrates was determined by heating the samples at 50, 60 or 80°C for 30 min. The cytotoxins from all four isolates were heat-labile, retaining < 10% of the original cytotoxicity after treatment at 60°C for 30 min (fig. 2). Fractions of culture filtrates obtained by means of molecular sieving membranes were examined. Those of < 100 kDa were only slightly, if at all, cytotoxic. Those of > 100 kDa destroyed mammalian cell monolayers (table III).

To assess the effect of trypsin on cytotoxicity, the amount of protein in culture filtrates was decreased by

Table III. Cytotoxicity of culture filtrates fractionated by ultrafiltration

Isolate	Mean (SEM) percentage cytotoxicity of culture filtrate fractions on HeLa cells	
	< 100 kDa	> 100 kDa
B	0.0 (0.0)	98.0 (2.8)
G	11.5 (2.1)	89.2 (3.1)
H	3.8 (2.0)	100.0 (4.5)
GP	0.0 (0.0)	89.8 (3.0)

growing the organisms in medium containing serum 1% supplement rather than 10% as routinely used in culture of mammalian cells. This did not adversely affect cytotoxinogenesis (data not shown). Treatment of culture filtrates with trypsin 2 mg/ml at 37°C for 30 min destroyed 70–80% of the cytotoxin from isolates B, G, H and GP. This effect was specifically blocked by pre-incubation of the trypsin with soy bean trypsin inhibitor.

Discussion

This study showed that four of seven *B. piliformis* isolates produced substances cytotoxic to 3T3 mouse fibroblasts and BRL 3A rat hepatocytes. Inability to propagate isolate B on BRL 3A may have been due to cytotoxin production; the rat hepatocytes became rounded and detached after inoculation, preventing bacterial growth. Possibly the lower cytotoxicity observed when isolates G, H and GP were grown on 3T3 rather than BRL 3A cells may reflect uptake of cytotoxin by the cell monolayers during propagation. Preliminary characterisation of the cytotoxin revealed thermal lability and sensitivity to trypsin treatment, suggesting that the active components were protein in nature.

Earlier investigators recognised heterogeneity in *B. piliformis*, with variable degrees of immunological cross-reactivity^{14,15} and differences in protein and antigenic profiles.¹¹ These findings have prompted speculation that *B. piliformis* has a number of biovars. In this study, cytotoxins produced by different *B. piliformis* isolates varied in mammalian cell specificity. The isolates could be classified as follows: (1) isolate B, cytotoxic for BRL 3A cells; (2) those cytotoxic for 3T3

cells but with little if any cytotoxicity for BRL 3A cells (isolates G, H and GP); and (3) those lacking cytotoxicity for both cell lines (isolates E, M and R1). Isolates G, H and GP, which each produced cytotoxin active against 3T3 cells, are antigenically the most similar of the seven isolates examined.¹¹ Further information on cytotoxins may facilitate taxonomic classification and delineation of biovars in *B. piliformis*.

Culture filtrates from *B. piliformis* were toxic for various mammalian cell types including fibroblasts, liver, kidney and epithelial cells. This may indicate that the toxic factor utilises a non-specific cytotoxic mechanism or that it binds to membrane receptors found on numerous cell types.

Further study of cytotoxicity of *B. piliformis* may yield information on the pathogenesis of Tyzzer's disease. Two forms of Tyzzer's disease are recognised in animals—an acute clinical form, mainly affecting weanling animals or those with reduced resistance, and a subclinical form, occurring mainly in adult animals and liable to develop into acute clinical diseases as a result of adverse environmental circumstances. It seems possible that non-cytotoxinogenic *B. piliformis* isolates are of reduced virulence and therefore more likely to cause subclinical disease.

The role, if any, of cytotoxins in the mucosal necrosis and diarrhoea associated with Tyzzer's disease remains to be elucidated. Possibly they assist the organism in invading enterocytes or hepatocytes, or cause lysis of the host cells after intracellular multiplication has occurred.

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