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Identification of a Cytotoxin Produced by *Legionella pneumophila*

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Culture filtrates of *Legionella pneumophila* were cytotoxic for Chinese hamster ovary cells. The cytotoxin was found to be methanol soluble, heat stable, and stable from pH 5 through 8.

Legionella pneumophila, a recently described human pathogen (4, 8), causes lower respiratory tract infections associated with significant morbidity and mortality (3, 5, 11). The acute bronchopneumonia caused by *L. pneumophila* is frequently accompanied by extrapulmonary disturbances (5). These include encephalopathy, chemical evidence of damage to muscle and pancreas, hepatic dysfunction, renal failure, blood loss in the gut, and urinary, respiratory, and gastrointestinal tract symptoms (3, 5, 11).

The mechanism by which *L. pneumophila* causes disease is unknown. The pulmonary and extrapulmonary disturbances accompanying Leionnnaires disease have prompted speculation that *L. pneumophila* may cause disease by producing a toxin (6, 11). *L. pneumophila* produces endotoxin-like material (12), hemolysin (2), and protease (9); however, their roles in disease are unknown.

This report describes a cytotoxin in culture filtrates of *L. pneumophila*. This cytotoxin was found to be a heat-stable, methanol-soluble, low-molecular-weight material that is distinct from the hemolysin or protease of *L. pneumophila*.

L. pneumophila Knoxville 1, Togus 1, and Bloomington 2 were obtained from the Center for Disease Control, Atlanta, Ga. Medium used for maintenance of cultures (GC-FC agar) contained GC medium base (Difco) supplemented with L-cysteine (0.4 g/liter) and soluble ferric pyrophosphate (0.25 g/liter). Cultures were grown at 37°C on GC-FC agar slants in a 5% CO_2 atmosphere and transferred weekly. Stock cultures of all strains were stored in tryptic soy broth containing 20% glycerol (vol/vol) at −70°C. Purity of cultures was monitored by Gram stain morphology, growth characteristics, pigment production, and absence of growth on blood or nutrient agar plates.

The complex liquid medium of Warren and Miller (10) without ferric pyrophosphate was used for cytotoxin production. Complex media (30 ml in a 250-ml flask) was inoculated to an initial turbidity of 30 to 35 Klett units with a bacterial cell suspension obtained from 3- to 4-day GC-FC agar slant cultures. Flasks were incubated at 35°C on a rotary shaker water bath at 200 rpm for 48 h. Cultures were centrifuged at 9,000 rpm for 15 min, and the supernatant fluids were filter sterilized with 0.22-μm membrane filters (Millipore Corp.). Culture filtrates were frozen at −70°C.

Chinese hamster ovary (CHO) cells were grown in 75-cm^2 flasks (Falcon) in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal calf serum; 2 mM glutamine; 1% nonessential amino acids, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Flow Laboratories, Inc., Inglewood, Calif.); and carbenicillin (100 μg/ml). Cells were grown at 37°C in 7% CO_2. Confluent monolayers were harvested, and cells were suspended in fresh DME to a final density of 10^5 cells per ml. Cells were transferred to new flasks or microtiter assay plates (Limbro TS-FB-96, Flow Laboratories). The CHO cell cytotoxicity assay was used as described by Iglewski and Sadoff (7). Volumes of culture filtrate, ranging from 10 to 70 μl, were added to microtiter wells, and 2×10^4 CHO cells in 200 μl of DME were added. Cytotoxicity was scored colorimetrically (7). The minimal cytotoxic dose (MCD) was defined as the minimal amount of bacterial culture filtrate (microliters) required to inhibit a color change of the media in 3 to 4 days.

The culture filtrates from all three strains of *L. pneumophila* tested demonstrated cytotoxicity in the CHO cell cytotoxicity assay (Table 1). The MCD of culture filtrates obtained on different days varied from 20 to 40 μl. However, a given culture filtrate had the same MCD when tested on various days. Sterile complex media (20 to 100 μl per well) showed no toxic effect on CHO cells.

The onset of cytotoxicity was observed micro-

TABLE 1. *Production of a cytotoxin by three different strains of L. pneumophila*

Strain	MCD[a] (ul)
Knoxville 1	20
Togus 1	20
Bloomington 2	20
Media control	ND[b]

[a] Minimal amount of bacterial culture filtrate required to inhibit color change of the media from pink to yellow in 3 days.

[b] ND, Not detectable. Complex media at 20 to 100 μl per well was not toxic for CHO cells.

scopically as early as 24 h after the addition of 40 μl of Knoxville strain culture filtrate (Fig. 1). The earliest morphological changes observed were the rounding up of cells and poor development of a monolayer (Fig. 1B) as compared to control cells (Fig. 1A). After 48 h of incubation with culture filtrate, additional CHO cells became rounded and many degenerated and lysed, releasing cellular debris into the tissue culture media (Fig. 1D). Control cells formed almost a complete monolayer by 48 h (Fig. 1C). Seventy-two hours after the addition of bacterial culture filtrate, all the cells degenerated while control cells formed a confluent monolayer (Fig. 1E and F). The Togus and Bloomington strain filtrates showed similar effects in the CHO cytotoxicity assay.

Culture filtrate samples (2 ml) heated at 37 and 56°C for 1 h or 100°C for 30 min showed no loss of cytotoxin activity. The cytotoxic activity in *L. pneumophila* culture filtrates was also stable at 4°C and stable to freezing (−70°C) and thawing. Thus the cytotoxin is resistant to heating and freezing.

The effect of pH on cytotoxin activity in culture filtrates was determined by adjusting the pH of samples (2 ml) by addition of either 2 M HCl or 2 M NaOH. After incubation for 2 h at 37°C, the solutions were neutralized and brought back to the original volume by use of Amicon Minicon filters treated with 1% gelatin. The samples were filter sterilized and assayed for cytotoxic activity. The initial pH of the culture filtrate was pH 7.4. Cytotoxic activity was stable over the pH range of 5 to 8 (Table 2). A modest loss in cytotoxic activity occurred after incubation at pH 1 to 3 and at pH 9. Above pH 9 the cytotoxin lost considerable activity. After incubation at pH 10 and 11, the MCD increased from 20 to 50 μl.

L. pneumophila culture filtrates were methanol extracted by adding absolute methanol to culture filtrate at a ratio of 10:1 with vigorous stirring. The suspension was allowed to stand at room temperature for 30 min and then centrifuged at 28,000 × *g* for 30 min at 4°C, and the supernatant was removed. The methanol-soluble fraction was evaporated to dryness at 37°C with a rotary evaporator while the methanol-insoluble fraction was dried by incubation in a 37°C water bath. The methanol-soluble and -insoluble fractions were reconstituted to original volume with distilled water, filter sterilized, and assayed for cytotoxic activity. Sterile complex media was similarly extracted with methanol, and its methanol-soluble and -insoluble fractions were used as controls. Cytotoxic activity was present only in the methanol-soluble fraction of the culture filtrate. No cytotoxic activity was lost upon methanol extraction (100% recovered). The cytotoxin retained its heat stability after methanol extraction. No cytotoxicity was observed in the methanol-soluble or -insoluble fractions of the complex media control.

L. pneumophila culture filtrate was incubated with various proteolytic enzymes which were then inactivated by the addition of methanol (Table 3). The cytotoxic activity in the methanol-soluble fraction of culture filtrates was unaffected by trypsin treatment. Papain and pronase treatment increased the MCD 58 and 54%, respectively (Table 3). The cytotoxin's sensitivity to papain and pronase digestion suggests that it is a protein.

Culture filtrate (2 ml) was placed in Spectra/Por 6 dialysis tubing with a molecular weight cutoff of 1,000 (Spectrum Medical Industries Inc., Los Angeles, Calif.) and dialyzed against 4 ml of a potassium phosphate buffer (pH 7.4) for 20 h. Both the retentate and dialysate were filter sterilized and tested for cytotoxic activity. The cytotoxic activity of the retentate was equal to that of the dialysate. The original level of cytotoxic activity was recovered by combining the activity in the dialysate and retentate. Dialysis buffer had no cytotoxic effect on CHO cells. The cytotoxin remained dialyzable after methanol extraction, which indicates that both the crude and methanol-soluble activities are of low molecular weights.

This study reports that *L. pneumophila* produces a cytotoxin which causes inhibition of growth and death of CHO cells (Table 1; Fig. 1). A previous paper by Baine et al. (2) reported that culture filtrates of *L. pneumophila* had no cytotoxicity for CHO cells. This discrepancy may be due to the use of different bacterial media. Baine et al. (2) used Mueller-Hinton media, whereas we used the complex media of Warren and Miller (10). Production of many bacterial toxins is known to be media dependent (1).

L. pneumophila has been shown to produce a

hemolysin (2), protease (9; M. Thompson, B. H. Iglewski, and R. Miller, manuscript in preparation), and an endotoxin-like material (12). The CHO cell cytotoxic activity of *L. pneumophila* culture filtrate is distinct from these other products. The cytotoxin was found to be heat stable,

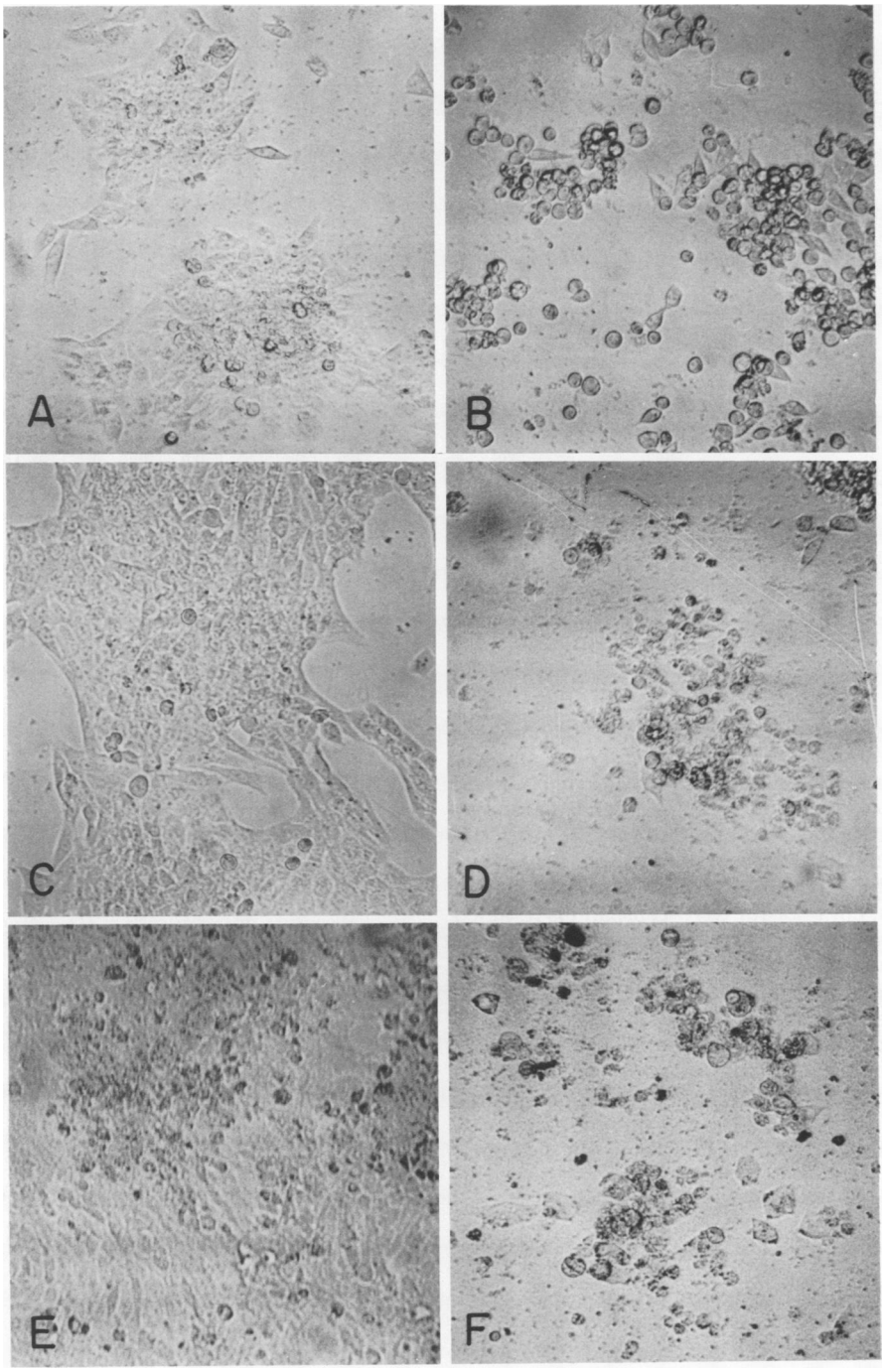

FIG. 1. *CHO cell growth in the absence (A, C, and E) and the presence (B, D, and F) of 40 μl of L. pneumophila culture filtrate. (A) and (B) 24 h; (C) and (D) 48 h; (E) and (F) 72 h. L. pneumophila culture filtrate was added at 0 h.*

TABLE 2. *Effect of pH on L. pneumophila cytotoxin activity*[a]

pH	MCD[b] (μl)
1	30
3	30
5	20
7	20
8	20
9	30
10	50
11	50
Untreated[c]	20

[a] The pH of culture filtrates was adjusted and held for 2 h at 37°C, neutralized, and tested for cytotoxic activity.

[b] Defined in footnote *a* of Table 1.

[c] The pH of untreated culture filtrate was 7.4.

TABLE 3. *Effect of proteolytic enzymes on L. pneumophila cytotoxin activity*[a]

Enzyme tested	Enzyme concn[b]	% MCD of control
None	0.0	100
Trypsin	0.6	100
Papain	0.4	58
Pronase	0.5	54

[a] Culture filtrates were treated with enzymes at 37°C for 1 h and methanol extracted, and the soluble fraction was assayed for cytotoxic activity.

[b] Milligrams of enzyme per milliliter of culture filtrate.

methanol soluble, dialyzable, sensitive to pronase and papain, and insensitive to trypsin. These properties suggest the cytotoxin is a small polypeptide. In contrast, the hemolysin is partially heat labile and has no toxic effect on CHO cells (2). The protease activity was heat labile (60°C, for 20 min), destroyed by methanol extraction, not toxic for CHO cells, and is retained in dialysis tubing (M. Thompson et al., manuscript in preparation). The protein-like nature and apparent size of the CHO cell cytotoxin suggests that it is not related to the organism's endotoxin-like material (12).

The pulmonary and extrapulmonary disturbances which reportedly accompany Legionnaires disease suggest the possible involvement of a bacterial toxin (6, 11). The cytotoxin described in this report might be considered as a potential candidate for such a virulence factor; however, its role in *L. pneumophila* infections awaits further study.

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