Legionella pneumophila Inhibits Protein Synthesis in Chinese Hamster Ovary Cells

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Legionella pneumophila is a gram-negative facultative intracellular parasite that causes Legionnaires disease. To explore the interactions between L. pneumophila and host cells, we have developed a continuous cell line model of infection. We show that about 80% of Chinese hamster ovary (CHO) cells were associated with L. pneumophila after incubation for 3 h at a multiplicity of infection of 20 bacteria per cell. Within 3 to 4 h of incubation with L. pneumophila, protein synthesis of CHO cells was markedly inhibited, as shown by the reduction of incorporation of radiolabeled amino acids into proteins. L. pneumophila did not inhibit transport of amino acids or cause degradation of newly synthesized proteins in CHO cells. Cytochalasin D blocked internalization of L. pneumophila by CHO cells, yet CHO cell protein synthesis was inhibited. These results indicated that L. pneumophila could inhibit host protein synthesis from the cell exterior. L. pneumophila that had been killed with antibiotics prior to incubation with CHO cells still inhibited protein synthesis, indicating that the inhibition of CHO cell protein synthesis occurred in the absence of de novo protein synthesis by L. pneumophila.

Legionella pneumophila is the gram-negative bacterial pathogen which causes legionellosis in humans (36). L. pneumophila is a facultative intracellular parasite of monocytic phagocytic cells (14). It has been postulated that the survival of L. pneumophila within phagocytic cells may be the result of a bacterial product which inhibits phagolysosomal fusion (14, 15). In addition, the multiple systemic symptoms associated with legionellosis have prompted speculation that L. pneumophila may produce toxins. Friedman and coworkers (10) demonstrated that a 1.2-kDa peptide from Legionella culture supernatant was cytotoxic for Chinese hamster ovary (CHO) cells, as evidenced by a pH change of the medium after prolonged incubation with fractions containing the peptide. In later reports, this peptide from Legionella culture supernatant was shown to inhibit respiratory burst in human neutrophils (11).

Although L. pneumophila appears to release a small toxic peptide, it is possible that this pathogen expresses additional toxic molecules. To explore this possibility, we have developed a continuous cell line model of Legionella infection that allows synchronous infection of a high percentage of CHO cells at a low multiplicity of infection (MOI). We have utilized this model to show that L. pneumophila inhibits protein synthesis in CHO cells.

MATERIALS AND METHODS

Bacterial strains and media. L. pneumophila serogroup 1 Philadelphia strain 1 was obtained from the Centers for Disease Control and serially passaged in embryonated hen eggs until a 50% lethal dose for eggs of approximately 10 bacteria was attained (6). L. pneumophila were inoculated from egg yolk suspensions onto buffered charcoal-yeast extract agar (BCYE) plates (8). L. pneumophila retains virulence for many passages on BCYE (16). A virulent stock of L. pneumophila was maintained by suspending bacteria in yeast extract broth containing 40% glycerol and freezing 0.10-ml aliquots at −70°C. Purity of Legionella cultures was intermittently assessed by demonstrating that the bacteria grew on BCYE but not on Luria-Bertani plates (22) and by immunostaining.

Continuous cell lines. Wild-type CHO cells (WTB CHO) were a gift from April Robbins (Genetics and Biochemistry Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md.). HeLa cells were a gift from Jerry Kaplan (University of Utah), and Vero cells were a gift from Spotswood Spruance (University of Utah). Chinese hamster ovary cells which were auxotrophic mutants for glycine, adenosine, and thymidine were a gift from Stuart Arfin (University of California, Irvine). Vero and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 5% calf serum. CHO cell strains were incubated in medium A, which is minimal essential medium (Auto Mod; Sigma Chemical Co., St. Louis, Mo.) supplemented with proline, glycine, glutamine, adenosine, thymidine, and 5% heat-inactivated fetal calf serum (HyClone Laboratories, Inc., Logan, Utah).

Experimental infections of continuous cell lines. CHO, Vero, and HeLa cells were seeded at 104 cells per well into 24-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, N.J.) for 36 h. Cells were incubated overnight in antibiotic-free medium prior to exposure to the bacteria. L. pneumophila was suspended in phosphate-buffered saline (PBS) at a density of 109 bacteria per ml and added to monolayers in antibiotic-free tissue culture medium to achieve an MOI of 20:1. After incubation in antibiotic-free medium for 3 h, cell supernatants were removed and the monolayers were washed three times with 1 ml of sterile PBS to remove unattached bacteria. After further incubation in

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tissue culture medium, the percentage of cells infected was determined by immunostaining as described below.

**Growth curves of *L. pneumophila* in CHO cells.** CHO cells were seeded in 35-mm tissue culture dishes (Becton Dickinson Labware). Monolayers were incubated with *L. pneumophila* at an MOI of 2:1 for 3 h and then washed and maintained in antibiotic-free medium A. At each time, supernatant fluid was removed, diluted in sterile PBS, and inoculated onto BCYE plates. CHO cells were lysed with 1 ml of sterile distilled water to release intracellular *L. pneumophila*, and aliquots of the cell lysates were immediately diluted in distilled water and inoculated onto BCYE agar plates. *L. pneumophila* was shown to remain viable but did not multiply in sterile distilled water for 30 min. In antibiotic-free medium A, the bacteria remained viable for 8 h but did not multiply (data not shown).

**Peroxidase-conjugated antibody staining.** Peroxidase-conjugated antibody staining was used to examine cell monolayers in 24-well plates. Cells were washed three times with PBS and then fixed for 30 min with 3% Formalin in PBS. Following fixation, the cells were washed with PBS (1 ml per well) and incubated (1:300, 10 min), permeabilized with 0.1% of 100% methanol per well for 10 min, and then washed again with PBS (1 ml per well, three times). Rabbit antisera against *Legionella* serogroups 1 through 6 (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) was diluted 1:100 in PBS, and 200+μl was added to each monolayer for 45 min at 37°C. The cells were washed three times with 1 ml of PBS, and 200 μl of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.), diluted 1:100 in PBS, was added and incubated with the cells at 37°C for 45 min. Monolayers were washed with PBS, exposed to dianaminobenzidine (0.5 mg/ml in PBS) and H₂O₂ (0.001%, vol/vol) in PBS for 30 min, and then washed with PBS prior to examination with an inverted microscope.

**Fluorescence microscopy.** Intracellular bacteria were distinguished from extracellular bacteria by using immunofluorescence staining of cells cultured on 10-mm glass cover slips (13, 31). Following experimental infections, CHO cell monolayers were washed with PBS and incubated at 37°C for 30 min with rabbit anti-*L. pneumophila* antiserum diluted 1:100 (vol/vol) in PBS. All subsequent incubations with antibodies were at 37°C. After the monolayers were rinsed with PBS, they were incubated with rhodamine-conjugated goat anti-rabbit antibody (1:300, vol/vol, in PBS) for 30 min and then washed and fixed with 3.7% Formalin in PBS for 15 min at room temperature. Following permeabilization of monolayers with 100% methanol for 5 min, cells were washed with PBS and then incubated with rabbit anti-*Legionella* antiserum for 30 min. Monolayers were then washed with PBS and incubated with fluorescein-conjugated goat anti-rabbit antibody for 30 min. After a final wash in PBS, fluorescence was visualized by using a Zeiss fluorescence microscope with appropriate filters to distinguish the rhodamine and fluorescein conjugates.

**Protein determinations.** Protein concentration was measured with Bradford reagent (Pierce Chemical Company, Rockford, Ill.) by using bovine serum albumin as the standard. Aliquots were obtained after lysis of the CHO cell monolayers with 0.1% sodium dodecyl sulfate (SDS) or 0.1% Triton X-100 in water.

**Radiolabeling experiments.** Radiolabeling studies were done by adding 0.5 to 1.0 μCi of [³⁵S]methionine or [¹⁴C]leucine (ICN Radiochemicals, Irvine, Calif.) to each well in 0.5 ml of medium A and incubating for 1 h at 37°C. The medium was removed, and the monolayers were washed three times with 1 ml of PBS and lysed by addition of 0.5 ml of 0.1% SDS in water. A 0.1-ml aliquot was removed for protein measurement. A 0.35-ml aliquot was brought to a final concentration of 20% trichloroacetic acid, frozen at -20°C for 1 h, and then thawed, and the resultant precipitates were collected on fiberglass filters (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using a Hoefer filter apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The filters were placed in 10 ml of Optifluor (Packard Instrument Company, Inc., Downers Grove, Ill.), and radioactivity was measured by using a Hewlett Packard scintillation counter (Packard Instrument Company, Inc.).

**L. pneumophila culture conditions and cell toxicity.** To determine the effect of culture density on *L. pneumophila* toxicity to CHO cells, bacteria were inoculated onto BCYE solid medium (100-mm dishes; Becton Dickinson Labware) at different initial concentrations. Aliquots (100 μl) of *L. pneumophila* were thawed and spread on a 2-cm² area on BCYE plates and then cultured for 16 h. The bacteria were then scraped from the plates and suspended in PBS. Quantitation of CFU was estimated by measuring the absorbance (optical density at 550 nm) of 100 ml of 550 μl of 1:10 dilution of growing cultures in PBS, and 3 to 10² CFU of *L. pneumophila* were inoculated and spread over the entire surface of separate BCYE plates with a sterile Dacron swab and then incubated at 37°C for 16 h. Bacteria from each plate were suspended in sterile PBS, and the total number of bacteria per plate was estimated by measuring absorbance and by colony counting. Bacteria from each plate were diluted in PBS and incubated with CHO cells (MOI of 20:1) for protein synthesis measurements as described above.

**Degradation of proteins in CHO cells infected with *L. pneumophila*.** CHO cells were incubated with 1 μCi of [³⁵S]methionine per ml in medium A for 2 h to label proteins. Following removal of the radiolabel by washing, the monolayers were incubated with *L. pneumophila* at a MOI of 20:1 for 3 h. The monolayers were washed and then incubated in medium A for 1 h. Radioactivity was determined after lysis with SDS (0.1%) and trichloroacetic acid (TCA) precipitation, as described above.

**Treatment of *L. pneumophila* with washing, trypsin, or heat.** *L. pneumophila* was suspended in PBS and adjusted to an optical density at 550 nm of 0.2. One milliliter of the suspended *L. pneumophila* was added to a 0.2 μM nitrocellulose filter, and 30 ml of sterile PBS was forced over the filter. Washed *L. pneumophila* was removed from the filter by gentle agitation in sterile PBS, and the optical density at 550 nm was measured. These *L. pneumophila* cells were incubated with CHO cells at an MOI of 20:1, and the collected effluent from the wash was incubated with CHO cells for 4 h before protein synthesis was measured.

Trypsin treatment was effected by suspension of *L. pneumophila* in PBS with 0.25% trypsin (GIBCO Laboratories, Life Technologies, Inc., Grand Island, N.Y.) for 15 min. After addition of an equimolar amount of soybean trypsin inhibitor (Sigma Chemical Co.), CHO cells were infected at an MOI of 20:1. Controls were uninfected cells and cells infected with *L. pneumophila* which were neither washed nor trypsin treated. CHO cell protein synthesis was then measured as described above.

**L. pneumophila** was resuspended in PBS at an optical density at 550 nm of 0.0. Aliquots were placed in sterile capped polypropylene microcentrifuge tubes, and immersed in boiling water for 2 min. The tube was removed and cooled to room temperature with tap water. After this treatment, the bacte-
ria were incubated with CHO cells (MOI of 20:1) as described above.

Cytochalasin D treatment. Uptake of bacteria was inhibited by cytochalasin D by using a modification of the method of Elliot and Winn (7). CHO cell monolayers were incubated with medium A containing 0.5 mg of cytochalasin D (Sigma Chemical Co.) per ml for 16 h before the addition of *L. pneumophila* at an MOI of 20:1 for 3 h. Cytochalasin D (0.5 mg/ml) was included in the PBS and in medium A for subsequent washes and incubation. The CHO cells were incubated for 1 to 2 h in medium A before protein synthesis was measured or immunolocalization studies were performed.

Chromium uptake and release. Release of $^{51}$Cr and intracellular concentrations of $^{51}$Cr were compared in control cells and *L. pneumophila*-infected cells. CHO cells were incubated overnight with medium A containing 1 $\mu$Ci of $^{51}$Cr per ml. Cells were washed with PBS and then incubated with *L. pneumophila* at an MOI of 20:1 in medium A for 3 h. The monolayers were washed to remove unattached bacteria and incubated in medium A without antibiotics for 1 h. Radioactivity was measured in aliquots of supernatants to determine spontaneous release of label from cells and in aliquots from cells lysed with 0.1% SDS.

**Methodine uptake.** Transport of methionine into cells was measured by using a modification of the method of Foster and Pardee (9). CHO cells were infected with *L. pneumophila* at an MOI of 30:1 and then washed and incubated in PBS for 1 h at 37°C. Cells were then incubated in PBS containing [35S]methionine (1 $\mu$Ci/ml). After 5 and 15 min of incubation, cells were chilled by placing the tissue culture plates on an ice bath. The culture supernates were removed, and cell monolayers were rapidly washed three times with 1 ml of PBS (4°C). After the cells were lysed with 0.5 N NaOH, aliquots were removed for assay of radioactivity and for determination of protein concentration.

**Antibiotic killing of L. pneumophila.** *L. pneumophila* was cultured on BCYE and suspended at a concentration of approximately $10^9$ bacteria per ml in PBS or in PBS containing 100 $\mu$g of gentamicin (Sigma Chemical Co.) per ml and 500 $\mu$g of erythromycin (Sigma Chemical Co.) per ml. Protein synthesis was measured by adding [3H]methionine (1 $\mu$Ci/ml) and incubating for 1 h. Aliquots were removed at 15-min intervals, and bacteria were separated from extracellular [3H]methionine by spinning for 2 min at 8,000 $\times$ g in a microcentrifuge. The pellets were washed by suspending them in 1 ml of PBS followed by centrifugation in the microcentrifuge. The bacteria were solubilized with 0.5 ml of 0.1% SDS and heated at 95°C for 2 min. A 0.1-ml aliquot was removed for protein determination, and a 0.35-ml aliquot was adjusted to 20% TCA and assayed for radioactivity as described above.

Viability of antibiotic-treated *L. pneumophila* was assessed by using bacteria which had been incubated in PBS or in PBS with gentamicin (100 $\mu$g/ml) and erythromycin (500 $\mu$g/ml) for 1 h. Bacteria were diluted and incubated on BCYE for 4 days before determination of colony numbers.

**RESULTS**

Initially, we compared the ability of *L. pneumophila* to adhere to different continuous cell lines. *L. pneumophila* was incubated for 16 h on BCYE solid medium, suspended in PBS, and then incubated for 3 h with cell monolayers in antibiotic-free tissue culture medium at an MOI of 20:1. After unattached bacteria were removed by washing, 80 to 90% of the CHO cells were associated with at least one *L. pneumophila* bacterium, as evidenced by peroxidase antibody staining. In contrast, only 10% of HeLa and 35 to 45% of Vero cells were associated with *L. pneumophila*. Therefore CHO cell lines were used for the experiments described below.

We next determined if CHO cells could support the growth of *L. pneumophila*. *L. pneumophila* was incubated with CHO cells at a low MOI (2:1) since a higher MOI (20:1) caused disruption of CHO cell monolayers 8 to 10 h after incubation (data not shown). Immunostaining showed that approximately 5% of the CHO cells were associated with *L. pneumophila* after 3 h of incubation (data not shown) at an MOI of 2:1. There appeared to be a lag phase in *Legionella* growth of about 6 to 9 h (Fig. 1). Between 12 and 24 h, *Legionella* numbers increased logarithmically, with a doubling time of about 2 h. Quantitation of *L. pneumophila* in the tissue culture medium indicated that after 24 h of incubation, significant numbers of bacteria were released and many CHO cells were lysed. Since tissue culture medium and CHO cell conditioned culture medium did not support the growth of *L. pneumophila*, these results indicated that *L. pneumophila* multiplied within CHO cells.

CHO cells incubated with *L. pneumophila* at an MOI of 20:1 exhibited cytopathic changes after 8 to 10 h (data not shown). One mechanism by which bacteria can cause cytotoxicity is by inhibiting host cell macromolecular synthesis. To test this hypothesis, we measured the rate of incorporation of radiolabeled amino acids into TCA-precipitable material in both infected and uninfected CHO cells. After extracellular *L. pneumophila* was removed from the infected CHO cells by washing, host cell protein synthesis was found to be inhibited by 70 to 80%. Protein synthesis in infected cells did not appear to recover (Fig. 2). Similar results were found by using [3H]leucine. These results indicated that infection of CHO cells with *L. pneumophila* resulted in a marked reduction in the ability of CHO cells to incorporate radiolabeled amino acids into proteins.
To determine if host cell amino acid transport was inhibited by *L. pneumophila* infection, we examined methionine uptake in both infected and uninfected CHO cells as described in Materials and Methods. Our results showed that over 15 min, the rate of methionine uptake was similar for both infected and uninfected CHO cells (Fig. 3). Under these same experimental conditions, CHO cell protein synthesis was blocked by 80% in infected cells. Thus, CHO cells infected with *L. pneumophila* appeared to transport methionine at the same rate as uninfected cells. Assays of CHO cell permeability were performed by incubating CHO cells overnight with 51Cr, followed by rinsing the monolayers with PBS and incubating them in medium A with *L. pneumophila* for 3 h. The extracellular bacteria were removed by washing with PBS and cells incubated for 1 h in medium A. Quantitation of cell-associated and extracellular 51Cr showed 13% spontaneous release of 51Cr from uninfected control cells and 10% release from cells infected with *L. pneumophila*. These results indicated that *L. pneumophila* did not disrupt the integrity of CHO cell membranes.

The toxic effect of *L. pneumophila* on CHO cell protein synthesis was dependent on the conditions used for growth of the bacteria. *L. pneumophila* incubated at starting concentrations of $3 \times 10^6$, $3 \times 10^7$, and $3 \times 10^{10}$ CFU per plate yielded approximately $7 \times 10^6$, $5 \times 10^9$, and $1.2 \times 10^{11}$ CFU per plate, respectively, after incubation for 16 h at 37°C. *L. pneumophila* from each of these plates was incubated with CHO cells at an MOI of 20:1. Only bacteria from plates with approximately $10^{11}$ CFU per plate caused significant inhibition of protein synthesis in CHO cells. Error bars show standard deviation from the mean.

![FIG. 2. Inhibition of CHO cell protein synthesis by *L. pneumophila*. $[^{35}S]$methionine incorporation into TCA-precipitable material (1-h pulses) was measured at times indicated after a 3-h incubation of bacteria with CHO cells as described in Materials and Methods. Symbols: □, control cells; △, cells incubated with *L. pneumophila*. Error bars represent standard error of the mean.](https://iai.asm.org/article-figures/)

![FIG. 3. Effect of *L. pneumophila* infection on transport of $[^{35}S]$methionine into CHO cells. $[^{35}S]$methionine uptake after 5- and 15-min incubations was measured in uninfected and infected CHO cells (see Materials and Methods). Symbols: ▲, *L. pneumophila*; □, uninfected cells. Error bars show standard deviation from the mean.](https://iai.asm.org/article-figures/)

![FIG. 4. Dependence of inhibition of CHO cell protein synthesis by *L. pneumophila* on bacterial culture conditions. Bacteria seeded at different initial concentrations were incubated for 16 h at 37°C, suspended in PBS, and incubated with CHO cells at an MOI of 20:1. Only bacteria from plates with approximately $10^{11}$ CFU per plate caused significant inhibition of protein synthesis in CHO cells. Error bars show standard deviation from the mean.](https://iai.asm.org/article-figures/)
L. pneumophila inhibited protein synthesis after extensive washing of the bacteria with PBS prior to infection. The effluent from the washed bacteria did not cause reduction of CHO cell protein synthesis. Treatment of L. pneumophila with 0.25% trypsin for 15 min at 37°C prior to infection of CHO cells had no effect on protein synthesis inhibition (data not shown). Moreover, bacteria which had been heated to 95°C for 2 min prior to incubation with CHO cells did not cause inhibition of protein synthesis. These results indicated that the toxic Legionella component which inhibited CHO cell protein synthesis did not appear to be secreted in an active form, that it was trypsin resistant, and that it was heat sensitive.

The CHO cell protein synthesis inhibition described above could be due to a molecule produced by L. pneumophila after contact with CHO cells. To examine this possibility, we determined whether treatment of L. pneumophila with antibiotics prior to incubation with CHO cells would alter the toxic activity on cell protein synthesis. L. pneumophila was treated with high levels of gentamicin and erythromycin in PBS for 1 h. After this treatment, Legionella protein synthesis was completely inhibited (Fig. 7A) and there was about a 5-log reduction in viable L. pneumophila. Antibiotic-killed L. pneumophila inhibited protein synthesis of CHO cells by 80%, as did bacteria incubated in PBS without antibiotics (Fig. 7). Immunofluorescence showed that antibiotic-killed L. pneumophila was associated with and internalized by CHO cells. If 5% of the Legionella cells were still viable after this antibiotic treatment, the effective MOI would be approximately 2 bacteria per cell. At this MOI CHO cell protein synthesis was reduced by only 15% (Fig. 7). These results indicated that L. pneumophila retains its toxic activity even after being killed with antibiotics and strongly suggested that ongoing Legionella protein synthesis was not required for toxicity.

**DISCUSSION**

Our results indicate that L. pneumophila causes inhibition of protein synthesis in CHO cells. The inhibition did not appear to result from diminished amino acid transport (Fig. 3) or from disruption of CHO cell membranes, as measured by 51Cr release. Inhibition of CHO cell protein synthesis occurred under conditions in which internalization of bacteria was blocked by cytochalasin D treatment, suggesting that extracellular L. pneumophila could inhibit protein synthesis of CHO cells. However, L. pneumophila did not appear to release an active toxic factor(s) into the culture medium. Inhibition of protein synthesis was not observed after L. pneumophila had been boiled for 2 min, suggesting that the toxic factor is not endotoxin, which is heat stable (20).

Interactions between L. pneumophila and continuous cell
lines such as Vero, HeLa, MRC-5, Hep-2, U937, and HL-60 have been reported (19, 25, 28, 37). However, the percentage of cells infected after the initial incubation period with L. pneumophila was not reported. In these studies, peak numbers of L. pneumophila were reached after long periods of incubation (2 to 5 days), which suggests that initially only a small fraction of cells were infected with bacteria. In our studies, the efficient infection of a high percentage of cells in the monolayer suggests that specialized mechanisms of adherence of L. pneumophila to CHO cells may exist. Uptake of L. pneumophila by human monocytes is facilitated by engagement of complement receptors (27). Perhaps uptake in CHO cells occurs through a structure analogous to the complement receptor. We are currently investigating this possibility.

The findings presented here should facilitate further studies of the cell biology of the host-parasite relationships in L. pneumophila infections. The CHO cell model represents a valuable tool for further studies of basic mechanisms of L. pneumophila infection. We demonstrate that a high percentage of CHO cells in monolayers are infected synchronously after a 3-h incubation with L. pneumophila. Synchronous infections allow investigation of host cell responses to infection with this intracellular parasite. Mutant CHO cell lines have been used to investigate the cell biology of other intracellular parasites (30) and to examine mechanisms of bacterial toxins. For example, CHO cells which are deficient in endosomal acidification have been used to demonstrate that diphtheria toxin requires access to an acidified compartment to be activated (32).

We cannot directly extrapolate our findings with CHO cells to monocytes or macrophages, which are primary sites of infection in humans. We have recently found that L. pneumophila inhibits protein synthesis in U937 cells and human monocytes (21a), which suggests that the CHO cell is a valid model for further study. In addition, recent reports of L. pneumophila infections of soft tissue and prosthetic valves suggest that these bacteria may invade nonphagocytic cells (2, 35).

The conditions used for Legionella growth affected the ability of the bacteria to inhibit protein synthesis in CHO cells. Only bacteria obtained from high-density cultures (approximately 10^11 CFU per plate) caused significant inhibition of CHO cell protein synthesis (Fig. 4). Previous investigations have shown that expression of diphtheria toxin (26) and invasiveness of Salmonella spp. are dependent on the conditions used for bacterial growth (18). Possibly at higher concentrations of bacteria, nutrient depletion induces expression of the factor(s) required for inhibition of protein synthesis. Alternatively, the toxic factor(s) may be expressed only during a specific phase of growth, as occurs in exotoxin expression by Staphylococcus aureus during stationary phase (29). To test this hypothesis, we will need to examine L. pneumophila obtained from broth cultures at different growth phases.

L. pneumophila inoculated onto BCYE plates at lower initial concentrations did not inhibit protein synthesis. Antibody staining showed that a much lower percentage of bacteria from these cultures adhered to CHO cells (data not shown). Thus, it is possible that bacterial growth conditions influence the expression of an adhesive molecule required for attachment to CHO cell surfaces.

Our results indicated that inhibition of CHO cell protein synthesis occurred even after extensive washing of L. pneumophila. Further, the eluant from washed L. pneumophila did not inhibit protein synthesis in CHO cells. Thus, the factor causing inhibition of CHO cell protein synthesis reported here does not appear to be soluble in tissue culture medium or PBS. In contrast, the 1.2-kDa peptide previously shown to have cytotoxic effects on CHO cells (10) and to inhibit respiratory burst in neutrophils (11) was present in Legionella culture supernates.

Most bacterial toxins that inhibit protein synthesis, such as diphtheria toxin, Pseudomonas exotoxin A, and colicin E, are soluble peptides which enter the cytosol of the affected cell (23, 33). The inhibition of CHO cell protein synthesis by L. pneumophila reported here, however, appears to be tightly associated with bacterial cells. Clostridium perfringens enterotoxin causes inhibition of protein synthesis, RNA synthesis, and DNA synthesis in Vero cells by inducing leakage of low-molecular-weight molecules as shown by sensitive assays of cell permeability utilizing ^115^Rb and [3H]uridine (17, 21). The enterotoxin appears to remain tightly associated with the Vero cell membrane, distinguishing its mechanism of action from soluble toxins. We are currently attempting to isolate the factor which inhibits CHO cell protein synthesis from L. pneumophila as a first step in understanding its mechanism of action.

Intracellular parasites depend on continued host cell function for nutrients (24). Since L. pneumophila utilizes amino acids rather than carbohydrates for its energy source (34), it is possible that inhibition of protein synthesis of the host cell would increase availability of nutrients to the bacteria. Intracellular growth of Chlamydia sp. is facilitated when host protein synthesis is inhibited by cycloheximide (12). Of course, severe inhibition of protein synthesis by L. pneumophila will eventually kill host cells. Further studies are in progress in our laboratory to determine whether L. pneumophila inhibits protein synthesis in human macrophages.

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REFERENCES