Characterization of a *Legionella pneumophila* Extracellular Protease Exhibiting Hemolytic and Cytotoxic Activities

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A preliminary screening of selected *Legionella* species for proteolytic and hemolytic phenotypes suggested a correlation between these activities. To investigate the relationship of these phenotypes, a mutant strain of *Legionella pneumophila* deficient in the expression of a 38-kilodalton (kDa) exoprotease was isolated and characterized. This strain, designated PRT8, was also found to be nonhemolytic when screened on blood agar composed of 5% canine or guinea pig erythrocytes. Strain PRT8 was serologically and biochemically identical to the parental strain with the exception of the expression of the exoprotease. Immunoblot analysis of concentrated culture filtrates from PRT8 probed with polyclonal anti-38-kDa exoprotease serum revealed no cross-reactive peptides. To resolve the role of the exoprotease in the hemolytic phenotype, the exoprotease was purified from the culture supernatant of the parental strain by a combination of ion-exchange and hydrophobic interaction chromatography steps. The hemolytic activity was found to copurify with the proteolytic activity, and analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot revealed a single protein species exhibiting an apparent molecular mass of 38 kDa. Finally, the purified exoprotease and concentrated culture supernatant from the parental strain, but not from PRT8, exhibited cytotoxicity (minimum cytotoxic activity of 0.17 U of protease activity) in a Chinese hamster ovary cell assay. These data suggest that the exoprotease is responsible for the hemolytic and cytotoxic phenotypes described for this species and therefore may be one of several determinants associated with virulence.

*Legionella pneumophila* is a facultative intracellular parasite that invades and grows in human alveolar macrophages, leading to an acute pneumonitis referred to as legionellosis. To date, no single factor has been identified as sufficient for the production of this disease, suggesting that virulence may be multifactorial. The cytopathology of infected lung tissue, as well as the extrapulmonary manifestations associated with this disease, suggests the involvement of a cytotoxin (11, 28). Several extracellular factors are elaborated by *L. pneumophila*, including exoprotease, lipase, hemolysin, cytotoxin, and acid and alkaline phosphatases (1, 3, 4, 9, 11, 23, 24). Recent studies have demonstrated that the lung cytopathology could be reproduced experimentally in guinea pigs after aerosolization of purified *L. pneumophila* exoprotease into the lungs (2, 4). Subsequent work demonstrated that lung tissue from guinea pigs infected with *Legionella* bacteria contained the exoprotease (5, 27). Purified exoprotease from *L. pneumophila* has also been shown to have dermal ulcerative activity and cytotoxicity for Chinese hamster ovary (CHO) cells (20). These studies strongly suggest that the *Legionella* exoprotease plays a role in the pathogenesis of legionellosis.

The purified *L. pneumophila* exoprotease requires zinc for enzymatic activity (metalloendopeptidase) and exhibits an apparent molecular mass of 38 to 40 kilodaltons (kDa) (6). The enzyme exhibits both gelatinase and caseinolytic activities (23). In the present study we attempted to isolate, by transposon mutagenesis, mutant strains of *L. pneumophila* deficient in the expression of several extracellular enzymes, including the exoprotease. In the course of this study, an exoprotease-deficient mutant strain (*L. pneumophila* PRT8) was isolated and characterized. This strain was nonhemolytic, and concentrated supernatants from strain PRT8 were no longer cytotoxic for CHO cells. This study confirms and extends recent reports that the *Legionella* extracellular protease is a cytotoxin and presents evidence that this protein is also responsible for the hemolytic phenotype that has been described for this species (1, 24).

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and serological reagents.** A virulent strain of *L. pneumophila* serogroup 1 (Philadelphia 1) and all other *Legionella* strains listed in Table 1 were obtained from either J. C. Feeley or Barry Fields (Centers for Disease Control, Atlanta, Ga.) and maintained on ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal-yeast extract (BCYE) agar medium (18). A spontaneous streptomycin-resistant mutant strain was isolated on BCYE agar containing 100 μg of streptomycin per ml and passed intraperitoneally through guinea pigs to confirm and maintain virulence. *Legionella* strains were screened for purity by plating onto brucella agar, on which they do not grow, and serogroups were confirmed by immunofluorescence with serotyping reagents obtained from the Centers for Disease Control. Matched pairs of human acute- and convalescent-phase sera from confirmed cases of legionellosis were obtained from Jayne Baker (North Carolina State Laboratory of Public Health, Raleigh).

**Determination of proteolytic and hemolytic activities.** Protease activity was screened on BYE agar supplemented with 1% powdered milk (Cost Cutter, Kroger), and hemolytic activity was similarly screened on BYE agar medium supplemented with 5% defibrinated canine or guinea pig erythrocytes. Protease activity was assayed using Azo-coll (Calbiochem-Behring, La Jolla, Calif.) as described previously (6). One unit of protease activity was defined as the amount...
TABLE 1. Analysis of protease and hemolysin activities in selected Legionella species

<table>
<thead>
<tr>
<th>Species</th>
<th>Activitya</th>
<th>Protease</th>
<th>Hemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. hacklei Lansing 2</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. micdadei P1-12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. oakridensis OR-10</td>
<td>W</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. pneumophila group 1</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. pneumophila group 2</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. bozemanii VIC</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. dumoffi NY-23</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. gormanii L-10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. jordanis BL-540</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. anisa WA-316</td>
<td>++</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>L. feeleii WO-44-C3</td>
<td>++</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>L. feelei C9815</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. longbeachae Tucker 1</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Proteolytic activity was determined on BYE agar medium supplemented with 1% powdered milk as zones of clearing surrounding bacterial colonies. Hemolytic activity was determined on BYE agar medium supplemented with 5% canine erythrocytes as zones of hemolysis surrounding bacterial colonies. Symbols and abbreviation: +++, strong positive reaction; +++, positive reaction; W, weak reaction; −, negative reaction.

producing a change in A595 of 1.0 in 30 min with shaking (200 rpm) at 37°C.

Isolation of protease-deficient Legionella mutants. The broad-host-range plasmid pRK530 (temperature sensitive for replication) was used to deliver Tn3 into the chromosome of a streptomycin-resistant virulent strain of L. pneumophila. This plasmid is a derivative of pRK340::Tn3 (13) which was constructed in this laboratory by the introduction of Tn3 in a mating between Escherichia coli HB101::Tn3 (pRK340::Tn3) and X1849 (Nal'). The plasmid was mobilized from E. coli X1849 to an avirulent L. pneumophila Sm+ strain, and this strain served as a donor in matings with an L. pneumophila Sm+ strain. Matings were performed at 30°C overnight with antibiotic selection against the donor strain. The pin-point colonies on BCYE-streptomycin medium were then shifted to 42°C and grown overnight. Kanamycin-resistant colonies were then selected on BCYE agar supplemented with 50 μg of kanamycin per ml at 37°C. Colonies were picked and transferred onto BYE milk agar plates supplemented with 50 μg of kanamycin per ml and incubated at 37°C. Zones of proteolysis were scored after 24 to 48 h of incubation. Aproteolytic colonies were screened for the presence of a plasmid as described previously (13) and by plating onto BCYE agar containing ampicillin (50 μg/ml).

Purification of the exoprotease. The extracellular protease was purified essentially as described by Dreyfus and Iglewski (6) from broth cultures of L. pneumophila grown for 16 to 24 h in 2-liter flasks containing 1 liter of BYE medium. After clarification (10,000 × g for 15 min) the medium (3 liters) was batch absorbed with anion-exchange resin (DE-52, Whatman, Inc., Clifton, N.J.). All fractions from each step in the purification were examined for proteolytic and hemolytic activities. The primary purification step involved hydrophobic interaction chromatography on octyl-Sephacrose (Sigma Chemical Co., St. Louis, Mo.). This step readily removes the brown melaninlike pigment and most of the contaminating proteins (6). The concentrated and dialyzed fraction containing both proteolytic and hemolytic activities was applied to a 20-cm DE-52 column, and the proteins were eluted with a NaCl gradient (50 to 300 mM). Purity was judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the specific activity was determined by the Azocoll procedure.

Cytotoxicity assay. CHO cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Suspensions of CHO cells (200 μl containing 106 cells per ml) were pipetted into wells of a 96-well microdilution test. Test samples of either purified exoprotease or concentrated culture supernatants were added at various concentrations to the wells, and all volumes were adjusted to a final volume of 300 μl with Dulbecco modified Eagle medium. The microdilution plates were incubated at 37°C in 5% CO2, and cytotoxicity was judged colorimetrically as previously described (12). The wells were examined by phase-contrast microscopy and photographed. The estimation of protein in the spent culture medium was complicated by the presence of a melaninlike pigment, as noted in previous studies (6, 10). Therefore, the units of protease tested are reported as units per milliliter of concentrated culture medium.

Radiolabeling and autoradiography. The synthesis of exoprotease, as well as other extracellular proteins, was monitored after a 500-μCi pulse of [35S]methionine (1,011 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) into a 100-ml BYE broth culture of either PRT8 or the parent L. pneumophila protease-positive strain. Samples (5 ml each) were collected from the late log or early stationary phases of growth and centrifugated at 11,000 × g, and the proteins in the supernatant fraction were precipitated with 10% ice-cold trichloroacetic acid (TCA). The precipitates were washed once in cold acetone, and the pellets were solubilized in 2% Triton X-100-50 mM Tris hydrochloride-150 mM NaCl-0.1 mM EDTA (21). The samples were then diluted in SDS sample buffer, applied to a 12% SDS-polyacrylamide gel, and subjected to electrophoresis in the discontinuous buffer system of Laemmli (14). After staining with Coomassie brilliant blue R250 and destaining, the SDS-polyacrylamide gel was prepared for autoradiographic analysis by first soaking the gel in En3Hance (Dupont, NEN) for 1 h, drying, and exposing to Kodak XAR X-ray film for 2 to 5 days.

Immunological techniques. Purified exoprotease (400 μg/ml) was suspended in Freund incomplete adjuvant and administered intramuscularly in multiple sites in New Zealand White rabbits. Booster doses were similarly prepared and administered at 2 and 4 weeks. Titters were determined by an enzyme-linked immunosorbent assay, as described by Engvall (7). Specific antiprotease antibodies were affinity purified from the serum by adsorption to purified exoprotease, as described by Olmstead (17). Immunoblot analysis was performed essentially by the procedure of Towbin et al. (25). Proteins were separated on a 7.5 to 15% gradient SDS-polyacrylamide gel and subsequently electroeluted to nitrocellulose at a constant voltage (15 V, 16 h). Affinity-purified horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika, Malvern, Pa.) was used to identify antigen-antibody complexes on nitrocellulose sheets, using 4-chloro-1-naphthol (Sigma).

RESULTS

Both virulent and avirulent strains of L. pneumophila and most other Legionella species (exceptions, L. micdadei and L. feeleii) produce an extracellular protease(s), as indicated by zones of proteolysis on BYE agar plates supplemented with 1% powdered milk. Of a limited number of species tested, we noted an apparent correlation between the expression of protease activity and hemolytic activity for
canine or guinea pig erythrocytes (Table 1). Since it was widely believed that the exoprotease and hemolysin were distinct entities, we set out to identify the genes and gene products responsible for these phenotypes.

Isolation of *L. pneumophila* aprotolytic mutants. The broad-host-range plasmid pRK300::Tn5, which contained Tn3, permitted the screening of Tn3 insert-containing transconjugants for the presence of the plasmid (i.e., ampicillin resistance) since the plasmid containing the tetracycline resistance determinant was poorly expressed in *L. pneumophila* (13). We had also noted previously that matings between *Legionella* strains or from *Legionella* strains back to *E. coli* occurred at much higher frequencies than did matings from *E. coli* to *Legionella* strains (data not included). Therefore, pRK530 was first moved into an *L. pneumophila* Sm' strain from a diaminopimelic acid-requiring strain of *E. coli* X1849, as previously described (13).

After the mating between *Legionella* strains on streptomycin-containing medium, plates were incubated for up to 2 days at 42°C and then replica plated onto kanamycin-containing medium at 37°C. Approximately 4,800 of the Km' colonies were screened for loss of exoprotease activity on BYE agar containing 1% powdered milk. Eight protease-negative colonies were identified, and all but one eventually reverted to the proteolytic phenotype. The aprotolytic strain was designated PRT8 and confirmed as *L. pneumophila* by use of serogroup 1 immunofluorescent reagents obtained from the Centers for Disease Control and by an inability to grow on brucella agar medium. PRT8 and the parental strain hydrolyzed hippurate and expressed a 28-kDa major outer membrane protein in the outer membrane fraction (data not presented). Interestingly, strain PRT8 still contained pRK530, as indicated by growth on ampicillin-containing BYE medium and by conjugating pRK530 to *E. coli* HB101. PRT8 was then grown on BCYE agar containing 5 μg of novobiocin per ml (16), and a resulting colony was found to be ampicillin sensitive. This isolate was confirmed serologically to be *L. pneumophila*, and analysis for a plasmid by matings with *E. coli* and by agarose gel electrophoresis showed that this strain had been cured of pRK530.

Interestingly, this strain of PRT8 was also sensitive to kanamycin, suggesting that Tn3 had also been lost during this procedure. Since the isolate retained the aprotolytic phenotype, experiments aimed at resolving whether the mutation was caused by a Tn3 insertion and imprecise excision were not pursued. The aprotolytic phenotype of PRT8 was stable and no revertants were obtained after repeated plating on milk agar medium. When PRT8 was examined on canine erythrocyte-containing medium, the strain was no longer hemolytic (Fig. 1).

Examination of extracellular proteins by SDS-PAGE, immunoblot analysis, and autoradiography. *L. pneumophila* expresses a variety of extracellular proteins, and many enzymatic functions have been described (24). Concentrated supernatants from PRT8 were compared with those of the parental strain for these various enzymatic activities. Both strains were positive for lipase (TWEEN and phosphatidylidyinositol hydrolysis), pigment production, and alkaline phosphatase (data not presented). A comparison of growth rates between the PRT8 and parental strain grown in BYE broth revealed no differences (data not presented). When the concentrated culture supernatants obtained from the early stationary phase of growth were examined by SDS-PAGE for proteins, both fractions contained numerous proteins (Fig. 2A). However, only the concentrated supernatant fraction from the parental strain exhibited a polypeptide of comparable molecular mass with the purified exoprotease seen in lane A. In the lane containing PRT8 supernatant a prominent protein band at 33 kDa was observed. To resolve whether the 33-kDa peptide of PRT8 was a truncated form of the exoprotease, monospecific polyclonal antibodies were used to probe SDS-PAGE-fractioned proteins by immunoblot analysis for immunoreactive peptides. The monospecific antiserum reacted with the purified exoprotease and with a polypeptide of similar molecular mass in the concentrated supernatant fraction from the parental strain (Fig. 2B). No cross-reactive peptides were noted in the concentrated supernatant from PRT8. The failure to detect a truncated protein suggests that the defect might be in the structural gene or possibly in a regulatory gene affecting the expression of both the exoprotease and the hemolysin.

To examine the possibility that PRT8 expresses a prominent polypeptide which is rapidly degraded, we radiolabeled proteins with [35S]methionine and precipitated the extracellular proteins with TCA. Figure 3 depicts the autoradiogram of radiolabeled proteins separated by SDS-PAGE. The TCA-precipitated supernatant from the parental strain exhibited a prominent 38-kDa protein (lane a) which was absent in TCA-precipitated supernatant from PRT8 (lane b). Moreover, there was no indication that PRT8 expressed a polypeptide which rapidly degraded. The protein profiles matched more closely between the parental strain and PRT8 than did the protein profiles obtained with concentrated supernatants. The TCA precipitation step appears to circumvent the apparent proteolysis of the other supernatant proteins which occurs during the concentration step. The fact that PRT8 expresses all proteins observed in supernatants from the parental strain, as judged by autoradiography, diminishes the possibility that the defect in PRT8 leads to a pleiotropic effect on the secretion of protease and hemolysin.

Cell-free extracts (prepared by sonication) of both strains were examined for the expression of proteolytic activity to...
determine whether the defect in PRT8 leads to an intracellular accumulation of exoprotease. However, both cell-free extracts exhibited minimal proteolytic activity, as determined by the Azocoll assay and by zones of proteolysis on milk agar plates. These results suggest that in the case of the parental strain, synthesis of exoprotease is tightly coupled to protein export and for PRT8 the aproteolytic phenotype was not due to a deficiency in protein export.

**Purification of proteolytic and hemolytic phenotypes.** Proteolytic and hemolytic activities were purified from spent culture medium by batch absorption onto DE-52 ion-exchange resin. The void material and fractions eluted from the column up to 1 M NaCl were examined for proteolytic and hemolytic activities. Interestingly, all fractions exhibiting proteolytic activity on milk agar also exhibited hemolytic activity on BYE-canine erythrocyte agar medium. Fractions containing these activities were pooled, concentrated, made 1 M with respect to ammonium sulfate, and applied to an octyl-Sepharose hydrophobic interaction chromatography column. Both activities were detected in the same set of fractions eluted from the column by 45 to 50% ethylene glycol. After a final DE-52 ion-exchange step, the fractions containing the proteolytic-hemolytic activity were concentrated and examined by SDS-PAGE. Figure 2A (lane A) depicts the electrophoretic profile of the purified protease-hemolysin. The specific activity of the exoprotease, as determined from the Azocoll assay, was 660 U/mg of protein.

The possibility that the hemolysin and exoprotease were distinct proteins of similar molecular mass was investigated by examining the fraction for proteolytic and hemolytic activities in the presence of various protease inhibitors and antiserum. From the Azocoll protease assay, we noted that 5 mM EDTA inhibited proteolysis. To ensure that proteolysis was completely inhibited, 25 mM EDTA was used; 25 mM EDTA completely abolished proteolytic and hemolytic activities (Table 2). In contrast, 5 mM phenylmethylsulfonyl fluoride had no effect on the proteolytic and hemolytic activities. Hyperimmune serum inhibited proteolysis by 96% and hemolysis by 82%. Heat inactivation of the purified exoprotease also abolished both activities (data not presented). These results are consistent with the idea that a single protein is responsible for both phenotypes.

**Cytotoxic activity.** The hemolytic properties of the exoprotease led us to examine the possibility that this protein might be cytotoxic for various tissue culture cell lines. Concentrated culture filtrates from the parental strain, PRT8, and purified exoprotease were examined for cytotoxicity in a CHO cell assay. Purified exoprotease, as well as concentrated culture filtrates from the parental strain of *L. pneumophila*, exhibited cytotoxicity (Table 3). In contrast, culture filtrates from PRT8, as well as heat-inactivated purified exoprotease, were not cytotoxic for CHO cells. The estimated minimum cytotoxic dose for the purified protease was determined to be 0.17 U (0.3 μg of protein). Microscopic examination of control CHO cells (exposed to heat-inactivated exoprotease) and CHO cells exposed to purified exo-
TABLE 2. Effect of inhibitors and antiserum on proteolytic and hemolytic activities of the purified exoprotease-hemolysin

<table>
<thead>
<tr>
<th>Sample vol (µl), inhibitor</th>
<th>Activitya (zone diam [cm])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proteolytic</td>
</tr>
<tr>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>50, 25 mM EDTA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>50, 10 mM PMSFb</td>
<td>0.8</td>
</tr>
<tr>
<td>100, Preimmune rabbit serum</td>
<td>1.2</td>
</tr>
<tr>
<td>100, Antiprotease rabbit serum</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Proteolytic activity was measured as zones of clearing surrounding wells cut out of BYE agar medium containing 1% powdered milk and incubated at 37°C for 24 h. The protein concentration was estimated at 220 µg/ml. Hemolytic activity was similarly measured for BYE agar medium containing 5% canine erythrocytes and incubated at 37°C for 24 h.

b PMSF, Phenylmethylsulfonyl fluoride.

Protease revealed that the protease action had either prevented the attachment of the CHO cells or destroyed those cells that had become adherent (data not shown).

Immunoblot with human convalescent-phase serum. Two matched pairs of acute- and high-titer convalescent-phase sera obtained from confirmed cases of legionellosis (convalescent-phase serum titer, <1,024) were examined for antibodies reactive with the purified L. pneumophila exoprotease. Figure 4 depicts the results of an immunoblot with one of these pairs of serum. The acute-phase serum (lane a) was negative, whereas the convalescent-phase serum contained antibodies specifically reactive with the purified exoprotease. The other paired sera exhibited no reactivity with the exoprotease (data not presented). Whether human immune responses to the exoprotease represent common or isolated consequences of legionellosis will require screening of additional paired acute- and convalescent-phase sera.

DISCUSSION

In this study we have characterized the hemolytic and cytotoxic properties of the L. pneumophila Zn-metalloendopeptidase. This 38-kDa exoprotease, purified by the methods of Dreyfus and Iglewski (6), was found to reproduce the hemolytic phenotype described by many investigators (1, 24) and to be cytotoxic, as determined by the CHO cell assay. Additionally, the systematic examination of all column fractions for proteolytic and hemolytic activities diminishes the possibility that multiple proteases and hemolysins are expressed in any appreciable quantity by L. pneumophila Philadelphia 1. Furthermore, the isolation and characterization of a mutant strain of L. pneumophila deficient in the expression of exoprotease, hemolysin, and cytotoxic activity, also support the idea that the exoprotease is responsible for these additional phenotypes. Our work confirms that of other investigators who have also demonstrated cytotoxicity by the CHO cell assay for L. pneumophila exoprotease (20). Conlan and co-workers (2, 4, 5, 27) have used a guinea pig animal model to demonstrate the cytotoxic properties of the exoprotease. In these studies, purified exoprotease aerosolized into the lungs of guinea pigs induced rapid tissue destruction and death in many of the animals. Moreover, the cytopathology of the lung lesions resembled those from experimental legionellosis infections of guinea pigs and from human lung material. These investigators have demonstrated the presence of the exoprotease in the lung tissue of experimentally infected guinea pigs (5, 27). Both the cytotoxic nature of the exoprotease against CHO cells and the detec-
tion of antibodies in human convalescent-phase serum reactive with the exoprotease, in conjunction with the results from animal and human cytopathology studies, strongly suggest that this protein is associated with virulence.

Our initial goal for this study was to isolate and characterize the factors responsible for the proteolytic and hemolytic phenotypes. In a preliminary screening of selected Legionella species, we noted a correlation between proteolytic and hemolytic phenotypes. When further study revealed that purified exoprotease could reproduce the hemolytic phenotype, we reexamined our results and those of others in an effort to determine the mechanism of erythrocyte destruction. Interestingly, both canine and guinea pig erythrocytes had been reported as more sensitive substrates than human or sheep erythrocytes to the action of the L. pneumophila hemolysin (1, 24). In retrospect, this observation is consistent with the relative stability of the erythrocytes from these species (15). We have noted that canine and guinea pig erythrocytes are much more fragile than sheep erythrocytes and age more quickly. Sheep erythrocytes can be rendered highly sensitive to the action of the exoprotease after incubation of the medium for several days at 37°C. Aging of canine and guinea pig blood agar medium can be readily observed as an increase in the transparency of the medium without destruction of the hemoglobin. Such aged media are sensitive to proteolysis by trypsin, proteinase K, and pronase, whereas media containing fresh erythrocytes are more resistant to the action of trypsin. Therefore, the mechanism of action of the L. pneumophila exoprotease in hemolysis appears to require some degree of degeneration of the erythrocyte membrane. Once this occurs, the exoprotease exhibits a broad spectrum of activity against hemoglobin and other major proteins found in erythrocytes (unpublished observations). Since L. pneumophila colonies require 3 days to appear on blood agar medium, it is reasonable to assume that the observed hemolysis in previous works could be attributed to the action of the exoprotease.

The cytoxicity of the L. pneumophila exoprotease toward CHO cells supports previous work demonstrating the role of the exoprotease as the extracellular cytoxin (4, 20). The tissue-destructive nature of the exoprotease may be due in part to the hydrophobicity of this protein, as noted by its affinity for octyl-Sepharose (6). Furthermore, studies by Gul’nik et al. (10) indicated that the exoprotease exhibited a preference for hydrophobic amino acid sequences. These results are consistent with the idea that the exoprotease exhibits a preference for membrane proteins, although additional studies will be required to confirm this possibility.

Finally, we would like to propose a role for the exoprotease in pathogenesis. First, it is unlikely that the exoprotease is involved in the invasion of human or guinea pig macrophages. A study by Payne and Horwitz has clearly shown that both virulent and avirulent strains of L. pneumophila adhere to and are internalized by macrophages by complement-mediated endocytosis or by Fc receptor-mediated endocytosis (19). Second, the fact that avirulent Legionella strains also express the exoprotease and are killed by macrophages diminishes the role of the exoprotease in colonization. Third, the nonproteolytic Legionella species (i.e., L. micdadei and L. feeleii) are also able to invade and colonize host cells (8, 26). On the basis of these observations, the cytoxic exoprotease could be considered necessary for, but not sufficient for, the production of acute pneumonitis. In this view, the exoprotease would be a persistence factor that enhances virulence through the destruction of macrophages and surrounding tissue. In contrast, self-limiting legionellosis infections, such as Pontiac fever, may result from proteolytic organisms colonizing lung macrophages, but failing to elicit the cytotoxic damage necessary for the development of the more acute disease. One such candidate for Pontiac fever is L. feeleii, which is a proteolytic (22). In addition, L. micdadei, which is also proteolytic, produces disease most frequently in immunocompromised hosts (18, 26). Cloning and characterization of the structural gene for the L. pneumophila exoprotease, along with an analysis of mutants generated in a virulent background by recombinational mutagenesis, should further establish the role of the exoprotease in pathogenesis.

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LITERATURE CITED


