Demonstration of the intracellular production of tissue-destructive protease by *Legionella pneumophila* multiplying within guinea-pig and human alveolar macrophages

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The major extracellular enzyme of *Legionella pneumophila*, a metalloprotease, has been proposed as a pathogenic factor in Legionnaires' disease due to its cytotoxic, tissue-destructive, and phagocyte-inhibitory properties. The relevance of these activities depends on the production of the protease during infection, i.e. by *L. pneumophila* multiplying intracellularly. In this study, *L. pneumophila* was demonstrated to produce protease in guinea-pig and human alveolar macrophages infected *in vitro*. After 24 h infection, approximately 0.1 to 0.2 µg of protease per 10⁶ bacteria was measured by ELISA in culture supernatants and lysates of the infected cells, whereas no protease could be detected immediately after infection. Immunogold labelling using anti-protease antibody showed the enzyme to be located within phagosomes and distributed throughout the macrophages. Recent observations have shown that this protease could modify host defence mechanisms through inhibition of bacterial killing by neutrophils and monocytes. The intracellular production of the enzyme in infected macrophages demonstrated here further supports a role for the protease in the pathogenesis of Legionnaires' disease.

Introduction

Legionella pneumophila, a facultatively intracellular Gram-negative bacterium, appears to be the most common cause of Legionnaires' disease. This bacterium is able to multiply intracellularly within a variety of cells, e.g. human monocytes (Horwitz & Silverstein, 1980), monocyte-derived macrophages (Rajagopalan *et al.*, 1987) and alveolar macrophages (Nash *et al.*, 1984; Mintz *et al.*, 1988), as well as alveolar and peritoneal macrophages of guinea-pigs (Elliott & Winn, 1986; Kishimoto *et al.*, 1981; Yamamoto *et al.*, 1987; Yoshida *et al.*, 1987). The dominant extracellular enzyme produced by *L. pneumophila* is a zinc metalloprotease also called tissue-destructive protease, cytolysin or major secretory protein (Conlan *et al.*, 1986; Dreyfus & Iglewski, 1986; Belyi *et al.*, 1988; Blander & Horwitz, 1989). This protease is toxic to different types of cells and causes tissue destruction and pulmonary damage (Baskerville *et al.*, 1986; Conlan *et al.*, 1986; Rechnitzer *et al.*,

1989a; Rosenfeld *et al.*, 1986), which suggests its involvement in the pathogenesis of Legionnaires' disease. In further support of a role for protease as a pathogenic factor, we have recently described the inhibitory effect of the enzyme on the function of human neutrophils and monocytes, affecting particularly the microbicidal activity of these cells (Rechnitzer & Kharazmi, 1992). The relevance of these *in vitro* observations largely depends on the presence of the protease at the site of infection where the phagocytes are concentrated. *In vivo* production of the protease has been demonstrated by Conlan *et al.* (1988) in whole-lung macerates taken from guinea-pigs with experimental Legionnaires' disease. Whilst immunocytochemical studies have shown the presence of protease within guinea-pig alveolar macrophages *in vivo* (Williams *et al.*, 1987), production of the enzyme by *L. pneumophila* multiplying intracellularly has not been demonstrated. The present study was designed to investigate, by ELISA, the intracellular production of protease in guinea-pig and human alveolar macrophages infected *in vitro* with *L. pneumophila*.

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Abbreviation: FCS, foetal calf serum.

Methods

Organism. *L. pneumophila*, Corby strain, is a serogroup 1 human isolate (kindly provided by Dr R. A. Swann, John Radcliffe Hospital, Oxford, UK). Stock cultures, stored at -70°C , were thawed and grown on buffered charcoal yeast extract agar plates supplemented with 2-oxoglutarate (BCYE). Bacterial suspension was adjusted spectrophotometrically to 10^8 bacteria ml^{-1} .

Guinea-pig alveolar macrophages. Lungs taken post mortem from normal guinea-pigs were washed out in Eagle's minimal essential medium (MEM) containing 10 mM-sodium bicarbonate, 20 mM-HEPES, 20 mM-L-glutamine and 5 U heparin ml^{-1} (MEMa) using a procedure described by Jepras *et al.* (1985). Cells were harvested from the lavage by centrifugation at 200 *g* for 10 min and resuspended in MEM containing 5% (v/v) autologous guinea-pig serum (MEMb). Cells were counted in a Neubauer haemocytometer chamber, adjusted to 10^6 macrophages ml^{-1} and 200 μl aliquots added to wells of a 96-well tissue culture plate (Nunc). Cells were allowed to settle at 37°C for 30 min. Non-adherent cells were then washed away with MEMb.

Human alveolar macrophages. Bronchoalveolar lavage (BAL) was performed under local anaesthesia in uninfected patients undergoing diagnostic bronchoscopy. Informed consent had been obtained prior to bronchoscopy. A flexible fibreoptic bronchoscope was wedged in a middle or lower lobe bronchus of the normal lung and 200 ml of prewarmed sterile saline instilled in aliquots of 50 ml. After each instillation, the BAL fluid was aspirated and collected in polyethylene tubes. On average, 70% of the instilled fluid was recovered. BAL fluids used in the study were found free of tumour cells and microbiological examination was performed to exclude infected samples. The cells were harvested by centrifugation and washed twice with RPMI 1640 medium containing 25 mM-HEPES and 2 mM-L-glutamine, pH 7.4 (hereafter called RPMI). Total and differential cell counts were done using methyl violet and nonspecific esterase staining, respectively. Macrophages represented 90–95% of the total cell population. Cell suspensions were adjusted to 5×10^5 ml^{-1} in RPMI containing 20% foetal calf serum (FCS; Bodinco), penicillin ($500 \mu\text{g ml}^{-1}$) and streptomycin ($50 \mu\text{g ml}^{-1}$). One millilitre of cell suspension was layered onto each well of a 24-well multidish (Nunc) and left for adherence for 90 min at 37°C in 5% (v/v) CO_2 . The non-adherent cells were then removed by gentle washing twice with RPMI. The monolayers were further incubated for 24 h in medium containing 20% (v/v) FCS and antibiotics, then washed twice and incubated for 48 h without antibiotics.

Infection of guinea-pig alveolar macrophages. Bacterial suspensions were quantified by viable count on BCYE, suspended in MEMb and added to macrophages at a concentration which yielded 100 organisms per macrophage. A 2 h period of incubation was allowed for ingestion of organisms, then extracellular bacteria were killed by incubating for 2 h with $50 \mu\text{g}$ gentamicin ml^{-1} in MEMb followed by three washes with MEMb, a procedure which does not kill intracellular *L. pneumophila* (Fitzgeorge, 1985). To count intracellular bacteria the macrophages were disrupted using 200 μl 0.8% (w/v) digitonin in sterile distilled water per well for 10 min at 37°C . This detergent does not affect the viability of *L. pneumophila* (Fitzgeorge, 1985). The contents of each well were aspirated and added to 1.8 ml distilled water. This suspension, diluted further as necessary, was plated onto BCYE agar plates. Extracellular organisms were enumerated by plating out serial dilutions of culture supernatants. Intracellular growth of organisms was investigated by incubating infected macrophages at 37°C for 24 h followed by digitonin treatment and enumeration. Colonies were counted after 3 d incubation at 37°C in all cases. Protease in the culture supernatants and the cell lysates was assayed by ELISA. Controls were

included to determine the effects of digitonin, MEMb, and supernatants and lysates of non-infected macrophages on the assay and growth of bacteria in MEMb. During the course of infection macrophages were examined by phase-contrast microscopy.

Infection of human alveolar macrophages. Infection of the monolayers was performed as described by Rajagopalan *et al.* (1987). The monolayers were washed twice with RPMI, and bacterial suspension in RPMI with 20% (v/v) pooled normal human serum was added to each well at a ratio of 10 organisms per macrophage. The multidishes were then incubated for 60 min, rotating at 150 r.p.m., in 5% (v/v) CO_2 at 37°C . Following infection, the cells were washed 10 times in order to remove any extracellular bacteria. Supernatants from triplicate wells were harvested and adherent cells lysed with 0.5 ml cold sterile distilled water per well. Serial dilutions of supernatants and lysates were plated onto BCYE plates and c.f.u. enumerated after 72–96 h incubation at 37°C . The multidishes were further incubated for 24 h at 37°C in 5% (v/v) CO_2 . Triplicate supernatants and lysates were harvested as above. Supernatants and lysates of non-infected cells identically treated were harvested to be used as control in the ELISA. Control cultures consisted of 0.5 ml bacterial suspension in RPMI-FCS placed in triplicate wells containing no cells. Macrophages were examined by phase-contrast microscopy as above.

ELISA for protease. The protease and anti-protease antibody used in this study have been used and validated previously by Conlan *et al.* (1986, 1988). Briefly, protease was purified from broth culture supernatants by gel filtration followed by ion-exchange chromatography. Purity was verified on Coomassie-blue and silver-stained SDS-PAGE gels. Anti-protease antibody was prepared by immunization of New Zealand White rabbits with the purified protease. The IgG fraction was obtained from high-titre antisera by protein A affinity chromatography and anti-protease IgG further purified by affinity chromatography on a protease-Sepharose column. The specificity of this antibody has been demonstrated by Western blots (Conlan *et al.*, 1988).

Supernatants and lysates were titrated by a sandwich ELISA as described by Conlan *et al.* (1988) with minor modifications. Briefly, 96-well immunoplates (MaxiSorb, Nunc) and 100 μl volumes of the different reagents were used at room temperature. PBS containing 0.1% Tween 20 was used as wash solution and wash solution with 10% (v/v) FCS as diluent. Coating was performed overnight with anti-protease IgG at $4 \mu\text{g ml}^{-1}$. Horseradish-peroxidase-conjugated anti-protease IgG was used at $4 \mu\text{g ml}^{-1}$. The substrate used was 3,3',5,5'-tetramethylbenzidine dissolved in DMSO and diluted to 0.1 mg ml^{-1} in 50 mM-sodium acetate/acetic acid buffer, pH 6.0, with H_2O_2 added to 0.007%. The reaction was stopped by addition of 25 μl 1 M- H_2SO_4 and absorbance was read using an automatic ELISA reader (Bio-Rad) with a 450 nm filter. A twofold dilution row of purified tissue-destructive protease (at $5.5 \mu\text{g ml}^{-1}$) was included on each plate to generate a standard curve from which the apparent concentration of protease in each sample was derived.

Immunocytochemistry. Immunolabelling of organisms and protease in guinea-pig macrophages by electron microscopy was performed as previously described (Williams *et al.*, 1987). Briefly, macrophages were incubated with bacteria for 2 h in siliconized glass tubes and following removal of extracellular organisms, were incubated for 24 h at 37°C . Cells were pelleted, then resuspended and fixed in 1 ml 0.1% glutaraldehyde in phosphate buffer for 1 h at 4°C , then pelleted in agar. The pellet was cut into 1–2 mm cubes and processed to Araldite as described by Williams *et al.* (1987). Sections 80 nm thick were immunogold labelled using an indirect method with the affinity-purified rabbit anti-protease described above, followed by goat anti-rabbit IgG gold (10 nm; Seralab). Controls using rabbit IgG in place of specific antibody were included.

Table 1. *Production of tissue-destructive protease by L. pneumophila multiplying within guinea-pig alveolar macrophages*

Results of four independent experiments are presented. Viable counts and protease concentration were determined in duplicate. ND, Not detected (the limit of detection of the ELISA was 5 ng of protease); NT, not tested.

Time (h)	Expt	Supernatant		Lysate	
		Viable count (c.f.u. ml ⁻¹)	Concn of protease (µg per 10 ⁶ bacteria)	Viable count (c.f.u. ml ⁻¹)	Concn of protease (µg per 10 ⁶ bacteria)
0	1	0	ND	2.4-2.5 × 10 ³	ND
	2	NT	NT	2.4-2.7 × 10 ⁵	ND
	3	NT	NT	2.2-3.6 × 10 ⁵	ND
	4	NT	NT	4.1-7.1 × 10 ³	ND
24	1	NT	NT	NT	NT
	2	8.3-9.1 × 10 ⁶	0.074 (0.071-0.078)	5.0-6.9 × 10 ⁶	0.221 (0.210-0.232)
	3	0.4-8.0 × 10 ⁷	0.146 (0.022-0.270)	1.1-1.6 × 10 ⁷	0.106 (0.051-0.162)
	4	1.5 × 10 ⁶	0.267	1.1-2.3 × 10 ⁶	0.133 (0.085-0.182)

Table 2. *Production of tissue-destructive protease by L. pneumophila multiplying within human alveolar macrophages*

Results of five independent experiments are presented. Viable counts and protease concentration were determined in duplicate. ND, Not detected (the limit of detection of the ELISA was 5 ng of protease); NT, not tested.

Time (h)	Expt	Supernatant		Lysate	
		Viable count (c.f.u. per well)	Concn of protease (µg per 10 ⁶ bacteria)	Viable count (c.f.u. per well)	Concn of protease (µg per 10 ⁶ bacteria)
0	1	3.5-8.5 × 10 ²	ND	0.6-0.7 × 10 ⁴	ND
	2	3.0-4.5 × 10 ²	ND	0.3-1.0 × 10 ⁴	ND
	3	5-10 × 10 ²	ND	0.8-1.0 × 10 ⁴	ND
	4	5-8 × 10 ²	ND	1.9-2.5 × 10 ⁴	ND
	5	10-12 × 10 ²	ND	1.1-1.2 × 10 ⁴	ND
24	1	4.6-6.8 × 10 ⁶	0.029	3.1-5.5 × 10 ⁵	0.064
	2	3.8-11.2 × 10 ⁶	0.029	2.4-3.7 × 10 ⁵	0.088
	3	0.8-11.1 × 10 ⁶	0.109 (0.088-0.125)	NT	NT
	4	3.4-6.1 × 10 ⁶	0.105 (0.095-0.125)	1.7-2.2 × 10 ⁶	0.104 (0.086-0.141)
	5	3.0-4.6 × 10 ⁶	0.180 (0.176-0.188)	1.2-1.4 × 10 ⁶	0.066 (0.040-0.086)

Results and Discussion

In this study, we have shown that a highly virulent clinical isolate of *L. pneumophila* produces tissue-destructive protease intracellularly in similar quantities in guinea-pig and human alveolar macrophages infected *in vitro*. Within 24 h of infection, bacterial counts increased by 2-3 orders of magnitude in both guinea-pig and human alveolar macrophages (Tables 1 and 2). Approximately 0.1-0.2 µg protease per 10⁶ *L. pneumophila* organisms was measured in culture supernatants

and cell lysates at 24 h. Protease was not detected immediately after infection, in uninfected cells, or in bacterial suspensions incubated in the absence of cells. Tissue culture media and digitonin had no effect on the ELISA assay.

Since the majority of extracellular bacteria were removed following infection of the cells and bacteria were unable to grow in tissue culture medium alone, bacterial multiplication and protease production must have occurred intracellularly. Large quantities of protease were detected in culture supernatants as a result of

Fig. 1. Phase-contrast micrographs of human alveolar macrophage monolayers: (a) uninfected; (b) after 24 h infection with *L. pneumophila*. Bars, 50 μ m.

lysis of the infected macrophages, which was evident on examination of the cells by phase-contrast microscopy (Fig. 1 *a, b*). Immunogold labelling of infected guinea-pig alveolar macrophages using anti-protease antibody is shown in Fig. 2 (*a, b*). Specific labelling for protease showed the enzyme to be located within phagosomes and distributed throughout the macrophages. No gold particles were seen on cells incubated with rabbit IgG in place of anti-protease antibody (Fig. 2*c*).

The concentrations of protease quoted in this paper were calculated from comparisons with purified, enzymically active Corby strain tissue-destructive protease used as a standard in the ELISA. Since it is an immunoassay, these figures are not necessarily related to the functional activity of intracellular protease. It would be difficult to estimate protease activity in these samples owing to the presence of endogenous macrophage proteolytic en-

zymes. However, since the *L. pneumophila* tissue-destructive protease has previously been shown to be responsible for the severe tissue damage seen *in vivo* (Baskerville *et al.*, 1986; Conlan *et al.*, 1988; Williams *et al.*, 1987), it is probable that the protease is produced and released from macrophages in a highly active form. Further studies are required to demonstrate and define the functional enzyme activities of this and other proteases produced by *Legionella* bacteria in the *in vitro* assay and those produced *in vivo* in experimentally infected guinea-pigs.

The question of whether the quantities of tissue-destructive protease produced in these *in vitro* experiments can be related to those found *in vivo* should be addressed since this has relevance to the role of protease produced during Legionnaires' disease. Numbers of intra- and extracellular bacteria found in the present *in*

Fig. 2. Immunogold labelling for tissue-destructive protease in sections of guinea-pig alveolar macrophages infected *in vitro* with *L. pneumophila*. (a, b) Sections of cells incubated with anti-protease antibody showing numerous gold particles (►) both within phagosomes and distributed throughout the cell; (c) section of cells incubated with rabbit IgG in place of anti-protease antibody showing no gold particles. Bars, 1 μ m.

vitro experiments were 10–100-fold lower than those enumerated *in vivo* following aerosol infection of guinea-pigs with this strain (Conlan *et al.*, 1988). Assuming that the rate of protease production would remain relatively

constant, then a further 10–100 fold multiplication of the *Legionella* would result in quantities of protease of the same order, if not greater, than those shown to be inhibitory to phagocytes by Rechnitzer & Kharazmi

(1992). These projected levels of protease also equate to those detected *in vivo* in lung macerates.

The location of protease *in vivo* should also be considered. The macrophage is well-established as the site of replication of *L. pneumophila in vivo*. A. Williams & R. B. Fitzgeorge (unpublished data) have shown that the strain used in this study was unable to grow in cell-free guinea-pig lung lavage, even when this was obtained from animals with experimental Legionnaires' disease. Since this strain is also killed by polymorphonuclear neutrophil leukocytes (Fitzgeorge *et al.*, 1988; Jepras *et al.*, 1985), it would suggest that the macrophage is the principal site of protease production *in vivo*. Immunocytochemical studies support this suggestion, since the protease was seen concentrated at the sites of lung lesions and only in association with specifically labelled *L. pneumophila* (Williams *et al.*, 1987). Up to 22 µg of protease were detected by the same ELISA in total guinea-pig lung macerate by Conlan *et al.* (1988). Assuming the macrophages to be the major site of protease production, high concentrations of the enzyme would be reached in their vicinity.

A number of previous studies concerning *L. pneumophila* metalloprotease have suggested an important role of the enzyme in the pathogenesis of Legionnaires' disease. More recently, Szeto & Shuman (1990) described a transposon mutant of *L. pneumophila* Philadelphia 1 strain which was deficient in protease (major secretory protein, MSP) production. When tested in an aerosol infection guinea-pig model, the mutant was as lethal as its parent strain (Blander *et al.*, 1990). It was thus concluded that the MSP is not a virulence factor of *L. pneumophila*.

In general, virulence may be regarded as being multifactorial and, whilst the protease may not be a major virulence factor, its tissue-destructive properties and recent observations that the enzyme could modify the host inflammatory response (Reznitzner *et al.*, 1989b; Diamant *et al.*, 1991; Reznitzner & Kharazmi, 1992) suggest that the protease should not be excluded from having a role in the pathogenesis of Legionnaires' disease.

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