BACTERIAL PATHOGENESIS

Inhibition of oxidative burst and chemotaxis in human phagocytes by *Legionella pneumophila* zinc metalloprotease

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Legionella pneumophila produces several extracellular proteins, but their role in the pathogenesis of Legionnaires' disease is unclear. This study examined the effects of the *L. pneumophila* major secretory protein (Msp), a zinc metalloprotease, on the oxidative burst and chemotaxis of human phagocytes. Polymorphonuclear leucocytes (PMNLs) and adherent monocytes treated with sublethal amounts of Msp protease were stimulated with formyl-leucyl-methionyl-phenylalanine (fMLP) and opsonised zymosan particles (ZAP). A dose-dependent inhibition in superoxide anion production in response to both stimuli was seen, and complete inhibition was achieved in PMNLs and monocytes treated with Msp at concentrations of 1500 and 1000 U/ml, respectively. ZAP-induced chemiluminescence by PMNLs and mononuclear cells and fMLP-induced PMNL nitroblue tetrazolium dye reduction were both significantly inhibited. The chemotactic response of PMNLs to fMLP was inhibited in a dose-dependent manner and substantial inhibition (11% of control) was achieved with Msp 1200 U/ml. These results suggest that the *L. pneumophila* Msp protease alters human phagocyte functional responses significantly and may contribute to the pathogenesis of Legionnaires' disease.

Introduction

Legionella pneumophila is the aetiological agent of Legionnaires' disease, which may present as a life-threatening pneumonia. The pathogenesis of Legionnaires' disease involves the intracellular multiplication of the organism in alveolar macrophages, as demonstrated in lung specimens from patients with Legionnaires' disease [1] and experimentally infected guinea-pigs [2], as well as within alveolar macrophages [3] and monocytes [4] cultured *in vitro*. The major feature of *L. pneumophila* replication in monocytes is the formation of a specialised phagosome [5] and inhibition of phagosome–lysosome fusion [6]. Although the molecular mechanisms by which these responses are induced have not been elucidated completely, several *dot* and *icm* genes that are required for intracellular growth have been identified [7–10]. Some of the predicted Dot and Icm proteins resemble components of type IV secretion systems identified in other bacterial pathogens [11], but a secreted product

required for intracellular growth of *L. pneumophila* has not been identified.

L. pneumophila produces several extracellular enzymes and toxins [12–17], but the role of these secreted products in the pathogenesis of Legionnaires' disease is unclear. The major secretory protein (Msp, also referred to as tissue-destructive protease or cytotoxic protease), is a 38-kDa zinc metalloprotease which also exhibits haemolytic and cytotoxic properties [18, 19]. The Msp protease has been cloned and sequenced [19] and is structurally and functionally homologous to the *Pseudomonas aeruginosa* elastase [20]. Most recently, the Msp protease has been shown to be secreted by a type II general secretion pathway [21, 22], utilising an open reading frame containing general secretion pathway genes *lspFGHIJK* [21] plus the prepilin peptidase (*pilD*) gene [22]. Mutants defective in *lspGH* were unable to grow in amoebae, yet were still cytotoxic for macrophages [21]. Mutants in *pilD*, on the other hand, have been shown to be unable to replicate in macrophages and were avirulent in guinea-pigs [22].

Based on studies with Msp protease-deficient mutants, the protease itself appears not to be required for intracellular growth or cell killing in macrophages [23]

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and it is also reportedly not a required virulence factor in guinea-pig pneumonia models of Legionnaires' disease [24, 25]. Nevertheless, the Msp protease has been shown to be produced in the lungs of guinea-pigs during infection [26, 27] and the administration of purified protease can produce lesions similar to those documented in both the guinea-pig and human lungs as a result of *L. pneumophila* pneumonia [28, 29]. Guinea-pigs immunised against the Msp protease are also protected against lethal challenge with the organism [30]. Other relevant activities of the Msp protease include inactivation of human α -1-antitrypsin [31], inhibition of natural killer cell activity [32] and cleavage of CD4 on human T cells [33].

Thus, the role of the Msp protease in human infections has yet to be fully examined, particularly as regards the early inflammatory events that occur following exposure to the bacteria. Rechnitzer and Kharazmi [34] have shown that low levels of protease had inhibitory effects on the chemotaxis and bactericidal activity of human polymorphonuclear leucocytes (PMNLs), but had no effect on the oxidative burst or phagocytosis by PMNLs and monocytes. In contrast, another study reported that total exoproducts from a protease-deficient mutant of *L. pneumophila* were unable to inhibit superoxide anion generation and chemotaxis (by PMNLs), compared with the inhibitory activity demonstrated by total exoproducts from the wild-type strains [35]. These results suggested that the *L. pneumophila* protease was responsible for the inhibitory effects on the oxidative burst and chemotaxis of PMNLs. Because of conflicting results, as well as the renewed interest in secreted products of *L. pneumophila*, the present study was designed to examine further the effect of a wide range of sublethal concentrations of purified Msp protease on two functions of human phagocytes, the oxidative burst of PMNLs and monocytes (as measured by superoxide anion generation, chemiluminescence (CL) and nitroblue tetrazolium dye reduction) and the chemotaxis of PMNLs in response to the chemotactic peptide fMLP.

Materials and methods

Bacterial strain and growth media

L. pneumophila strain Knoxville-1 (serogroup-1) is an extensively laboratory-adapted (avirulent) isolate originally obtained from R. Weaver (Centers for Disease Control, Atlanta, GA, USA) and was stored at -70°C in Tryptic Soy Broth (Difco Laboratories, Detroit, MI, USA) containing glycerol 20% w/v. It was cultured on buffered charcoal yeast extract (BCYE) agar prepared from Legionella Agar Base (Difco) supplemented with L-cysteine 0.01% w/v and ferric pyrophosphate 0.05%. Casein agar (screening medium for protease) contained Difco agar 1.0%, casein (Sigma) 1.0% and sodium azide (Sigma) 0.001% in 100 mM sodium phosphate buffer (pH 6.0).

Purification of *L. pneumophila* protease

Protease was purified by a modification of the method described by Rechnitzer *et al.* [36]. Briefly, *L. pneumophila* was grown in ACES yeast extract (AYE) broth containing yeast extract (Difco) 1.0% and ACES (Research Organics, Cleveland, OH, USA) 1.0% supplemented with L-cysteine and ferric pyrophosphate as described above. The isolate was inoculated into 200 ml of AYE broth at 75 Klett units and incubated in a rotary shaker for 24 h. Cells were then removed by centrifugation at 10 000 *g* for 15 min at 4°C .

Culture supernate was precipitated with 60% saturated solution of ammonium sulphate (Sigma) for 16 h at 4°C and centrifuged at 12 500 *g* for 30 min at 4°C . The precipitate was collected and dialysed in 50 mM Tris-HCl, pH 8.0, containing 0.25 M NaCl, for at least 24 h. Dialysed supernate was centrifuged at 48 000 *g* for 15 min at 4°C and then applied to a MonoQ Sepharose (Pharmacia-LKB, Uppsala, Sweden) column (3 ml) equilibrated at 0.5 ml/min. After washing the column, protease was eluted at 1 ml/min with a step-wise gradient (0.25–1.0 M Na) and fractions of 1 ml were collected on ice.

Fractions were tested for protease by adding 10 μl into wells (3 mm) made in casein agar plates and zones (mm) of caseinolytic activity were measured after 24 h. Fractions with maximum zones were pooled and concentrated by ultrafiltration (10 $\times$) in a Centricell with a 30 000 relative molecular mass cut-off. Concentrated protease was then dialysed in Hanks's Balanced Salts Solution without phenol red (HBSS; Gibco BRL, Life Technologies, Grand Island, NY, USA). Criteria for purity of the final protease preparations were based on a single band on SDS-PAGE migrating at 38 kDa visualised by silver staining.

Enzyme assays

Protease activity was measured by adding 100 μl of sample to 20 mg of hide powder azure (Sigma) suspended in 5 ml of 100 mM sodium phosphate buffer (pH 6.0) and incubated at 35°C for 60 min on a rotary shaker. Samples were filtered (Whatman no. 1 filter) and the absorbance of the soluble fraction was measured at 595 nm in an Ultraspec spectrophotometer (LKB, Biochrom, Cambridge). Each unit was arbitrarily defined as proteolysis resulting in a change of absorbance of 0.001/h.

Protein content was estimated by the Bichinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. The average specific activity of the various protease preparations was 3200 units/mg of protein.

Neutrophil isolation

Whole blood from healthy volunteers (75 ml) was collected in tubes containing heparin 10 units/ml. PMNLs were isolated by buoyant density centrifugation with Mono-Poly resolving medium (ICN Biomedicals, Irving, CA, USA) and washed twice in HBSS. The final pellet containing PMNLs was suspended in HBSS to a cell density of 4.0×10^6 cells/ml. Cell viability was determined by trypan blue dye exclusion and was always >99%. Cell purity was determined by Giemsa stain and was always >99%.

Monocyte isolation and preparation of adherent monocytes

Peripheral blood mononuclear cells were isolated from whole blood with Mono-Poly resolving medium as described above. Mononuclear cells were washed twice and the final pellet was suspended in HBSS to a cell density of 3×10^6 cells/ml. Cell viability was assessed as above and was always >99%. Adherent monocytes were prepared by adding 1 ml of sample to 24-well tissue-culture plates (Costar, Cambridge, MA, USA) and incubating at 37°C for 2 h in air with CO₂ 5%. Non-adherent cells were removed by washing with HBSS (2×). In this laboratory this procedure consistently yields a monolayer of adherent monocytes (*c.* 2.5×10^5 cells/well) of >95% purity, as determined by non-specific esterase staining.

Superoxide anion assays

Levels of superoxide dismutase (SOD)-inhibitable superoxide anion generation by PMNLs and adherent monocytes were assayed as described by Summersgill *et al.* [37]. Briefly, protease was added in increasing amounts to 0.5 ml of PMNLs suspended in HBSS, or adherent monocytes, in the presence of 240 µM cytochrome C (Type III, Sigma). SOD (Sigma) was added to matched control tubes to a final concentration of 50 µg/ml. After the addition of either 100 µl of ZAPTM (opsonised zymosan A; Cardinal Associates, Santa Fe, NM, USA) or 10^{-6} M formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma), tubes and tissue-culture plates were incubated at 37°C for 90 min with rotation at 8 rpm (tubes) or gently shaken on a rocker (plates). Ice-cold HBSS was then added to each tube or well to stop the reaction. Tubes were centrifuged at 6900 *g* for 5 min and plates were centrifuged at 200 *g* for 10 min, both at 4°C. Absorbance of the supernates was determined at 550 nm. Values were corrected for the amounts of cytochrome C reduced in the presence of SOD and converted to nanomoles of reduced cytochrome C with the extinction coefficient $E_{550} = 2.1 \times 10^4$ M/cm [38]. Superoxide anions generated were calculated as nanomoles of superoxide anions released/min/ 2.0×10^6 PMNLs or as per well of adherent monocytes (*c.* 2.5×10^5 cells/well) and all results were expressed as a percentage of control (for

each stimulus used). PMNL superoxide anion generation in response to ZAP and fMLP was also measured with bovine serum albumin (Sigma) in amounts (µg of protein) that were either equal to or exceeding ($10\times$) that of the protease.

A slightly modified assay was used to measure superoxide anions generated by PMNLs which had been pre-incubated with protease; the assay time was reduced to 10 min after cells had been exposed to fMLP. This modified assay was also used to compare the effect of protease with a commercially available elastase (Type II-a, from porcine pancreas; Sigma) dissolved in HBSS (5 mg/ml) on PMNL superoxide anion generation in response to ZAP and fMLP. The amount of elastase used above was predetermined by comparing its caseinolytic activity to that of *L. pneumophila* protease that brought about a 50% inhibition in superoxide anion production by PMNLs.

To determine the direct effects of protease on the indicator systems used for the superoxide anion assay, PMNLs were incubated with protease for 90 min at 37°C and the amount of residual reducible cytochrome C present was measured at 550 nm after the addition of sodium dithionite (Sigma) 50 mg. Degradation of ferricytochrome C was determined by incubating it with protease or HBSS for 90 min, followed by the addition of sodium dithionite. The maximal reduction exhibited was measured at 550 nm as described above. The viability of PMNLs was determined by trypan blue dye exclusion after cells had been treated with protease 1500 units/ml for 90 min.

Chemiluminescence

The CL of PMNLs and mononuclear cells was determined as described by Summersgill *et al.* [37]. Briefly, PMNLs and mononuclear cells at a concentration of 2.0×10^6 and 3.0×10^6 cells/ml, respectively, were exposed to different amounts of protease or HBSS in the presence of 200 µM bis-N-methylacridinium nitrate (Lucigenin; Sigma). Cells were stimulated with ZAP and CL was measured at 37°C with a Picolite B6500 automated luminometer (Los Alamos Diagnostics, Los Alamos, NM, USA), interfaced with an IBM-PC utilising software supplied by the manufacturer. CL was measured over 120 min, and the cumulative chemiluminescent response (total area under the curve) was expressed as a percentage of HBSS-treated controls. In some experiments, cells were pre-incubated with protease for 1 h at 37°C, before assaying for CL. Preliminary experiments had demonstrated that the mononuclear cell CL was due solely to the monocyte population, with no measurable contribution by lymphocytes.

Nitroblue tetrazolium dye reduction (NBT)

PMNLs at a final concentration of 2.0×10^6 cells/ml

were pre-incubated with different amounts of protease or HBSS for 15 min at 37°C with rotation at 8 rpm. Cells were stimulated at room temperature with fMLP for 5 min (to decrease the sensitivity of the assay) before the addition of NBT reagent. Tubes were incubated at 37°C for 10 min. NBT reagent was prepared by dissolving 20 mg of NBT powder (Sigma) in 10 ml of saline 0.9% followed by the addition of 10 ml of 0.15 M phosphate-buffered saline (pH 7.2). This mixture was shaken rapidly at room temperature, filtered through a 0.02- μ m Millipore filter and stored at 4°C. Cells (200 μ l) were centrifuged at 400 *g* for 10 min on to slides using a Cytospin 2 (Shandon, Pittsburgh, PA, USA). Slides were air-dried and stained with NBT stain, which was prepared by adding Fast green FCF (394 mg), Neutral red (291 mg) and Eosin Y (57.5 mg) (Eastman Kodak, Rochester, NY, USA) to 100 ml of ethanol 95%. The stain was shaken vigorously in a closed container, filtered as above and stored at room temperature. Slides were stained for 15 min and then washed with distilled water, air-dried and examined microscopically under oil immersion. The numbers of cells containing blue-black formazan deposits/100 cells were counted and results were expressed as a percentage of control (HBSS only).

Chemotaxis

Under-agarose chemotaxis of PMNLs was assayed according to the method described by Nelson *et al.* [39]. Agarose medium (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) (0.024 g/ml) was prepared and 5-ml volumes were dispensed into 60 $\times$ 15-mm tissue-culture plates (No. 3002, Falcon Plastics, Oxnard, CA, USA) and wells (3 mm diameter) were punched. Plates were pre-incubated in air with CO₂ 5% for 1 h at 37°C, followed by the aspiration of all moisture from the wells. PMNLs (2.5 $\times$ 10⁷ cells/ml) were dispensed in 100- μ l amounts, centrifuged at 400 *g* for 5 min and reconstituted with protease or HBSS. PMNLs (10 μ l), HBSS (10 μ l) and fMLP (10⁻⁷ M; 7 μ l) were added to appropriate wells. Plates were incubated for 2 h in air with CO₂ 5% at 37°C, fixed with methanol and Giemsa stained. PMNL migration was measured by light microscopy at a magnification of 400 $\times$ with an ocular micrometer. The chemotactic differential was calculated as directed migration minus spontaneous migration and expressed as a percentage of HBSS-treated controls. The viability of PMNLs after pretreatment with protease was determined by trypan blue dye exclusion. In some experiments, PMNLs were pre-incubated with protease for 1 h and assayed for chemotaxis directly, or after two washes with HBSS. In a separate experiment, heat-inactivated fMLP, or heat-inactivated fMLP and protease (72°C for 1 h) or fMLP alone were used to determine chemotaxis of untreated PMNLs.

Statistical analysis

Data were compared for significance by repeated measures ANOVA followed by orthogonal contrast analysis for linear trend and Bonferroni post-hoc tests, and expressed in *p* values. Differences were considered significant at the level of *p* < 0.05.

Results

Superoxide anion generation

Superoxide anion generation by PMNLs in response to two stimuli, ZAP and fMLP, was measured following the addition of increasing amounts of purified protease. Superoxide anion production by PMNLs in response to both stimuli was inhibited in a dose-dependent manner as compared with untreated controls (linear downward trend, *p* < 0.05 (Fig. 1a)). Virtually complete inhibition was seen in PMNLs treated with protease at a concentration of 1500 U/ml. However, statistically significant inhibition of superoxide anion production was observed in PMNLs treated with 400 U of protease/ml when exposed to ZAP (14.7 SD 12.9% of control; *p* < 0.05) and with 800 U/ml when exposed to fMLP (12.1 SD 10.8% of control; *p* < 0.05). No inhibition of superoxide anion generation by PMNLs with either stimulus was observed in the presence of a negative control protein (bovine serum albumin) in amounts (i.e., μ g/ml) equal to or exceeding that of the protease.

Superoxide anion production by adherent monocytes also exhibited a dose-dependent decrease (linear downward trend; *p* < 0.05) following treatment of monocytes with protease (Fig. 1b). Virtually complete inhibition was achieved at the highest concentration of protease tested (1000 U/ml) with monocytes exposed to either stimulus.

To determine whether the inhibition of superoxide anion generation by phagocytes in the presence of protease was reversible, PMNLs were treated with protease 1200 U/ml and then washed. Superoxide anion production was measured by a modified 10-min assay after exposure to fMLP. PMNLs treated with protease without any pre-incubation were significantly inhibited in this modified protocol (54.7 SD 12.8% of control; *p* < 0.05), as were cells pre-incubated for 2 h (44.5 SD 25% of control; *p* < 0.05) (Fig. 2). PMNLs pre-incubated with protease for 2 h and then washed were also significantly inhibited when stimulated with fMLP (53.9 SD 29.5% of control; *p* < 0.05). Experiments with ZAP demonstrated similar irreversibility (data not shown).

PMNL viability after treatment with protease 1500 U/ml for 90 min was 90.4% of control viability. Control experiments showed that degradation of cytochrome C by protease was minimal during the assay

Fig. 1. Effects of *L. pneumophila* Msp protease on superoxide anion generation by human phagocytes. Superoxide anion production by PMNLs (a) and adherent monocytes (b) in the presence of different amounts of protease, in response to ZAP (100 μ l; □) and 10^{-6} M fMLP (■). Results are expressed as the percentage of untreated controls (12.6 SD 1.4 and 11.0 SD 2.7 nanomoles of superoxide anions produced by PMNLs and 9.3 SD 1.8 and 2.9 SD 0.3 nanomoles of superoxide anions produced by adherent monocytes, in response to ZAP and fMLP, respectively). Results are expressed as the mean and SD of three experiments.

period (data not shown). Furthermore, *L. pneumophila* protease and a commercially available elastase (porcine pancreas) had similar inhibitory effects on superoxide anion production in PMNLs pre-incubated with either product for 30 min and then stimulated with ZAP or fMLP (data not shown).

Chemiluminescence

Cumulative CL response was measured in PMNLs treated with protease and stimulated with ZAP over a 2-h period. Cells showed a significant inhibition of the CL response when treated with 500 and 1500 U of protease/ml ($p < 0.05$) (Fig. 3a). However, complete inhibition was achieved only in cells pre-incubated with 3000 U/ml for 1 h (5 SD 4% of control; $p < 0.05$). CL response in mononuclear cells stimulated with ZAP was inhibited in a similar fashion to that seen with PMNLs (Fig. 3b), with a significant inhibition occurring with 500 U of protease/ml (54 SD 35% of control; $p < 0.05$). Maximal inhibition occurred with protease 3000 U/ml (14 SD 6% of control; $p < 0.05$), but did

not require pre-incubation with protease, as was observed with PMNLs.

Nitroblue tetrazolium dye reduction

A third confirmatory assay of oxidative burst was performed with PMNLs stimulated with fMLP, followed by the measurement of their nitroblue tetrazolium (NBT) dye reducing ability. Cells exposed to 1000 U of protease/ml showed a substantial loss in dye reduction in response to this stimulus (8% of control; $p < 0.05$) (data not shown).

Chemotaxis

The second functional parameter examined was the under-agarose chemotaxis of PMNLs in response to fMLP. Cells were exposed to increasing concentrations of protease and the chemotactic differential was then measured and compared to HBSS buffer-treated controls. A dose-dependent decrease in the chemotactic ability of PMNLs was seen (linear downward trend;

Fig. 2. Irreversibility of the effect of *L. pneumophila* Msp protease on superoxide anion generation by PMNLs as measured by a modified protocol. Superoxide anion production by PMNLs exposed to protease 1200 units/ml (no pre-incubation, pre-incubation with and without a wash) and stimulated with 10^{-6} M fMLP for 10 min. Results are expressed as a percentage of untreated controls (11.0 SD 4.1 nanomoles superoxide anions).

Fig. 3. Effect of *L. pneumophila* Msp protease on PMNLs and mononuclear cell chemiluminescence (CL). Mean cumulative CL is calculated as the total area under the curve for PMNLs (a) and mononuclear cells (b) in the presence of varying concentrations of protease, in response to ZAP (100 μ l) and expressed as a percentage of untreated control. Raw data for untreated controls were $7.2 \pm 0.4 \times 10^9$ and $4.6 \pm 1.6 \times 10^9$ total counts/120 min for PMNLs and mononuclear cells, respectively. Data shown represent the mean and SD of three separate experiments.

Fig. 4. Effects of *L. pneumophila* Msp protease on the under-agarose chemotaxis of PMNLs. In response to 10^{-7} M fMLP, in the presence of different amounts of protease. PMNLs were added directly (a) or after pre-incubation for 1 h with or without a wash step (b). Results are expressed as a percentage of untreated controls (42.8 SD 1.7 and 42.0 SD 5.2 chemotaxis units for (a) and (b) respectively). Data shown represent mean and SD of three separate experiments.

$p < 0.05$) (Fig. 4a). Significant reduction in chemotaxis occurred in PMNLs treated with protease 500 U/ml (37 SD 12% of control; $p < 0.05$). The chemotaxis of PMNLs was substantially reduced with protease 1200 U/ml (11 SD 9% of control).

To determine if the inhibitory effects of protease on the chemotactic ability of PMNLs were reversible, cells were pre-incubated with protease for 1 h and then washed, before performing the chemotaxis assay. As shown in Fig. 4b, PMNLs showed a normal chemotactic response to fMLP at all concentrations of protease (linear downward trend; $p < 0.05$), whereas cells that were not washed were significantly inhibited, similar to the experiments described above. Control experiments showed that the degradation of the chemotactic peptide by the protease was minimal, as no significant difference was seen in chemotaxis in

response to fMLP treated with protease when compared with fMLP alone (data not shown).

Discussion

Human PMNLs and adherent monocytes treated with protease exhibited a dose-dependent decrease in oxidative burst ability in response to two receptor-mediated stimuli, ZAP and fMLP. Significantly, complete inhibition of superoxide anion production by phagocytic cells was observed with concentrations of protease that did not affect their viability. The inhibitory effect on superoxide anion generation was not due to a limitation in substrate availability (i.e., cytochrome C degradation), as determined by the excess reducible cytochrome C present at the end of the assay. Furthermore, normal superoxide anion

generation by PMNLs in the presence of excess bovine serum albumin suggests indirectly that the loss of superoxide anion radicals may not be due to a scavenging effect by proteins. PMNLs pre-incubated with protease for 2 h and then washed free of protease still demonstrated a comparable inhibition in superoxide anion generation in response to fMLP, suggesting that the effect on PMNLs was irreversible.

Two further assays were also used to measure the phagocyte oxidative burst. The cumulative CL response of both PMNLs and mononuclear cells stimulated with ZAP was significantly inhibited by protease treatment. This inhibition was observed with concentrations of protease comparable to those used in the superoxide anion assays. However, complete inhibition of CL response required a substantially higher concentration of protease. As CL is an amplified response assay, it may require a more significant alteration of the cell surface for inhibition to be complete. There was also a significant loss in NBT dye reducing ability in PMNLs exposed to protease in concentrations comparable to those required to inhibit superoxide anion generation by PMNLs.

Chemotaxis of PMNLs treated with protease was inhibited in a dose-dependent manner, with maximum inhibition occurring with protease concentrations comparable to those used for oxidative burst assays. This inhibition was not due to degradation of chemotactic peptide fMLP. However, in contrast to the oxidative burst data, the inhibition was completely reversible, perhaps due to a recycling of fMLP receptors from an existing intracellular pool [40] during the 2-h chemotaxis assay, as compared with the 10-min oxidative burst assay.

Similar studies by Rechnitzer and Kharazmi [34] failed to show any significant inhibition of phagocytic oxidative burst as measured by superoxide anion generation and CL response following treatment with protease. This difference is probably due to the smaller amounts (*c.* 10-fold less) of protease used in their study compared with the present data. They also showed that while the chemotaxis of PMNLs was significantly inhibited in response to fMLP- and zymosan-activated serum, the effect was only partially reversible. In contrast, the chemotaxis of PMNLs was not inhibited in cells washed free of protease, even at concentrations 10-fold higher than those used in the present study. This may be due to the different chemotaxis assays, and possibly to recycling of fMLP receptors during the time of the assay.

L. pneumophila protease has been characterised as a zinc metalloprotease, which shows considerable homology to *Pseudomonas aeruginosa* elastase at the genetic level and may also share a similar molecular mechanism of proteolysis [20]. The results of the current study suggest that inhibitory

effects on superoxide anion generation, CL, nitro-blue tetrazolium dye reduction and chemotaxis are biological activities shared by these proteases [20,41,42].

Although *L. pneumophila* protease has been shown not to be required for intracellular growth in cultured monocytes [23] and is not a required virulence factor in guinea-pig models [24,25], its contribution to the pathogenesis of disease in the human lung has not been elucidated. The inhibitory effects of the protease on human phagocytic cells may be more pronounced in an extracellular milieu during the early inflammatory stages of disease. There is evidence that functionally active protease is detectable in microgram quantities in homogenised lung tissue from guinea-pigs experimentally infected with virulent *L. pneumophila* [26, 27]. Concentrations of protease at local areas of infection within the lung might be expected to be significantly higher. Overall, these data suggest that the Msp protease may play a role in the pathogenesis of human Legionnaires' disease. Further studies are required to establish the actual concentrations of protease present in the lungs of human patients infected with *L. pneumophila* and to elucidate the mechanism(s) by which normal phagocyte functions are altered by the protease. The observation that the *L. pneumophila* protease is capable of cleaving CD4 on human T cells [33] suggests that the inhibitory activity on PMNLs and monocytes may be due to cleavage of surface receptors on the phagocytes.

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