Inhibition of rat alveolar macrophage phagocytic function by a *Pseudomonas cepacia* lipase

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Summary. The effects of purified *Pseudomonas cepacia* lipase on rat pulmonary alveolar function and morphology were examined. Lipase (2.5–20 µg/ml) adversely effected the phagocytic function of rat pulmonary alveolar macrophages in a dose-dependent manner. The lipase itself was not directly cytotoxic to these cells. Alveolar macrophages, in the absence of lipase, phagocytosed c. 35% of a given population of opsonised *P. cepacia* in 30 min when the ratio of bacteria:phagocyte was 10:1. Phagocytosis of *P. cepacia* by rat pulmonary alveolar macrophages was significantly reduced when the cells were either pre-incubated with the lipase or when phagocytosis occurred in the presence of the lipase. This was confirmed by transmission electronmicroscopy. These functional changes were associated with marked alterations of the macrophage morphology. Scanning electronmicroscopy showed that macrophages exposed to the *P. cepacia* lipase had fewer specialised surface structures and did not spread on plastic surfaces as well as untreated macrophages. The effects of the lipase were lost after heat inactivation, which indicates that the effects of the *P. cepacia* lipase were due to its enzymic activity. These results suggest that, if sufficient quantities of the enzyme are produced *in vivo*, lipase may be an important virulence factor for *P. cepacia*, allowing the organism to evade phagocytic cells.

Introduction

Pseudomonas cepacia was first described by Burkholder in 1950¹ as the causative agent of bacterial onion bulb rot. At first this organism was thought to be only a phytopathogen, but it is now recognised as an important cause of opportunist infection in man.^{2,3} It has recently received a great deal of attention because of its association with fatal pulmonary infections in patients with cystic fibrosis.^{4–6}

Relatively little is known about the virulence factors of this organism. McKeivitt and Woods⁷ demonstrated that *P. cepacia* produces an appreciable number of extracellular products including gelatinase, haemolysin, protease and lipase. Straus *et al.*^{8,9} demonstrated the production of a material they called the extracellular toxic complex (ETC) that was composed of capsular polysaccharide (CPS), lipopolysaccharide (LPS) and protein. This material caused extensive pulmonary pathology when deposited in the lungs of experimental animals. Lonon *et al.*¹⁰ recently characterised a lipase from *P. cepacia* and examined its toxicity in tissue culture and *in vivo*. The same authors showed that of 10 *P. cepacia* strains examined, all produced an immunologically similar lipase.¹¹

Extracellular lipase production by *P. cepacia* has

been studied with regard to physical, chemical and enzymic properties,^{10,11} but the role of lipase in infections has not been examined. Rollof *et al.*¹² recently demonstrated that an extracellular lipase produced by *Staphylococcus aureus* reduced phagocytic killing of the homologous organisms by granulocytes pre-incubated with the lipase, in a dose-dependent manner. Therefore, we examined the lipase produced by *P. cepacia* to find out whether it had a similar effect on pulmonary alveolar macrophages.

Materials and methods

Enzyme

Lipase from *P. cepacia* strain 90ee was purified as described previously.¹⁰ The lipase was homogeneous when tested by SDS-PAGE, yielding a single Coomassie Blue-staining band with an apparent M_r of 30 kDa. Gel chromatography on Sephadex G-200 also showed homogeneity. The lipase activity was c. 4387×10^{-3} units/µg of protein, with Tween 20 as substrate (1 unit of lipase activity was defined as that amount of enzyme that, after 2 h under the conditions of the assay, resulted in an increase of optical density at 400 nm of 0.01). The purified lipase preparation was dialysed extensively against Dulbecco's Modified

Eagles Medium (Sigma) containing bovine calf serum 10% (Hyclone, Logan, UT, USA) (DMEMS) and sterilised by filtration before use in phagocytic assays. Inactivated lipase was prepared by heating (100°C, 120 min) and the activity was checked at intervals to exclude reactivation.

Bacteria

P. cepacia strain 90ee (Pc 90ee) was used for all experiments. It was obtained from J. D. Klinger, Rainbow Babies and Children's Hospital, Cleveland, OH, and was maintained frozen in tryptic soy broth with glycerol 20% at -70°C. Cultures were grown for 24 h at 37°C with shaking at 200 rpm to an OD₅₄₀ of c. 1.0. This represented c. 8×10^8 cfu/ml. Bacteria were collected by centrifugation, washed three times in sterile phosphate-buffered saline (PBS) and diluted appropriately in Minimal Essential Medium (MEM; Sigma) before use.

Isolation of alveolar macrophages

Pulmonary alveolar macrophages (PAM) were obtained from the lungs of pentobarbitol-anaesthetised male Wistar rats. Lungs were lavaged five times intratracheally with 8 ml of saline. Cells were recovered by centrifugation at 800 g for 10 min. Macrophages were suspended in DMEMS and placed in microtitration-plate wells in 200 µl amounts containing 7×10^4 cells and maintained in a water-saturated atmosphere of air 95% and CO₂ 5%. Non-adherent cells were removed by five vigorous washings with culture medium.

Opsonophagocytic assay

Bacteria were pre-treated twice for 30 min at 25°C with 0.5 ml of fresh rat serum obtained on the day of use as a source of complement, and then washed three times in sterile PBS. Centrifugation of bacteria during these treatments was performed in a Beckman J2-21 centrifuge at 12000 g at 4°C for 30 min. Bacteria were added (7×10^5 cfu in 10 µl) to the macrophage cultures at a ratio of 10 bacteria to one macrophage. Cells were incubated for 5–120 min at 37°C in a CO₂ 5% environment. Assay medium was collected and dilutions in PBS were plated on brain heart infusion agar. Plates were incubated for 48 h at 37°C. Colonies were counted to determine the titre as colony forming units (cfu)/ml. Results were calculated as follows:

Percentage uptake =

$$\frac{\text{cfu}/10 \mu\text{l}(\text{x time}) - \text{cfu}/10 \mu\text{l}(0 \text{ time})}{\text{cfu}/10 \mu\text{l}(0 \text{ time})} \times 100$$

where x equals the time in min that the assay was incubated. All assays were run in triplicate and were repeated at least three times. The results presented were obtained from a representative experiment.

Electronmicroscopy

The macrophages were prepared as described above for the opsonophagocytic studies. For transmission electronmicroscopy (TEM), c. 1×10^7 macrophages were incubated with 1×10^8 Pc 90ee in 1 ml of DMEMS with and without active lipase 20 µg/ml. For scanning electronmicroscopy (SEM), 1×10^7 macrophages were incubated in 1 ml of DMEMS with and without active lipase 20 µg/ml. After 30 min, the macrophages were fixed in glutaraldehyde 3% in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 h at room temperature. The samples were then post-fixed in osmium tetroxide 1% and prepared for ultrastructural analysis by standard methods.¹³ Since it has been shown that lipase from one bacterial species (*S. aureus*) markedly alters the surface structure of human granulocytes,¹² we evaluated the effect of *P. cepacia* lipase on the surface of rat PAM. To determine the percentage of macrophages in a population that were "spread out" on a plastic surface, 20 separate fields for each preparation were examined by SEM by standard procedures.¹³ The percentage of spreading (spread cells/total cells) for each field was calculated and the Mann-Whitney test was used to determine whether lipase treatment caused a significant change in the cell population.

Serum and macrophage viability

Undiluted fresh rat serum was used in all bacterial opsonisation experiments. The viability of the rat PAM was tested by trypan blue dye exclusion. Macrophages were pre-incubated with lipase 20 µg/ml for 30 min at 37°C as well as in DMEMS alone and were examined for viability by light microscopy.

Results

Temporal phagocytosis of opsonised Pc 90ee by rat PAM

When Pc 90ee was opsonised by undiluted fresh rat serum and exposed to rat PAM at a ratio of 10:1, phagocytosis occurred rapidly (fig. 1). After 5 min, c. 11% of the bacteria were phagocytosed. Phagocytosis reached a maximum of 34% after incubation for 15 min and remained virtually unchanged for up to 2 h.

Effect of Pc 90ee lipase on rat PAM phagocytic killing

Pre-incubation of rat PAM with Pc 90ee lipase was associated with a dose-dependent decrease in phagocytosis of Pc 90ee (fig. 2). Exposure to lipase for 15 min at 2.5 µg/ml reduced Pc 90ee uptake to 22%, macrophages pre-incubated with DMEMS alone retained their phagocytic capacity of 32%. Macrophages pre-incubated with lipase 5 µg/ml had their phagocytic

Fig. 1. Phagocytosis by rat PAM of *P. cepacia* 90ee opsonised in undiluted fresh rat serum. Percentage uptake was determined after 5, 10, 15, 30, 60 and 120 min.

Fig. 2. Effects of pre-treatment with lipase or concomitant exposure to lipase on phagocytic killing by rat PAM. After pre-incubation with various concentrations of lipase (●—●) or concomitant exposure to lipase and bacteria (○—○), rat PAM were incubated with opsonised *P. cepacia* 90ee. Bacterial uptake was determined after 30 min at 37°C in a CO₂ 5% environment.

capability reduced to 12.5%. Macrophages pre-incubated with Pc 90ee lipase 10 or 20 µg/ml for 15 min were essentially unable to phagocytose Pc 90ee.

A similar effect was noted when the lipase was added to the phagocytic assay concomitant with the opsonised bacteria. Fig. 2 shows that when Pc 90ee lipase 2.5 µg/ml was added to the phagocytic assays along with the opsonised bacteria, phagocytosis by rat PAM was reduced from 32% to *c.* 18%. When the amount of lipase added was increased to 5 µg/ml, phagocytosis was decreased to 11.8%. The addition of lipase 10 or 20 µg/ml to the phagocytic assay along with the opsonised Pc 90ee totally prevented phagocytosis. The lipase (20 µg/ml) had no effect on the viability of the cells as determined by trypan blue dye exclusion. More than 95% of the macrophages remained viable after exposure to lipase for 30 min. Moreover, lipase inactivated by heat had no effect on the phagocytic capability of the rat PAM when the concentrations described above were used (data not shown).

Transmission electronmicroscopy

Fig. 3a shows a portion of a rat PAM that had been exposed to opsonised Pc 90ee for 30 min. Numerous phagocytosed Pc 90ee cells are visible inside the macrophage. Fig. 3b shows a higher magnification of phagocytosed *P. cepacia* inside a rat PAM. Fig. 3c illustrates a TEM of Pc 90ee cells that have not been phagocytosed. The size and shape of these bacteria are very similar to phagocytosed *P. cepacia* inside rat PAM.

Scanning electronmicroscopy

Because TEM studies indicated that rat PAM treated with lipase were incapable of phagocytosing Pc 90ee, we performed SEM to observe better macrophage surfaces. Pre-treatment with Pc 90ee lipase greatly affected macrophage morphology. The normal appearance of rat PAM incubated in DMEMS alone can be seen in fig. 4. These rat PAM appear to be in the

Fig. 3. TEM of a rat PAM incubated in DMEMS without lipase. (a) The macrophages were exposed to *P. cepacia* 90ee for 30 min and numerous bacteria (arrows) were internalised ($\times 13\,750$). (b) A section of a rat PAM containing internalised *P. cepacia* 90ee ($\times 68\,750$). (c) A pellet of *P. cepacia* 90ee treated as described for (b) ($\times 44\,000$).

process of spreading on the plastic surface. However, after treatment with Pc 90ee lipase $20\ \mu\text{g/ml}$, most macrophages remained round (fig. 5), which is their shape when they are isolated initially from the rat lung. Treated macrophages exhibited a morphology that clearly differed from the normal complex shape of the untreated rat PAM with its numerous pseudopodia, microvilli and other projections. Indeed, when 20 different fields of lipase-treated and normal rat PAM were examined by SEM, it was clear that the lipase-treated cells were significantly less capable of "spreading out" than the untreated rat PAM. The table shows that in an untreated rat PAM population, after incubation for 30 min at 37°C , c. 64% of the cells were beginning to spread out on the plastic surface. However, when lipase $20\ \mu\text{g/ml}$ was present, only 32% of the macrophages had begun to spread after incubation for 30 min.

Discussion

Many micro-organisms produce extracellular enzymes that are lipolytic. Whereas it has been specu-

lated that the physiological role of extracellular lipases produced by bacteria is probably nutritional,¹⁰ the hypothesis has also been put forward that the extraordinarily large amounts of lipase excreted by some strains may indicate that the enzyme performs other functions.¹² For example, Rollof *et al.*¹² demonstrated that phagocytic killing of *S. aureus* by human granulocytes pre-incubated with *S. aureus* lipase was reduced in a dose-dependent manner. Furthermore, Jaeger *et al.*¹⁴ have shown recently that an extracellular lipase of *P. aeruginosa* inhibited chemiluminescence by human peripheral blood monocytes. This inhibitory effect was dose dependent and was abolished by heating the enzyme at 100°C . These studies, together with our findings that demonstrate that *P. cepacia* lipase decreased phagocytic function of rat PAM, are consistent with the hypothesis that lipase may play a role in pathogenesis.

The *P. cepacia* lipase appears to exert its antiphagocytic effect on rat PAM by affecting the cell surface. Normal rat PAM tend to form pseudopodia and "spread out" on the plastic surface. Rat PAM exposed to lipase $20\ \mu\text{g/ml}$ for 30 min show greatly reduced

Fig. 4. SEM of rat PAM incubated in DMEM without lipase.

Fig. 5. SEM of rat PAM incubated for 30 min in DMEM plus *P. cepacia* 9000 lipase 20 µg/ml.

pseudopodia and diminished "spreading." Roloff *et al.* obtained similar results:¹² they demonstrated that pre-treatment with *S. aureus* lipase greatly affected human granulocyte morphology. After pre-incubation with *S. aureus* lipase 12 µg/ml, most of the granulocytes had denuded, flat surfaces. This was in marked contrast to the surface of normal granulocytes that had multiple pseudopodia and microvilli. We have provided data that demonstrate that rat PAM treated with the *P. cepacia* lipase show significantly less spreading than do normal macrophages incubated in DMEM alone. These drastic effects on the cell surface of the rat PAM could explain the reduction in their phagocytic capability, since the early stages of phago-

cytosis involve the envestment of the bacterium with cytoplasmic processes. These effects appear to be elicited by the enzymic activity of the lipase because heat denaturation of the lipase completely negated its ability to effect these changes.

In conclusion, *P. cepacia* lipase appears to act directly on rat PAM causing changes in surface morphology and a reduction in their phagocytic capability. Our electronmicrographs demonstrate surface changes in the phagocytic cells which appear to result from the enzymic activity of *P. cepacia* lipase. These results suggest that bacterial lipases may be important virulence factors allowing bacteria to evade the mammalian host defence system.

Table. Effect of lipase treatment on PAM morphology

Morphology	Control PAM		Lipase-treated PAM	
	Number of cells examined	Average (SD) number/field	Number of cells examined	Average (SD) number/field
Rounded	74	3.7 (1.56)	64	3.2 (2.76)
Spreading	134	6.7 (2.49)	36	1.8 (1.88)
Total	208	10.4 (2.87)	100	5

Treatment was 20 µg/ml for 30 min at 37°C. The beginning of pseudopodia formation was considered "spreading". The Mann-Whitney two-sample test was used to compare spreading cells with the total cell population for control versus lipase-treated PAM. There was significantly less spreading ($p = 0.0001$) of lipase-treated cells.

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