Inhibition of Human Polymorphonuclear Leukocyte Respiratory Burst, Bactericidal Activity, and Migration by Pneumolysin

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The in vitro effects of pneumolysin, a sulfhydryl-activated toxin produced by Streptococcus pneumoniae, on various functions of human polymorphonuclear leukocytes (PMNLs) was investigated. Treatment of PMNLs with highly purified toxin significantly inhibited respiratory burst (in response to stimulation), ability to kill opsonized pneumococci, chemotaxis, and random migration. These inhibitions were observed at very low toxin doses (≤1 hemolytic unit (2 ng) per 10⁶ PMNLs), which had no effect on PMNL viability. These results suggest that pneumolysin could function in pathogenicity by interfering with the ability of PMNLs to migrate toward and kill pneumococci.

In a recent report (10), we presented direct evidence for the involvement of pneumolysin, a sulfhydryl-activated cytolytic toxin produced by Streptococcus pneumoniae, in the pathogenicity of the organism. These studies showed that immunization of mice with a highly purified pneumolysin preparation significantly increased survival time after challenge with virulent S. pneumoniae.

Although it is known that pneumolysin binds to cholesterol in the plasma membrane of host cells (7), its function in pathogenicity is not understood. Interference with the bactericidal function of polymorphonuclear leukocytes (PMNLs), which have been shown to bind pneumolysin (6), is one possible mechanism whereby the toxin could enhance virulence. When PMNLs engulf microorganisms, they undergo a series of metabolic changes referred to as respiratory burst (11). These changes result in increased production of H₂O₂ and other reactive oxygen species, which are an important component of the oxygen-dependent microbicidal system (11). Consequently, any bacterial product that interferes with the respiratory burst of PMNLs could also affect their microbicidal function.

In the present study, we treated human PMNLs with low (sublethal) doses of highly purified pneumolysin and then examined their ability to undergo a respiratory burst in response to stimulation and their ability to kill pneumococci in vitro. The effects of toxin treatment on chemotaxis and random movement were also investigated.

MATERIALS AND METHODS

Purification of pneumolysin. Pneumolysin was purified from a type 1 strain of S. pneumoniae and assayed as previously described (10). The final preparation migrated as a single major protein species (which accounted for greater than 97% of total protein) when analyzed by polyacrylamide gel electrophoresis in either the presence or absence of sodium dodecyl sulfate. The purified material had a specific activity of at least 500,000 hemolytic units (HU) per mg of protein (10). (1 HU per ml will lyse 50% of a 1% suspension of human erythrocytes in 30 min at 37°C).

Preparation of PMNLs. Blood was drawn from healthy volunteers into tubes containing lithium-heparin. PMNLs were separated by the rapid single-step method described by Ferrante and Thong (4). Briefly, blood was layered onto a solution of Ficoll-Hypaque of specific gravity 1.144. After centrifugation for 20 min at 400 × g, the leukocytes resolved into two distinct bands. The mononuclear leukocytes appeared at the interface and the PMNLs banded ca. 1 cm below. The erythrocytes sedimented to the bottom. PMNLs were recovered in high yields at greater than 96% purity. The cells were washed three times in medium 199 and suspended to the required concentration. Viability was always greater than 99% as determined by the trypan blue exclusion technique.

Treatment of PMNLs with pneumolysin. PMNLs were suspended in the appropriate medium at a concentration of 4 × 10⁷/ml. The cells were incubated for 15 min at 37°C with 0 to 100 HU of pneumolysin per ml (which is equivalent to 0 to 2.5 HU/10⁶ PMNLs) before being analyzed for the various activities as described below.

HMP shunt assay. PMNLs to be used in the hexose-monophosphate (HMP) shunt assay were washed twice in glucose-free Earle medium before pneumolysin treatment. The assay was conducted under previ-
ously described conditions (13), except that in lieu of Warburg flasks, we adapted 20- and 5-ml scintillation vials to make up containers with inner and outer vessels. To each outer vessel was added 2 × 10⁶ PMNLs, 0.12 μCi of [1-C¹⁴]-l-glucose (3.96 mCi/mmol), and 4% human serum, with or without 0.4 mg of opsonized zymosan. All solutions were made up in glucose-free Earle medium, and the final volume in each outer vessel was 1.0 ml. The inner vessels contained 0.1 ml of 5 M NaOH to absorb the ¹⁴CO₂ evolved. The vessels were incubated in a shaking water bath at 37°C for 90 min, after which the radioactive content in the NaOH was counted by liquid scintillation.

Hydrogen peroxide assay. H₂O₂ production by PMNLs was measured by a microtechnique using 96-well (flat-bottomed) microtiter plates (Linbro; Flow Laboratories, Inc.). PMNLs (1 × 10⁶) in phosphate-buffered saline (15 min). 37°C, 106 PMNLs (final volume) of Hanks balanced salt solution supplemented with 8% human serum. The tubes were gassed with 5% carbon dioxide–95% air and incubated at 37°C with end to end mixing. Samples were taken at 0, 30, and 60 min, appropriately diluted in water, and plated onto blood agar to determine the number of viable bacteria.

Chemotaxis. PMNL chemotaxis and random movement were measured by the agarose technique (9) with some modifications (3). Six milliliters of 1% agarose in medium 199 was poured into tissue culture petri dishes (60 mm). After they had solidified, sets of three wells, 2.5 mm in diameter each and 2.5 mm apart, were cut (on a straight axis) with the aid of a template. To the center, outer, and inner wells was added 5 μl of PMNL suspension (containing 2 × 10⁵ cells), 5 μl of chemotactant, and 5 μl of chemotactant diluent, respectively. The chemotactant was N-formyl-L-methionyl-L-phenylalanine which was dissolved in dimethyl sulfoxide and diluted 2,500-fold in medium 199 to give a concentration of 2.5 × 10⁻¹⁷ M. The plates were incubated at 37°C in an atmosphere of 5% carbon dioxide–95% air with high humidity for 90 min. The distance migrated by the 10 fastest moving cells toward the chemotactant and the diluent wells was measured with the aid of a grid on the eye piece of an inverted phase-contrast microscope.

Statistical analysis. Differences between pneumolysin-treated and control PMNLs in the various experiments were analyzed with (two-tailed) paired t tests.

RESULTS

We examined the effects of pneumolysin on PMNL function over the concentration range of 0 to 100 HU/ml, which is equivalent to 0 to 2.5 HU per 10⁶ PMNLs under our incubation conditions. It is probably more appropriate to express pneumolysin dosage in the latter manner, since it has been shown by Johnson et al. (6) that 10⁶ human PMNLs can rapidly bind ca. 25 HU of toxin. In the present experiments, a maximum of 2.5 HU per 10⁶ PMNLs was used, and it is therefore unlikely that appreciable amounts of free, soluble toxin remained after the preincubation period. At the maximum toxin level used, greater than 97% of PMNLs remain viable as assessed by ability to exclude trypan blue.

Effect of pneumolysin on the respiratory burst of PMNLs. PMNLs possess an extremely active oxidative metabolism connected with their microbicidal activity. During phagocytosis, oxygen consumption is increased severalfold resulting in production of H₂O₂ and superoxide via the HMP shunt (11).

Initial experiments were set up to examine the effects of pneumolysin on the HMP shunt in nonstimulated PMNLs and in cells stimulated with zymosan particles (Fig. 1). In the presence of zymosan, the HMP shunt activity of control PMNLs (those preincubated without pneumolysin) was 7.0 times greater than in nonstimulated cells. However, the presence of zymosan resulted in only a 2.1-fold stimulation of HMP shunt activity in PMNLs treated with 2.5 HU of pneumolysin per 10⁶ cells. There was a progressive decrease in HMP shunt activity in the presence of zymosan with increasing pneumolysin dose, and this became statistically significant at 0.25 HU per 10⁶ PMNLs. In nonstimulated PMNLs, there was a slight increase in HMP shunt activity after pneumolysin treatment. This was statistically significant at 1.25 HU per 10⁶ PMNLs but not at any other dose.

The effect of pneumolysin treatment on the respiratory burst of PMNLs was further investigated by measuring H₂O₂ production (Fig. 2). For control PMNLs stimulated with PMA, the concentration of H₂O₂ achieved was 10.2 times greater than in nonstimulated cells, whereas for PMNLs treated with 2 HU of pneumolysin per 10⁶ cells the stimulation factor was only 5.5 fold. In the presence of PMA, there was a progressive decrease in final H₂O₂ concentration with increasing toxin dose; this became significantly different from that of control cells at 1.0 HU per 10⁶ PMNLs. There was no apparent effect of
 pneumatic treatment on the final concentration of H₂O₂ in nonstimulated cells.

**Effect of pneumolysin on PMNL bactericidal activity.** The above studies showed that pneumolysin inhibited the respiratory burst of PMNLs, an event that might lead to impairment of oxygen-dependent antimicrobial activity. The effect of pneumolysin on the ability of human PMNLs to kill opsonized pneumococci was therefore examined. Three different ratios of bacteria to PMNLs were used. At an S. pneumoniae:PMNL ratio of 2.5:1 the bactericidal activity was slightly inhibited by the toxin. After 60 min of incubation, 8.1% of the S. pneumoniae remained viable with control PMNLs, but in the presence of 2.5 HU of toxin per 10⁶ cells, 13.1% of the bacteria survived (Fig. 3a). The inhibitory effect of pneumolysin became more evident at the higher ratios of bacteria to PMNLs (Fig. 3b and c). At a ratio of 5:1, the survival rates after 60 min of incubation were 40.6, 44.8, 49.5, 60.3, and 80.3% for PMNLs treated with 0, 0.25, 0.5, 1.25, and 2.5 HU/10⁶ cells, respectively. The respective survival rates at a ratio of 7.5:1 were 85.6, 82.5, 101.0, 123.5, and 129.1%.

Statistical analysis of these results showed that the differences between the bacterial survival rates in the presence of control and pneumolysin-treated PMNLs, after 30 or 60 min of incubation, was significant (P < 0.05) at toxin doses of ≥1.25 HU per 10⁶ cells, for the bacter-

**Effect of pneumolysin on PMNL chemotaxis and random migration.** Although PMNLs have an extremely potent microbicidal machinery, its effective deployment depends on the ability of the cells to reach the bacteria. Thus, the effects of pneumolysin on PMNL chemotaxis and random migration were investigated (Fig. 4). At the maximum concentration of 0.5 HU per 10⁶ PMNL, the toxin caused a 43% inhibition of chemotaxis and a 59% inhibition of random migration. Significant inhibition of chemotactic responsiveness and random migration was observed at 0.25 and 0.125 HU per 10⁶ PMNL, respectively.

**Effect of cholesterol on inhibitory properties of pneumolysin.** To determine whether the inhibitory effects of purified pneumolysin on PMNL function were due to its specificity for cholesterol, the toxin was preincubated with 2 µg of cholesterol per 1,000 HU. Such treatment caused greater than 95% inhibition of toxin hemolytic activity. The experiments described in Fig. 1 to 4 were then repeated using the

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**FIG. 1.** Effect of pneumolysin on PMNL HMP shunt activity. PMNLs were preincubated for 15 min at 37°C with the indicated amounts of pneumolysin. HMP shunt activity was then measured in the presence (a) or absence (b) of zymosan, as described in the text. Results presented are the means of six experiments ± standard error. Significantly different from respective controls (no pneumolysin): (a) P < 0.05; (b) P < 0.01. kdpm, Kilodisintegrations per minute.

**FIG. 2.** Effect of pneumolysin on production of 

H₂O₂ by PMNLs. PMNLs were preincubated with the indicated pneumolysin dose. H₂O₂ production was measured with (a) or without (b) the addition of PMA as described in the text. Results presented are the means of three experiments ± standard error. Significantly different from respective controls (no pneumolysin): (a) P < 0.05; (b) P < 0.01.
maximum toxin dose or an identical volume of cholesterol-treated toxin. The inhibitory effects of pneumolysin on respiratory burst, bactericidal activity, and migration were not observed when PMNLs were treated with the cholesterol-inactivated toxin (results not shown).

**DISCUSSION**

In a recent report (10), we presented direct evidence for the involvement of pneumolysin in the pathogenicity of *S. pneumoniae*. These studies showed that immunization of mice with a highly purified preparation of pneumolysin increased survival time after challenge with virulent pneumococci. Results presented here suggest that pneumolysin could function in pathogenesis by interfering with the antimicrobial activity of PMNLs. The results presented also confirm the previous finding of Johnson et al. (6) that pneumolysin inhibits the chemotactic response and random migration of human PMNLs. From our studies, a toxin dose as low as 0.25 HU/10^6 PMNLs caused significant inhibition of both chemotaxis and random migration.

The oxygen-dependent antimicrobial system is the most potent microbicidal property of the PMNL. An important component of this system, the respiratory burst which accompanies phagocytosis, was inhibited by pneumolysin. This was demonstrated by inhibition of both the burst of HMP shunt activity and H_2O_2 production. The inhibition of this biochemical system was correlated with an inhibition of the ability of human PMNLs to kill *S. pneumoniae* in vitro. All these effects were observed at toxin concentrations that had no effect on the viability of PMNLs.

Andersen and Duncan (1) have previously reported that the related streptococcal toxin, streptolysin O, stimulated the metabolic activity of resting human PMNLs, as measured by chemiluminescence and O_2 consumption. This stimulation, which was only slight when compared with that induced by the addition of zymosan, may be analogous to the similarly slight but significant stimulation of HMP shunt activity observed in the present study when PMNLs were treated with 1.25 HU of pneumolysin per 10^6 cells in the absence of zymosan. Unfortunately, a comparison of the effects of pneumolysin and streptolysin O on the metabolic activity of stimulated PMNLs is not possible because data for the latter toxin are not available. Membrane-active toxins that are structurally unrelated to pneumolysin have also been shown to affect human PMNLs. Cavaliere and Snyder (2) have reported that *Escherichia coli* alpha-toxin stimulated chemiluminescence of PMNLs but inhibited chemotaxis, phagocytosis, and bactericidal activity. Conversely, staphylococcal alpha-toxin has been shown to enhance PMNL phagocytosis and bactericidal activity (5).

It has been suggested that release of reactive oxygen metabolites from PMNLs is a principal mediator in inflammatory tissue injury during a

**FIG. 3.** Effect of pneumolysin on killing of *S. pneumoniae* by PMNLs. Aliquots containing (a) 2.5 x 10^6, (b) 5.0 x 10^6, or (c) 7.5 x 10^6 *S. pneumoniae* were added to tubes containing no PMNLs (●) or 1.0 x 10^6 PMNLs that had been preincubated with 0 (○), 0.25 (■), 0.5 (□), 1.25 (▲) or 2.5 (△) HU of pneumolysin. Killing of the *S. pneumoniae* was measured as described in the text, and the results are expressed as the percentage of the original number of *S. pneumoniae* surviving after the indicated incubation time.
The findings indicate that pneumolysin inhibits PMNL antimicrobial function at an extremely low dose of $\leq 1$ HU (2 ng) per $10^6$ PMNLs, implying that this finding is likely to be of relevance in vivo.

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LITERATURE CITED


