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Pneumolysin Stimulates Production of Tumor Necrosis Factor Alpha and Interleukin-1β by Human Mononuclear Phagocytes

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Human peripheral blood monocytes and a human monocyte cell line were exposed to the toxin pneumolysin. Pneumolysin-exposed cells produced significantly larger amounts of tumor necrosis factor alpha and interleukin-1β than cells not exposed to the toxin. The viability of cells was not affected by the concentrations of pneumolysin used in the experiments.

Streptococcus pneumoniae is an important cause of disease in humans. The membrane-damaging toxin pneumolysin plays an important part in the ability of the pneumococcus to cause disease (3). This toxin belongs to a family of related toxins which are cytolytic for eukaryotic cells (1). However, pneumolysin has been shown to have other biological properties which, in vivo, may be important in the pathogenesis of pneumococcal infection. For example, pneumolysin alone is capable of causing the salient histological features of lobar pneumonia in rat lungs (4), is capable of causing a slowing of cilial beating in organ cultures of human respiratory epithelium (20), and is cytopathic for cultured endothelial (15) and epithelial (16) cells. Pneumolysin has been shown to activate the classical pathway of complement (12) in the absence of specific antibodies, and sublytic concentrations of the toxin cause an inhibition of antimicrobial activities of human phagocytes (9, 11, 19). Thus, pneumolysin may have a indirect role, via tissue damage and complement activation, in the toxemia associated with pneumococcal disease.

The aim of the present study was to examine if pneumolysin also could have a direct role in toxemia by induction of synthesis of relevant cytokines. Interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) were chosen as examples of relevant cytokines because they can induce meningeal pathology when given to experimental animals (18) and they have been detected in patients with pneumococcal meningitis (7).

Pneumolysin-induced cytokine production was measured with the human monocytic cell line U937 and human monocytes. U937 cells were maintained as described previously (2). For use, they were brought to 5×10^5 cells per ml. Human monocytes were isolated from the venous blood of healthy volunteers (14). Monocytes were allowed to form a monolayer by incubation for 2 h in eight-chambered LabTek slides (Miles Scientific). To each well, 5×10^5 monocytes were added. After 2 h, nonadherent cells were removed by washing the monolayers five times with RPMI 1640 medium. Cell viability was assessed by trypan blue exclusion and was routinely >90%.

For stimulation of production of cytokines, cells were incubated in 400 μl of RPMI 1640 medium supplemented with 2 mM glutamine and 6% (vol/vol) heat-inactivated fetal calf serum (complete RPMI 1640 medium) plus 5 mM indomethacin to prevent prostaglandin inhibition of cytokine production (17). The stimulus (pneumolysin or lipopolysaccharide [LPS]) was added in 50 μl of complete RPMI 1640 medium. After 24 h of incubation at 37°C in a 5% (vol/vol) CO_2–humidified air culture, the supernatants were removed for the TNF-α or IL-1β assay. TNF-α and IL-1β were measured by enzyme-linked immunosorbent assay (Quantikine cytokine assay kit; British Biotechnology) per the manufacturer's instructions. Release of hydrogen peroxide stimulated by phorbol myristate acetate was measured fluorimetrically as described previously (14).

Data were analyzed by two-way analysis of variance and by Scheffe's method for multiple comparisons of means.

Pneumolysin was purified by high-performance liquid chromatography (8). Endotoxin levels in the pneumolysin were measured colorimetrically with *Limulus* amoebocyte lysate (Coatest endotoxin test kit; Kabi Diagnostics, Stockholm, Sweden). The level of endotoxin detected was 1.04 pg/ml, which would result in a contaminating LPS concentration of 0.1 pg/ml in the cytokine assay mixtures. This level of endotoxin is substantially less than the amount expected to stimulate human monocytes (10).

The effects of sublytic concentrations of pneumolysin on the production of TNF-α by human monocytes and U937 cells are shown in Table 1. Analysis of variance showed a significant effect of pneumolysin on the amount of TNF-α produced by both cell types ($P < 0.001$ for U937 cells and $P < 0.05$ for monocytes). Further analysis of the individual means revealed that 1 ng of pneumolysin per ml significantly enhanced TNF-α release from both cell types ($P < 0.05$). A concentration of 10 pg of pneumolysin per ml significantly ($P < 0.05$) enhanced release from U937 cells. The extent of stimulation of TNF production by these concentrations of pneumolysin was the same as that by 50 ng of LPS per ml.

In contrast, 5 pg of toxin per ml significantly ($P < 0.001$) inhibited phorbol myristate acetate-stimulated release of hydrogen peroxide. Monocytes preincubated with pneumolysin released 0.9 ± 0.1 nmol of peroxide per h (mean ± standard deviation) compared with 3.7 ± 0.4 nmol/h in the absence of pneumolysin. With U937 cells, the levels released were 0.3 ± 0.1 and 3.3 ± 0.5 nmol/h, respectively. The viability of monocytes and U937 cells was not affected by the concentrations of pneumolysin and LPS used in these experiments. These data confirmed that the pneumolysin preparation used in this study would elicit a recognized effect on phagocyte behavior, namely, inhibition of the respiratory burst at sublytic concentrations.

To ensure that the observed stimulation of TNF production was not due to contamination by LPS, experiments were done

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TABLE 1. Production of TNF-α and IL-1β by human monocytes and the human monocytic cell line U937

Treatment	Amt produced (pg/h/5 × 10^5 cells)[a]			
	TNF-α		IL-1β	
	U937	Monocytes	U937	Monocytes
None	114 ± 12	112 ± 27	52 ± 19	50 ± 18
LPS (50 ng/ml)	199 ± 64	232 ± 114	318 ± 29	376 ± 10
Pneumolysin (1 ng/ml)	369 ± 125	258 ± 166	1,327 ± 24	525 ± 32
Pneumolysin (10 pg/ml)	229 ± 57	177 ± 110	627 ± 30	337 ± 48
Pneumolysin (3 pg/ml)	125 ± 25	Not done	305 ± 33	Not done

[a] Each value is the mean ± standard deviation of four experiments.

with pneumolysin that had been heated at 95°C for 10 min. In four experiments with 1 ng of heated pneumolysin per ml, U937 cells released 45 ± 13 pg of TNF-α per h per 5 × 10^5 cells. In these experiments, untreated cells released 70 ± 13 pg of TNF-α per h per 5 × 10^5 cells, while 50 ng of LPS per ml or 1 ng of unheated pneumolysin per ml again significantly ($P < 0.05$) stimulated U937 cells, which released 197 ± 18 and 224 ± 40 pg of TNF-α per h per 5 × 10^5 cells, respectively.

Pneumolysin also significantly ($P < 0.05$) stimulated IL-1β production by human monocytes and U937 cells at each concentration tested (Table 1), without an effect on cell viability. As before, the extent of stimulation was at least as great as that by 50 ng of LPS per ml.

The observations described here show that pneumolysin may mediate inflammation not only indirectly by damaging tissue and activating the complement system but also directly by stimulating TNF-α and IL-1β production from mononuclear phagocytes.

TNF-α and IL-1β are major mediators of inflammation during disease caused by gram-positive bacteria (7). Both IL-1β and TNF-α have been detected in cases of pneumococcal meningitis (7). Furthermore, the clinical features of meningitis can be induced in rabbits by administration of these cytokines, and antibodies against these cytokines decrease the extent of inflammation in pneumococcal meningitis (18). The presence of pneumolysin clearly is one route to the generation of these inflammatory agents.

We are not proposing that pneumolysin induction of TNF-α and IL-1β explains all of the inflammatory processes in pneumococcal disease. Pneumococcal cell wall components also play a role in inducing inflammation (5, 13), and other cytokines, such as IL-6, may be important (22). What we sought to do in this study was to establish the principle that the contribution of pneumolysin to inducing inflammation may be mediated, at least in part, via the stimulation of cytokine release. This we have done.

Addition of 10 pg of pneumolysin is equivalent to addition of about 10^3 pneumococci. This implies that pneumolysin is not as potent a stimulator of IL-1β release from monocytes as some pneumococcal cell wall components. Teichoic acid-containing cell wall fragments were reported to be effective in stimulating IL-1β release at a cell equivalent concentration of 1/ml (13). However, pneumolysin is more potent than cell wall components at stimulating TNF release. Even 10^8 cell equivalents of teichoic acid-containing cell wall fragments per ml did not stimulate TNF release (13).

Of course, even cytokine stimulation may not be the only method by which the toxin directly stimulates inflammation. Other mediators of inflammation may be directly induced by pneumolysin. Products of the cyclooxygenase and lipooxygenase pathways of arachidonic acid metabolism have been suggested as contributing to pneumococcal inflammation (21). The effect of pneumolysin on arachidonic acid metabolism is as yet unknown, but other thiol-activated toxins, streptolysin O and alveolysin, stimulate granulocytes, lymphocytes, and monocytes to release prostaglandins and leukotrienes (6).

The induction of inflammation by pneumolysin clearly has the potential to be a complex process. The next stage in the elucidation of the process will be an investigation, using inhibitors and anticytokine antibodies, of the role of each of the inflammatory mediators in vivo during pneumococcal infection and during the disease process induced when pneumolysin is administered alone.

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