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DOI

[10.1086/338008](https://doi.org/10.1086/338008)

Publication date

2002

Published in

The Journal of Infectious Diseases

[Link to publication](#)

Citation for published version (APA):

Rijneveld, A. W., van den Dobbelsteen, G. P., Florquin, S., Standiford, T. J., Speelman, P., & van der Poll, T. (2002). Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice. *The Journal of Infectious Diseases*, 185(1), 123-126. <https://doi.org/10.1086/338008>

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CONCISE COMMUNICATION

Roles of Interleukin-6 and Macrophage Inflammatory Protein-2 in Pneumolysin-Induced Lung Inflammation in Mice

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Pneumolysin (PLY), a toxin synthesized by *Streptococcus pneumoniae*, is an important virulence factor in pneumococcal disease. This study evaluated the effects of PLY in lungs of mice. Intranasal inoculation with PLY was associated with a dose-dependent influx of polymorphonuclear leukocytes (PMNL) in bronchoalveolar lavage fluid (BALF) and increased concentrations of interleukin (IL)-6, macrophage inflammatory protein (MIP)-2, and KC in BALF. PLY mutants with either reduced cytolytic activity or reduced cytolytic and complement-activating activities were less potent in inducing PMNL recruitment to the lung ($P < .05$), which suggests that PLY cytolytic activity is very important for the inflammatory response. IL-6 and MIP-2 also played a role in PLY-induced PMNL recruitment; this response was partially diminished in IL-6 gene-deficient mice and in mice treated with anti-MIP-2 antiserum. PLY may play an important role in the induction of an inflammatory response in the pulmonary compartment in the early phase of pneumococcal pneumonia.

Community-acquired pneumonia occurs in >4 million persons per year in the United States, and *Streptococcus pneumoniae* is isolated from 40%–75% of these patients. Because resistance of the pneumococcus to current antimicrobial agents is increasing and of concern, knowledge of pathogenetic processes during pneumococcal pneumonia is important for the development of alternative treatment modalities.

Pneumococcal virulence is largely attributable to the anti-phagocytic effects of its capsule. In addition, cell wall components and toxins are thought to play a role in the induction of an inflammatory response during pneumococcal infection [1]. Pneumolysin (PLY), a toxin produced by all *S. pneumoniae* clinical isolates, is a major determinant of virulence, as evidenced by findings in mice that infections induced by a PLY-deficient pneumococcal strain follow a less severe course than infections induced by a PLY-producing strain [2, 3].

The mechanisms by which PLY interacts with host defense are only partly elucidated. PLY is a pluripotent toxin with distinct cytolytic and complement-activating effects. At sublytic concentrations, PLY inhibits several functions of polymorphonuclear leukocytes (PMNL) in vitro, including respiratory burst, degranulation, chemotaxis, and bactericidal activity [4].

Knowledge of the in vivo effects of PLY in the pulmonary compartment is limited. In rats, instillation of PLY in the ligated apical lobe of the lung reproduced histologic features of pneumonia [5]. However, important inflammatory responses, such as recruitment of PMNL and production of cytokines, were not directly addressed in that study. In the present study, we further evaluated the effects of PLY in lungs of mice.

Materials and Methods

Animals. BALB/c mice were purchased from Harlan Sprague Dawley. Interleukin (IL)-6^{-/-} BALB/c mice were provided by M. Kopf (Basel Institute for Immunology). Experiments were done with 8-week-old female mice.

Reagents. PLY and PLY mutants were provided by the Laboratory for Vaccine Research, National Institute of Public Health and the Environment (RIVM). Three modified PLY preparations were used, of which one (PdB) lacks cytolytic properties, one (PdBD) lacks cytolytic and complement-activating activity, and one (PdT) shows no toxicity at all. PLY preparations were purified as described elsewhere [6,7]. Lipopolysaccharide (LPS) contamination was < 40 pg/μg of PLY for all PLY preparations, as determined by limulus amoebocyte lysate (LAL) assay (Laboratory of the Control of Biological Products, RIVM). Anti-mouse MIP-2 antiserum was gener-

Received 9 August 2001; revised 18 September 2001; electronically published 30 November 2001.

Presented in part: 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, September 1999 (abstract 1545).

All experiments were approved by the Institutional Animal Care and Use Committee, Academic Medical Center.

Financial support: Dutch Association for Scientific Research (grant to A.W.R.).

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The Journal of Infectious Diseases 2002;185:123–6

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 0022-1899/2002/18501-0018\$02.00

Table 1. Effects of pneumolysin (PLY) on cellular composition of and cytokine production in bronchoalveolar lavage fluid (BALF).

Variable	PLY, ng/mouse				
	0	100	250	500	1000
Total cells	3.5 ± 0.5	11.8 ± 2 ^a	18 ± 4.5 ^a	19.5 ± 3.8 ^a	77.5 ± 13.6 ^a
PMNL	0.8 ± 0.3	1.1 ± 0.3	4.6 ± 1.0 ^a	13.6 ± 3.9 ^a	62 ± 18.6 ^a
AMs	2.6 ± 0.7	10.0 ± 1.9 ^a	13.9 ± 4.1 ^a	5.5 ± 1.1	14.5 ± 6.0 ^a
Lymphocytes	0.04 ± 0.01	0.4 ± 0.14 ^a	0.1 ± 0.04	0.4 ± 0.1 ^a	0.7 ± 0.5
IL-6	0.03 ± 0.01	0.03 ± 0.0	0.2 ± 0.0	5.1 ± 0.5 ^a	2.24 ± 0.53 ^a
KC	0.38 ± 0.09	0.33 ± 0.04	0.62 ± 0.34	1.38 ± 0.36 ^a	2.08 ± 1.08 ^a
MIP-2	0.07 ± 0.02	0.11 ± 0.01	0.08 ± 0.01	0.59 ± 0.32 ^a	0.59 ± 0.32 ^a

NOTE. Data are mean ± SEM (×10⁴/mL of BALF for cell influx and ng/mL of BALF for cytokine concentrations) of 4–6 mice/dose group, 6 h after intranasal administration of PLY. Control mice (no PLY) received sterile isotonic saline. AMs, alveolar macrophages; IL, interleukin; MIP, macrophage inflammatory protein; PMNL, polymorphonuclear leukocytes.

^a $P < .05$ vs. control mice.

ated in rabbits, as described elsewhere [8]. Preimmune rabbit serum was used as control.

Experimental design. For intranasal inoculation, mice were anesthetized by inhalation of isoflurane (Upjohn), and wild-type (wt) or mutant PLY, diluted in 50 μ L of saline, was instilled intranasally. Control mice received 50 μ L of sterile saline or 40 pg of LPS (from *Escherichia coli* serotype O111 [B4]; Sigma) dissolved in 50 μ L of saline (i.e., the amount of LPS contamination that could maximally have been present in the PLY preparations). After 6 h, mice were anesthetized with fentanyl citrate and fluanisone (Hypnorm; Janssen Pharmaceutica) and midazolam (Roche), and bronchoalveolar lavage (BAL) was done or whole lungs were removed.

BAL. The trachea was exposed through a midline incision and was cannulated with a 22-gauge Abbocath-T catheter (Abbott). BAL was done by instilling 2 0.5-mL aliquots of saline. We retrieved 0.9–1.0 mL of BAL fluid (BALF) per mouse, and total cells were counted by hemocytometer (Emergo). Differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter).

Cytokine and chemokine determination. Cytokine and chemokine levels were measured by commercial ELISAs according to manufacturers' recommendations. Tumor necrosis factor (TNF)- α and IL-6 ELISAs were obtained from PharMingen and interferon (IFN)- γ , macrophage inflammatory protein (MIP)-2, and KC ELISAs from R&D Systems. Detection limits were 31, 37, 16, 47, and 12 pg/mL, respectively.

Histologic examination. Lungs were fixed in 10% formalin. After paraffin embedding, 4- μ m sections were cut and were stained with hematoxylin-eosin. Slides were coded before assessment by one pathologist without knowledge of the type of mice or treatment.

Statistical analysis. Data are expressed as mean ± SE unless indicated otherwise. Comparisons between groups were done by the Mann-Whitney *U* test. $P < .05$ was considered to be significant.

Results

Pulmonary inflammation induced by PLY. To determine the inflammatory properties of PLY in the pulmonary compartment

in vivo, we inoculated mice intranasally with increasing doses of PLY. PLY induced a dose-dependent increase in the number of PMNL in BALF. There were also increased alveolar macrophage and lymphocyte counts after PLY administration (table 1). In addition, PLY at doses of 500 or 1000 ng elicited increases in the concentrations of IL-6, MIP-2, and KC in BALF (table 1). By contrast, TNF- α and IFN- γ remained undetectable. On the basis of these results, further experiments were done with PLY at a dose of 500 ng. Recent work has shown that 500 ng of PLY is equivalent to 1.7×10^5 cfu of viable *S. pneumoniae* (D39 strain), as measured by the cytotoxic capacity of PLY and viable pneumococci [9]. The PLY preparation contained <40 pg of endotoxin by LAL assay analysis; neither 40 pg of LPS nor heated PLY induced any inflammatory response in mouse lungs.

Comparison of wt and PLY mutants. Earlier studies showed that PLY has cytolytic and complement-activating activity. To determine the extent to which these 2 activities contribute to PLY-induced lung inflammation, PMNL recruitment and cytokine production in lungs were determined after administration of PLY and 3 different PLY mutants: PdB (with reduced cytolytic activity), PdBD (with less cytolytic activity and reduced ability to activate complement), and PdT (with no toxicity) [6, 7].

PLY-induced PMNL influx was more pronounced than PMNL recruitment induced by PdB or PdBD, whereas PdT elicited no influx at all (all $P < .05$ vs. PLY). PMNL influx was similar after administration of PdB and PdBD (figure 1), suggesting that the cytolytic and not the complement-activating activity of PLY is responsible for PMNL attraction to the lung. We also measured cytokine and chemokine levels in BALF. PLY induced significantly more IL-6, KC, and MIP-2 than did mutated PLY ($P < .05$; figure 1).

Lung histology. When compared with the lungs from mice inoculated with saline or PdT, those from mice exposed to PLY showed accumulation of PMNL in the interalveolar septae, with formation of small intraparenchymal PMNL aggregates. Administration of PdB also resulted in PMNL influx into the lungs but was limited to the septae, without formation of PMNL aggregates. Lungs of mice exposed to PdBD displayed inter-

Figure 1. Reduced capacity of mutated pneumolysin (PLY) forms to induce influx of polymorphonuclear leukocytes (PMNL) into and production of interleukin (IL)-6, macrophage inflammatory protein (MIP)-2, and KC in mouse lungs. Bars: NaCl, saline controls; PLY, wild-type PLY; PdB, PLY lacking cytolytic properties; PdBD, PLY lacking cytolytic and complement-activating activity; and PdT, PLY without toxicity. Cell counts and cytokine levels were determined in bronchoalveolar lavage fluid (BALF) obtained 6 h after intranasal instillation of (mutated) PLY ($n = 6$ mice per group). Results are mean $\pm$ SEM. * $P < .05$ vs. NaCl controls; ** $P < .05$ vs. PLY-treated mice.

stitial inflammatory infiltrates composed predominantly of lymphocytes and monocytes (data not shown).

Roles of IL-6 and MIP-2 in PLY-induced PMNL recruitment. Having established that PLY induces PMNL influx concurrently with IL-6 and MIP-2 production, we determined the roles of these mediators in PMNL recruitment after administration of PLY. For this purpose, we compared PLY responses in lungs of IL-6^{+/+} and IL-6^{-/-} mice and in lungs of mice treated with preimmune or anti-MIP-2 rabbit antiserum. There tended to be fewer PMNL in lungs of IL-6^{-/-} mice than in lungs of IL-6^{+/+} mice (3.0 ± 0.9 vs. $6.2 \pm 1.4 \times 10^4$ PMNL/mL of BALF, respectively; P not significant). In addition, BALF KC levels were lower in lungs of IL-6^{-/-} mice than in those of IL-6^{+/+} mice (1.0 ± 0.1 vs. 0.5 ± 0.1 ng/mL, respectively; $P < .05$ vs. IL-6^{+/+} mice), whereas MIP-2 concentrations did not differ between groups (data not shown). In anti-MIP-2-treated mice, PMNL influx was significantly diminished when compared with that in control mice (1.9 ± 0.7 vs. $8.0 \pm 1.6 \times 10^4$ PMNL/mL; $P < .05$). KC levels tended to be lower in anti-MIP-2-treated mice (1.3 ± 0.4 vs. 0.5 ± 0.2 ng/mL; P not significant), whereas IL-6 levels did not differ between groups (data not shown).

Discussion

In this study, we demonstrated that PLY can induce lung inflammation in mice, characterized by PMNL influx and production of IL-6, MIP-2, and KC. By using PLY mutants, we demonstrated that the cytolytic activity of PLY likely is most important for PLY-induced inflammatory responses in the pulmonary compartment. We also showed that endogenously produced MIP-2 contributes to PMNL recruitment in response to PLY.

PLY is considered to be an important virulence factor in pneumococcal infections. PLY-deficient *S. pneumoniae* are markedly less virulent than wt *S. pneumoniae* in rodent models of intravenous, intraperitoneal, and pulmonary infection [2, 3, 10]. There are 2 known sets of PLY activities: cytolytic and complement activating. Earlier studies suggested that these properties of PLY differentially contribute to the pathogenesis of different forms of pneumococcal disease [3, 10].

We found that mutated forms of PLY with reduced cytolytic activity or with reduced cytolytic and complement-activating activity were equally less potent than wt PLY in eliciting PMNL recruitment to the lung. These data suggest that cytolytic activity is the main property contributing to the inflammatory response, whereas the ability of PLY to activate complement seems less important. However, since mutated PLY in which only complement-activating activity was reduced (and cytolytic activity was intact) was not available, it remains possible that both PLY activities are equally important but non-additive.

Pneumococcal pneumonia is associated with local production of cytokines and chemokines at the infection site. Earlier studies suggested that PLY may contribute to this response. PLY was found to stimulate the production of IL-1 and TNF by human monocytes in vitro [11]. In addition, serum IL-6 levels were relatively higher after intravenous injection of wt *S. pneumoniae* than after administration of PLY-negative mutant *S. pneumoniae* in mice [2]. We believe that our study is the first to describe the capacity of PLY to induce the production of IL-6 and the CXC chemokines, KC and MIP-2, in the pulmonary compartment. However, in contrast to earlier in vitro observations [11], PLY did not induce production of TNF in mouse lungs.

The cytolytic activity of PLY also seemed to be the most important factor for inducing the production of IL-6, MIP-2, and KC, as indicated by the fact that both PLY with reduced cytolytic activity and PLY with reduced cytolytic and complement-activating activities demonstrated a reduced capacity to induce these mediators, when compared with wt PLY. Since IL-6 and MIP-2 can influence PMNL influx into lungs during acute inflammation [12–14], we were interested in the involvement of these mediators in PLY-induced PMNL recruitment. IL-6^{-/-} mice displayed a reduced PMNL influx in BALF. In earlier work using rats, intratracheal instillation of IL-6 resulted in PMNL infiltration into the lung interstitium and alveoli and elevation of neutrophils in BALF [15]. Furthermore, endogenous IL-6 may have a proinflammatory role during bleomycin-induced lung injury [12]. However, other studies have indicated that endogenous IL-6 serves an anti-inflammatory role during

endotoxin-induced lung inflammation in mice. Indeed, IL-6^{-/-} mice had an enhanced influx of PMNL in BALF and higher lung MIP-2 concentrations than IL-6^{+/+} mice after intrapulmonary delivery of endotoxin [14]. Hence, the role of lung-derived IL-6 may differ, depending on the bacterial or inflammatory stimulus that is administered. Anti-MIP-2 reduces PMNL influx after PLY administration. In accordance, endogenous MIP-2 was important for PMNL recruitment during murine *Klebsiella pneumoniae* [8] and endotoxin-induced lung inflammation [13]. In the current study, both IL-6^{-/-} and anti-MIP-2-treated mice had diminished KC levels in BALF after inoculation with PLY, which suggests that PMNL contribute to KC production.

PLY contributes to the virulence of *S. pneumoniae*. In this study, we demonstrated that PLY can induce an acute inflammatory reaction in the mouse lung, characterized by PMNL influx, lung damage, and local IL-6 and CXC chemokine production, after intranasal instillation. PLY-induced PMNL influx likely is predominantly caused by its cytolytic activity and is mediated by MIP-2 and, to a lesser extent, by IL-6. PLY, conceivably together with cell wall constituents of *S. pneumoniae*, may play a significant role in mounting an inflammatory response in the pulmonary compartment during pneumococcal pneumonia.

Acknowledgments

We thank A. Maas and J. Daalhuisen for expert technical assistance.

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