Pneumonia: an investigation of host defence mechanisms

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Citation for published version (APA):
Rijneveld, A. W. (2003). Pneumonia: an investigation of host defence mechanisms

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Roles of interleukin-6 and macrophage inflammatory protein 2 in pneumolysin induced lung inflammation in mice
Pneumolysin (PLY), a toxin synthesized by *Streptococcus (S.) pneumoniae*, is an important virulence factor in pneumococcal disease. Intranasal inoculation with PLY was associated with a dose dependent influx of polymorphonuclear (PMN) cells in bronchoalveolar lavage fluid (BALF) and increases in BALF concentrations of interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2) and KC after 6 hours. PLY mutants with either reduced cytolytic activity or reduced cytolytic and complement activating activities were less potent in inducing PMN recruitment to the lung (P<0.05), suggesting that PLY cytolytic activity is most important for the inflammatory response. In addition, IL-6 and MIP-2 played a role in PLY-induced PMN recruitment considering that this response was partially diminished in IL-6 gene deficient mice (P = 0.1 vs. wild type mice), and in mice treated with anti-MIP-2 antiserum (P<0.05). PLY may play an important role in the induction of an inflammatory response in the pulmonary compartment in the early phase of pneumococcal pneumonia.

**Introduction**

Community-acquired pneumonia has a high incidence rate of more than 4 million patients per year in the United States. *S. pneumoniae* is isolated from 40-75% of these patients. Resistance of the pneumococcus to current antimicrobial agents is increasing and a reason for concern. Knowledge of pathogenetic processes during pneumococcal pneumonia is important for the development of alternative treatment modalities.

The virulence of the pneumococcus is largely attributable to the antiphagocytic effects of its capsule. In addition, cell wall components and toxins are considered to play a role in the induction of an inflammatory response during pneumococcal infection. PLY is a toxin produced by all clinical isolates of *S. pneumoniae* and a major determinant of the virulence of the pneumococcus, as evidenced by findings in mice that infections induced by a PLY deficient pneumococcal strain follow a less severe course than infections by a PLY producing strain.

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

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 histological features of pneumonia. However, important inflammatory responses, such as recruitment of PMNs and production of cytokines, were not directly addressed in that study. In the present study we sought to further evaluate the effects of PLY in lungs of mice.

**Material and methods**

*Animals.* BALB/c mice were purchased from Harlan Sprague Dawley Inc. (Horst, the
Netherlands). IL-6+/− BALB/c mice were kindly provided by M. Kopf (Basel Institute for Immunology, Basel, Switzerland). Experiments were done with 8 weeks old female mice.

Reagents. PLY and PLY mutants were kindly provided by the Laboratory for Vaccin Research, National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands). Three modified PLY preparations were used. PdB lacks cytolytic properties, PdBD lacks cytolytic and complement activating activity, and PdT shows no toxicity at all. PLY preparations were purified as described earlier. Lipopolysaccharide (LPS) contamination was less than 40 pg per 1 μg PLY, for all PLY preparations, as determined by the Limulus Amoeocyte Lysate (LAL) assay (Laboratory of the Control of Biological Products, RIVM, the Netherlands). Anti-mouse MIP-2 antiserum was generated in rabbits as described. Pre-immune rabbit serum was used as control.

Experimental design. For intranasal inoculation, mice were anaesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands), and wild type or mutant PLY, diluted in 50 μl saline, was instilled intranasally. Control mice received 50 μl sterile saline or 40 pg LPS (from Escherichia coli serotype 0111; B4; Sigma, St Louis, MO) dissolved in 50 μl saline, i.e. the amount of LPS contamination that could maximally have been present in the PLY preparations. After six hours, mice were anaesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), a bronchoalveolar lavage was performed or whole lungs were removed.

Bronchoalveolar lavage. The trachea was exposed through a midline incision and cannulated with a 22-gauge Abbochath-T catheter (Abbott, Sligo, Ireland). A bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of saline. 0.9-1 ml of lavage fluid was retrieved per mouse, and total cell numbers were counted in a hemacytometer. Differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, III).

Cytokine and chemokine determination. Cytokine and chemokine levels were measured by commercially available ELISA's according to manufacturers recommendations: Tumor necrosis facor-α (TNF) and interleukin-6 (IL) (Pharmingen, San Diego, CA), Interferon-γ (IFN), MIP-2 and KC (R&D systems, Abingdon, United Kingdom). Detection limits were 31 pg/ml, 37 pg/ml, 16 pg/ml, 47 pg/ml, 12 pg/ml, respectively.

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

Statistical analysis. Data are expressed as means ± SEM. Comparisons were conducted by the Mann Whitney U test. P-value <0.05 was considered to represent a statistically significant difference.

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 numbers of PMNs in BALF. There was also an increase in the numbers of alveolar macrophages (AM) and lymphocytes after PLY administration (Table 1). In addition, PLY at doses of 500 ng or 1000 ng elicited increases in the concentrations of IL-6, MIP-2 and KC in BALF (Table 1). By contrast, TNF and IFN remained undetectable. Based on these results, further experiments were performed with PLY at a dose of 500 ng. Recent work showed that 500 ng is equivalent to $1.7 \times 10^5$ CFU viable *S. pneumoniae* (D39 strain). The PLY preparation contained $<40$ pg endotoxin according to the LAL assay analysis; neither 40 pg LPS nor heated PLY induced any inflammatory response in mouse lungs.

### Table 1. Effect of PLY on cellular composition of and cytokine production in BALF

<table>
<thead>
<tr>
<th>PLY (ng/mouse)</th>
<th>0</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
<td>3.5 ± 0.5</td>
<td>11.8 ± 2*</td>
<td>18 ± 4.5*</td>
<td>19.5 ± 3.8*</td>
<td>77.5 ± 13.6*</td>
</tr>
<tr>
<td>PMN</td>
<td>0.8 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>4.6 ± 1.0*</td>
<td>13.6 ± 3.9*</td>
<td>62 ± 18.6*</td>
</tr>
<tr>
<td>AM</td>
<td>2.6 ± 0.7</td>
<td>10.0 ± 1.9*</td>
<td>13.9 ± 4.1*</td>
<td>5.5 ± 1.1</td>
<td>14.5 ± 6.0*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.04 ± 0.01</td>
<td>0.4 ± 0.14*</td>
<td>0.1 ± 0.04</td>
<td>0.4 ± 0.1*</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>5.1 ± 0.5*</td>
<td>2.24 ± 0.53*</td>
</tr>
<tr>
<td>KC</td>
<td>0.38 ± 0.09</td>
<td>0.33 ± 0.04</td>
<td>0.62 ± 0.34</td>
<td>1.38 ± 0.36*</td>
<td>2.08 ± 1.08*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>0.07 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.59 ± 0.32*</td>
<td>0.59 ± 0.32*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (x 10^4/ml BALF for cell influx and ng/ml BALF for cytokine concentrations) of 4-6 mice per dose, 6h after intranasal administration of PLY. Control mice (no PLY) received sterile isotonic saline. *P<0.05 vs. control mice.

### Comparison between wild type and PLY mutants.

Earlier studies showed that PLY has cytolytic and complement activating activity. To determine to which extent these two activities contribute to PLY-induced lung inflammation, PMN recruitment and cytokine production in lungs were determined after administration of PLY and PLY mutants. Three different PLY mutants were used. PdB, with reduced cytolytic activity, PdBD with less cytolytic and reduced ability to activate complement, and PdT with no toxicity.

PLY-induced PMN influx was more pronounced than PMN recruitment induced by PdB or PdBD, while PdT elicited no influx at all (all P<0.05 vs. PLY). PMN 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 PMN attraction to the lung. We also measured cytokine and chemokine levels in BALF, and PLY induced significantly higher levels of IL-6, KC and MIP-2 than mutated PLY (Figure 1).

### Histology of the lungs.

When compared to mice inoculated with saline or PdT, the lungs from mice exposed to PLY showed accumulation of PMNs in the interalveolar septae with formation of small intraparenchymal PMN aggregates. Administration of PdB also resulted in
PMN influx in the lungs, but limited to the septae without formation of PMN aggregates. Lungs of mice exposed to PdB displayed interstitial inflammatory infiltrates composed predominantly of lymphocytes and monocytes (data not shown).

Figure 1: Mutated PLY forms show a reduced capacity to induce influx of PMNs and production of IL-6, KC and MIP-2 in mouse lungs. NaCl=saline controls. PLY=wild type PLY. PdB=PLY lacking cytolytic properties. PdBD=PLY lacking cytolytic and complement activating activity. PdT=PLY without any toxicity. Cell counts and cytokines were determined in BALF obtained 6h after intranasal instillation of (mutated) PLY. N = 6 per group. Results are expressed as means ± SEM. *P<0.05 vs. NaCl controls. †P<0.05 vs. PLY treated mice

Roles of IL-6 and MIP-2 in PLY-induced PMN recruitment
Having established that PLY induces PMN influx concurrently with IL-6 and MIP-2 production, we determined the roles of these mediators in PMN recruitment after PLY administration. For this purpose we compared PLY responses in lungs of IL-6+/+ and IL-6−/− mice, and in lungs of mice treated with pre-immune or anti-MIP-2 rabbit anti-serum. The number of PMNs tended to be less in IL-6−/− mice when compared to IL-6+/+ mice (3.0 ± 0.9 vs. 6.2 ± 1.4 x 10^4/ml BALF resp.; non significant). In addition, BALF KC levels were lower in IL-6−/− than in IL-6+/+ (1.0 ± 0.1 vs. 0.5 ± 0.1 ng/ml respectively; P<0.05), whereas MIP-2 concentrations did not differ between groups (data not shown). In anti-MIP-2 treated mice, PMN influx was significantly diminished when compared to control mice (1.9 ± 0.7 vs. 8.0 ± 1.6 x 10^4 /ml;P<0.05). KC levels tended to be lower in anti-MIP-2 treated mice when compared to control treated mice (1.3 ± 0.4 vs. 0.5 ± 0.2 ng/ml;not significant), while IL-6 levels did not differ between the groups (data not shown).
Discussion

In this study, we demonstrated that PLY is able to induce lung inflammation in mice, characterized by PMN 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 PMN recruitment in response to PLY.

PLY is considered an important virulence factor in pneumococcal infections. PLY deficient *S. pneumoniae* has been found markedly less virulent than wild type *S. pneumoniae* in rodent models of intravenous, intraperitoneal and pulmonary infection.\(^2,3,10\) There are two known sets of activities of PLY: cytolytic and complement activating activity. Earlier studies have 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 wild type PLY in eliciting PMN 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 with only reduced complement activating activity (and intact cytolytic activity) 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 site of the infection. Previous studies have suggested that PLY may contribute to this response. PLY stimulated 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 wild type *S. pneumoniae* than after administration of PLY-negative mutant *S. pneumoniae* in mice.\(^2\) 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 previous in vitro observations,\(^11\) PLY did not induce production of TNF in mouse lungs.

The cytolytic activity of PLY also seemed most important 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 to wild type PLY. Since IL-6 and MIP-2 can influence PMN influx into lungs during acute inflammation,\(^12-14\) we were interested in the involvement of these mediators in PLY-induced PMN recruitment. IL-6\(^{-/-}\) mice displayed a reduced PMN influx in BALF. Previous work in rats showed that intratracheal instillation of IL-6 resulted in PMN infiltration into the lung interstitium and alveoli, and elevation of neutrophils in BALF.\(^15\) Further, 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 PMNs 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 was found to reduce PMN influx after PLY administration. In accordance, endogenous MIP-2 was important for PMN recruitment during murine *Klebsiella pneumonia* \(^8\) 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, suggesting that PMNs contribute to KC production.

PLY importantly contributes to the virulence of *S. pneumoniae*. We demonstrate here that PLY is able to induce an acute inflammatory reaction in the lung after intranasal instillation in mice, characterized by PMN influx, lung damage and local IL-6 and CXC chemokine production. PLY induced PMN 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.

References
