EXPERIMENTAL PSEUDOMONAS AERUGINOSA PNEUMONIA. EVALUATION OF THE ASSOCIATED INFLAMMATORY RESPONSE

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ABSTRACT

An abnormal inflammatory response (IR) in pneumonia is associated with poor outcomes and high mortality. Animal models could help to better understand the relationship between the pulmonary infection and the associated IR.

The aim of the study was to validate an experimental model of pneumonia induced by the inoculation of *Pseudomonas aeruginosa* in ventilated piglets and to study the associated IR over a long period of time (96 h).

Five Lagerwhite-Landrace piglets were ventilated for four days. After intubation, a solution containing 75 ml of *P. aeruginosa* (10⁶ cfu/ml) was bronchoscopically inoculated. Physiologic and laboratory parameters were monitored throughout the study. Proinflammatory cytokines were measured in serum and in bronchoalveolar lavage (BAL). Histopathology of the lungs and cultures from blood, BAL and lungs were performed.

All the animals developed histopathological evidence of pneumonia. Microbiological studies of both BAL and lung confirmed the presence of *P. aeruginosa* in all the samples. Throughout the study, an increase in IL-6 was observed in serum and in BAL.

In conclusion, this experimental model of pneumonia induced by the inoculation of high concentrations of *P. aeruginosa* in ventilated piglets is feasible and could be appropriate for the evaluation of different aspects of the associated IR.
INTRODUCTION:

Patients with severe community or hospital acquired pneumonia requiring mechanical ventilation have a very high mortality (1;2). Over the last decade, evidence has been collected demonstrating the relevance of the host inflammatory response in the evolution of bacterial infections. Several studies in severe pneumonia have shown increased levels of different inflammatory cytokines both in the lung and in serum (3;4), and recent publications have demonstrated that an increased inflammatory response (IR) is a strong predictor of non-response to treatment and mortality in these patients (5;6). In clinical practice, different factors such as use of antibiotics or presence of comorbidities may influence on the local and systemic IR, and the influence of these factors is difficult to assess accurately in humans. Animal models could help to clarify this issue. Marquette et al, have developed an animal model of pneumonia in mechanically ventilated piglets that closely resembles severe human pneumonia (7). This model has proved to be very useful for the proper evaluation of different aspects regarding diagnosis and treatment (8-13). Pseudomonas aeruginosa is among the most lethal causative microorganisms both in community-acquired pneumonia (CAP) and ventilator-acquired pneumonia (VAP) (1;2;6). The incidence of P. aeruginosa pneumonia is increasing and involves patients that usually receive different antibiotics and that suffer from diverse comorbidities and associated lung pathologies. These circumstances complicate the interpretation of the associated inflammatory response to this invasive microorganism. The availability of an animal model of severe pneumonia where potential confounders are excluded or controlled may improve our knowledge on the intimal mechanisms involving the associated IR. This animal model has been used for pharmacological studies (13) including the evaluation of the effects of antibiotics (14). In the present study we investigated the clinical and histopathological consequences of P. aeruginosa inoculation, as well as the characteristics of the associated inflammatory response in animals ventilated for long periods of time (96 hours).
We hypothesized that inoculation of high concentrations of *P. aeruginosa* in ventilated piglets induces severe pneumonia. By doing so and by maintaining piglets ventilated for a long period of time, the histopathological derangement of lung parenchyma as well as the associated inflammatory response can be investigated. The aim of the present study was to validate an experimental model of pneumonia induced by the inoculation of *Pseudomonas aeruginosa* in ventilated piglets and to assess the associated inflammatory response in the lung and in serum.
METHODS:

An experimental Intensive Care Unit (ICU) was set up at the Hospital de Clínicas, University of Buenos Aires. It is fully equipped with cardiovascular monitors (Hewlett-Packard Model 78353-A Andover, MA), ventilators (Luft 1 ®, Córdoba, Argentina) and electrical infusors (Abbott infusor Pumps, Abbott Park, IL).

Animal preparation.

Six healthy domestic bred Largewhite-Landrace piglets, aged 3 months and weighting 20 ± 2 kg were anesthesized and orotracheally intubated with a 7.0-mm low pressure cuff tube (Portex, Hythe, Kent, U.K.). They were pre-anesthetized with IM ketamine (Ketalar, Parke-Davis) 250 mg. Anesthesia was maintained with a continuous infusion of Midazolam (Dormicum®, Roche) 0.3 mg.kg⁻¹.h⁻¹, pancuronium bromide (Pavulon© 4 mg, Organon Argentina) 0.32 mg.kg⁻¹.h⁻¹ and phentanyl (Sublimaze© Janssen-Cilag) 0.07 mg, 5 µg.kg⁻¹.h⁻¹. A catheter was inserted in the femoral vein for continuous infusion of 10 % Dextrose (1.5 ml.kg⁻¹.h⁻¹) and Ringer Lactate (3 ml.kg⁻¹.h⁻¹) with an infusion pump. The femoral artery was cannulated with a 3F polyethylene catheter (Plastimed, St Leu la forêt, France) for pressure monitoring and blood sampling. An 8F suprapubic urinary catheter (Vesicoset, Angiomed, Karlsruhe, Germany) was placed in the bladder through surgical midline minipelvitomy. The piglets were then placed in a prone position and were mechanically ventilated. Piglets dying during the first 12 hours were excluded from the study.

Mechanical ventilation.

Animals were mechanically ventilated in a volume-controlled mode. Ventilator parameters consisted of a tidal volume (V₁) at constant inspiratory flow of 10 ml.kg⁻¹, a respiratory rate (RR) of 15 breaths per minute, an inspiratory time of 33%, with an initial FiO₂ of 100 % and positive end expiratory pressure (PEEP) = 0. Later, FiO₂ was set according to blood gas analysis in order to obtain 80 < PaO₂.
< 100 mmHg and PEEP was increased until a maximum of 5 cm H2O. Airway pressures, static pulmonary compliance (Cst), computed by dividing the Vt by the difference between the end-inspiratory plateau pressure and the total PEEP (15) and arterial blood gases (IL-1306; Instrumentation Laboraties, Milan, Italy) were determined every 6 hours. Throughout the protocol, PaCO2 was maintained at between 35 and 45 mmHg by increasing the respiratory frequency to the maximum level preceding the appearance of auto-PEEP. Above this limit, hypercapnia was tolerated.

**Bronchial inoculation.**

Animals were inoculated with 75 mL of a suspension of 10^6 colony forming units (cfu)/ml of pathogenic *Pseudomonas aeruginosa* ATCC 27853. 15 ml of the infusion were evenly distributed among every lobe of each lung through the bronchoscope’s channel.

The inoculums of bacteria were instilled once the animals were hemodinamically stable after sedation and mechanical ventilation.

**Samplings and procedures.**

Mechanical ventilation parameters (tidal volume, respiratory rate, airway pressures and FiO2), heart rate, blood pressure, body temperature, blood gases, serum electrolytes (sodium, potassium) and lactate concentrations were monitored at 0, 2, 6, 12, 24, 36, 48, 60, 72, 84 and 96 hours. Blood biochemistry (glucose, creatinine, urea, bilirubin, aspartat aminotransferase and alanine aminotransferase) and blood cells count were obtained at 0, 24, 48, 72 and 96 hours.

**Bronchoalveolar lavage:** five 20 mL aliquots of sterile saline solution (0.9% NaCl) were instilled and reaspirated through the bronchoscope’s channel in the right middle lobe at hour 0 (before the inoculation of *P. aeruginosa* suspension) and at hour 96.

**Inflammatory parameters.**

C-Reactive Protein (CRP): CRP was quantified in serum and BAL using C-reactive protein kit (Biosystems S.A., Barcelona, Spain).
Cytokines in blood and BAL fluid: TNF-alfa, IL-1 beta, IL-6 and IL-8 levels were measured in serum and BAL supernatant using ELISA method in specific porcine kits (R&D Systems Inc., Minneapolis, MN, USA).

BAL cytokines and CRP were determined at the time of intubation and at the end of the study. Serum cytokines and CRP were determined at the time of intubation and at 24, 48, 72 and 96 hours.

**Bacteriological studies.**

Quantitative cultures of BAL fluid, serum, and lung tissue were performed at the end of the study. Bronchoalveolar lavage and lung tissue specimens were processed for quantitative bacterial cultures as described elsewhere (16) according to recommended laboratory methods (17)

**Sacrifice and Post-mortem studies.**

Sacrifice was performed at hour 96 under general anesthesia by intravenous potassium chloride infusion.

**Collection of Lung Specimens:** after death, animals remained under mechanical ventilation until surgical samples of lung parenchyma were obtained for bacteriologic and histopathologic evaluation. Lungs were aseptically exposed through a cervico-thoracic midline incision. Thereafter, at least one lung tissue specimen (1 cm³) was taken from both the more preserved lobe (macroscopically) and the more involved lobe.

**Histopathologic assessment:** Lung tissue was processed according to standard methods. Analysis of vessels (thrombosis and endothelial lesions), pleural (acute or chronic pleuritis) and lung parenchyma was performed. The evaluation of lung parenchyma included severity of pneumonia and presence of other associated lesions (hyaline membranes and alveolar damage). Severity of pneumonia was graded according to previously published criteria (18) in grades 0: no pneumonia; 1: purulent mucous plugging; 2: bronchiolitis; 3: pneumonia (consolidation coexisting with significant accumulation of polymorphonuclear leukocytes, fibrinous exudates and cellular debris into the alveolar space); 4:
confluent pneumonia (extension along different secondary lobes); and 5: abscessed pneumonia (cellular necrosis coexisting with disruption of cellular architecture). Pneumonia was limited to the last 3 categories. Classification of each specimen was based upon the worst category observed.

Statistical Analysis.

All data were expressed as mean ± SD or SEM as appropriate. Non parametric tests for paired data were used; Wilcoxon and Friedman tests were used for comparison of two or more than two time points, respectively. A p value < 0.05 was considered statistically significant.

Approval by the institutional committee.

All animals were treated in compliance with the guidelines of the Ethics Committee and Direction of Investigation of the Hospital de Clínicas “José de San Martín”, University Buenos Aires and the Guide for the Care and use of Laboratory Animals (NIH Publication No. 93-23, revised 1985).
RESULTS

We studied a total of 5 piglets after excluding 1 that died during the first 12 hours (death attributable to the animal preparation).

Physiologic and laboratory data:

Physiologic and laboratory variables are shown in table 1. After bronchial inoculation, a rapid and persistent decrease of the PaO₂/FiO₂ ratio (p=0.01) and a rise in body temperature (p=0.03) was observed throughout the study. Static compliance decreased over time, although differences did not reach statistical significance. No differences in biochemical data were observed.

Inflammatory Response:

Serum IL-6 increased throughout the study, with a maximal increase at hour 48. IL-1 beta and IL-8 did not change significantly. Although not significant, levels of TNF-alfa decreased slightly at the end of the study (figure 1).

A significant increase in the concentration of IL-6 in BAL fluid at the end of the study was also observed. The increase in the concentration of the remaining cytokines evaluated did not reach statistical significance (figure 2). Levels of CRP in BAL fluid did not change over the study period (data not shown).

Microbiology:

Pseudomonas aeruginosa was present in all samples (20/20) from BAL fluid and lung tissue cultures from the 5 piglets evaluated. BAL cultures yielded P. aeruginosa in a concentration >10⁴ cfu/ml in four piglets and > 10⁵ cfu/ml in one piglet. Lung tissue cultures also showed growth of P. aeruginosa in a concentration >10⁴ cfu/gr in all the samples evaluated. Blood cultures were positive for P. aeruginosa in one piglet.
*Morganella Morgani* also grew in 2/20 samples evaluated (in a concentration of $10^3$ cfu/ml) and *Staphilococcus coagulase-negative* grew in 3/20 samples (in a concentration of $10^3$ cfu/ml).

**Histopathological Findings:**

All piglets developed pneumonia (grades 3 to 5). Pneumonia was present in all the samples taken from the most involved lobes and also in 3 out of the 5 samples taken from the most preserved lobes. Severe pneumonia, defined as abscessed pneumonia or confluent pneumonia (grades 4 and 5), was present in 60% of the pulmonary biopsies and in 3/5 piglets (table 2). Acute or subacute pleuritis was also present in all the piglets, diffuse alveolar damage (defined by the presence of hyaline membranes covering the alveolar epithelium) in 4/5 piglets and vascular lesions (including endothelial or thrombosis) in 3/5.
DISCUSSION

The results of the present study demonstrate that inoculation of *P. aeruginosa* into the lungs in ventilated piglets causes pneumonia. After a long period of mechanical ventilation (96 h) all the animals developed pneumonia by this microorganism and in the majority of piglets, severe histopathological lesions in the lungs were observed. Also, using this animal model, we were able to evaluate the dynamics of the associated inflammatory response that was characterized by a significant increase in IL-6 levels over time in both serum and BAL.

The availability of an experimental model of severe pneumonia without the interference of antibiotic treatment, in which animals are ventilated during 96 hours, allows the physiopathological changes taking place in the lung to be studied throughout the process. Inoculation of *P. aeruginosa* led to the development of clinical signs of pneumonia (fever and gas exchange impairment) and deterioration in lung mechanics (decrease in static compliance over time). Post mortem evaluation confirmed both, histopathologically and microbiologically, the presence of pneumonia in all the animals evaluated. Presence of pneumonia was evident in all those lobes that macroscopically were most involved but also in 60% of the most preserved ones, confirming the multilobar distribution of the infection.

Interestingly, there was also evidence of hyaline membranes in 80% of the piglets evaluated. Hyaline membranes appear lately on the evolution of pneumonia and their presence is associated with diffuse alveolar damage and irreversible fibrosis (19). Goldstein I et al (9) evaluated lesions associated to acute pneumonia in the same animal model and, as expected, no hyaline membranes or signs of diffuse alveolar damage were observed. By contrast, in the present study, since animals were ventilated for long periods of time, the development of late-stage lesions of pneumonia were common. This circumstance might be useful for the assessment of different types of treatment that have been claimed to be beneficial in these late stages such as glucocorticoids or other immunomodulators.
In the present study, we selected *P. aeruginosa* as the etiological agent because it is a common and often lethal cause of pneumonia in ventilated patients (2). Patients with *P. aeruginosa* pneumonia usually receive different types of antibiotics and suffer from several comorbidities, circumstances that may influence the associated inflammatory response. By using this animal model of pneumonia, we avoid some of the potential confounders, making the assessment of the inflammatory response more accurate.

The lung IR at the end of the study was characterized by an increase of different proinflammatory cytokines, mainly IL-6, in BAL fluid. Although an increase in IL-6 levels was also observed in serum, the dynamics of the remaining cytokines evaluated in blood did not show a consistent pattern, suggesting that the IR was mainly compartmentalized. Dehoux et al, in a study in humans with unilateral pneumonia, confirmed that the production of cytokines in response to local bacterial infection is compartmentalized (20). However, when the pneumonia is more severe, causes septic shock or is acquired during mechanical ventilation, the systemic IR is also evident (3;4;21). The lack of correlation between local and systemic inflammation and the inconsistent pattern of the different cytokines measured in serum may probably be explained by the absence of sepsis in the animals studied (as reflected by negative blood cultures in all but one animal, no hemodynamic disturbances and no requirement for vasopressive drugs). In the present study, IL-6 was the only of the cytokines evaluated that showed a significant increase (both in serum and in the lung). In fact, correlations between severity scores and serum IL-6 have been observed (22;23). In addition, IL-6 concentration in serum has been shown to be an independent predictive factor of mortality in different population groups (5;6). In this sense, measurement of IL-6 concentration in serum and in BAL, might be a very useful parameter to assess the magnitude of the IR and the potential effects of different antiinflammatory treatments.
Our study has limitations. First, the exogenous administration of a highly bacterial inoculum in a previously healthy animal does not necessarily reflect the complexities of pneumonia development in humans. Potential species differences in lung immunology between piglets and humans must also be considered. Also, for an accurate evaluation of the local IR, sequential BAL samples at different time-points would have been optimal. Although the “early phase” cytokines evaluated in the present study have been widely used in clinical practice for the assessment of the IR, other inflammatory mediators such as adhesion proteins, Rantes, or IL-10 might have been more appropriate since they are expressed in more advanced phases of the infection. Finally, we can not exclude that the injury of mechanical ventilation played some role in the observed inflammatory response.

In summary, the present animal model of pneumonia by *P. aeruginosa* has proved to be efficacious and reproducible, and may constitute a useful tool for evaluating the IR associated to pulmonary infections and the potential effects of immunomodulatory treatments.
REFERENCES


Table 1: Sequential measurements of physiologic and laboratory parameters. Data are expressed as mean ± SD. p values are according to Friedman test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 hour</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, ºC</td>
<td>36.4 ± 0.7</td>
<td>40.1 ± 0.7</td>
<td>39.0 ± 1.2</td>
<td>39.0 ± 1.7</td>
<td>39.1 ± 1.7</td>
<td>0.032</td>
</tr>
<tr>
<td>Heart Rate, bpm</td>
<td>146 ± 36</td>
<td>156 ± 39</td>
<td>138 ± 11</td>
<td>141 ± 20</td>
<td>140 ± 29</td>
<td>0.35</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>124 ± 4</td>
<td>114 ± 19</td>
<td>123 ± 22</td>
<td>118 ± 21</td>
<td>108 ± 27</td>
<td>0.58</td>
</tr>
<tr>
<td>Cst, ml/cm H₂O</td>
<td>16.9 ± 3.1</td>
<td>16.4 ± 3.2</td>
<td>14.8 ± 3.9</td>
<td>13.8 ± 4.0</td>
<td>13.2 ± 3.2</td>
<td>0.14</td>
</tr>
<tr>
<td>PaO₂/FiO₂, mmHg</td>
<td>350 ± 144</td>
<td>210 ± 98</td>
<td>150 ± 52</td>
<td>138 ± 50</td>
<td>132 ± 41</td>
<td>0.017</td>
</tr>
<tr>
<td>Hematocrit, L/L</td>
<td>30 ± 1</td>
<td>29 ± 2</td>
<td>27 ± 3</td>
<td>25 ± 1</td>
<td>22± 1</td>
<td>0.06</td>
</tr>
<tr>
<td>WBC, x10⁹/L</td>
<td>18160 ± 10046</td>
<td>21920 ± 12934</td>
<td>13160 ± 3922</td>
<td>10820 ± 5140</td>
<td>12340 ± 4160</td>
<td>0.09</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.8</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.83</td>
</tr>
<tr>
<td>ASAT, U/dL</td>
<td>61 ± 46</td>
<td>177 ± 285</td>
<td>255 ± 229</td>
<td>192 ± 269</td>
<td>130 ± 146</td>
<td>0.78</td>
</tr>
<tr>
<td>ALAT, U/dL</td>
<td>46 ± 15</td>
<td>64 ± 42</td>
<td>69 ± 56</td>
<td>79 ± 81</td>
<td>70 ± 62</td>
<td>0.99</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>2.9 ± 1.7</td>
<td>4.9 ± 3.9</td>
<td>5.5 ± 3.5</td>
<td>3.7 ± 0.9</td>
<td>3.6 ± 2.2</td>
<td>0.71</td>
</tr>
</tbody>
</table>

MAP= Mean Arterial Pressure; Cst= Static compliance; ASAT= Aspartat Amine Transferase, ALAT= Alanine Amine Transferase, WBC= White Blood Cell Count; CRP= C reactive protein.
**Table 2:** Histopathological findings

<table>
<thead>
<tr>
<th></th>
<th>More involved lobe</th>
<th>More preserved lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Piglet 1</strong></td>
<td>Abscessed pneumonia</td>
<td>Confluent pneumonia</td>
</tr>
<tr>
<td><strong>Piglet 2</strong></td>
<td>Confluent pneumonia</td>
<td>Confluent pneumonia</td>
</tr>
<tr>
<td><strong>Piglet 3</strong></td>
<td>Pneumonia</td>
<td>Purulent mucus plugging</td>
</tr>
<tr>
<td><strong>Piglet 4</strong></td>
<td>Abscessed pneumonia</td>
<td>Confluent pneumonia</td>
</tr>
<tr>
<td><strong>Piglet 5</strong></td>
<td>Pneumonia</td>
<td>Purulent mucus plugging</td>
</tr>
</tbody>
</table>
Figure 1

- **serum TNF-alfa**
  - Time (hours): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20
  - pg/ml: 0, 25, 50, 75, 100, 125, 150
  - p-value: 0.30

- **serum IL-8**
  - Time (hours): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20
  - pg/ml: 0, 25, 50, 75
  - p-value: 0.67

- **serum IL-6**
  - Time (hours): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20
  - pg/ml: 0, 25, 50, 75, 100, 125
  - p-value: 0.04

- **serum IL-1 beta**
  - Time (hours): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20
  - pg/ml: 0, 25, 50, 75
  - p-value: 0.97
Figure 2

**BAL IL-1 beta**

- Time (hours): 0, 96
- pg/ml: 0, 200, 400, 600, 800, 1000
- p-value: 0.06

**BAL IL-6**

- Time (hours): 0, 96
- pg/ml: 0, 50, 100, 150, 200
- p-value: 0.04

**BAL IL-8**

- Time (hours): 0, 96
- pg/ml: 0, 50, 100, 150
- p-value: 0.14

**BAL TNF-alfa**

- Time (hours): 0, 96
- pg/ml: 0, 50, 100, 150
- p-value: 0.46
LEGENDS TO FIGURES

**Figure 1**: Sequential determination of serum IL-1-beta, IL-6, IL-8 and TNF-alfa during the study. Data are expressed as mean ± SEM. p values are according to Friedman test.

**Figure 2**: Bronchoalveolar lavage (BAL) measurements at 0 hour and at 96 hours of the initiation of the study of IL-1 beta, IL-6, IL-8 and TNF-alfa. Data are expressed as mean ± SEM. p values are according to Wilcoxon test.