Augmented effect of early antibiotic treatment in mice with experimental lung infections due to sequentially adapted mucoid strains of *Pseudomonas aeruginosa*

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**Background:** Effects of treatment with tobramycin initiated 1 or 24 h post-infection were investigated in a new version of a pulmonary infection model in mice. The model reflects the differentiated behaviour of *Pseudomonas aeruginosa* mucoid strains isolated from the lungs of one chronically infected cystic fibrosis (CF) patient at different time periods during chronic lung infection.

**Methods:** BALB/c mice were challenged with alginate-embedded mucoid clinical isolates isolated in 1988, 1997 or 2003. Mice were euthanized on day 1, 2 or 3 post-infection for estimation of quantitative bacteriology, histopathology, and measurement of granulocyte colony-stimulating factor (G-CSF) and macrophage inflammatory protein 2 (MIP-2).

**Results:** There was a significant reduction of bacteria when comparing treatment initiated 1 h post-infection with treatment initiated after 24 h for isolates 1997 and 2003. Treatment initiated 1 h post-infection also resulted in a reduction of the pulmonary cytokines G-CSF, for all three isolates, and MIP-2, for isolates 1997 and 2003. Histological evaluation showed a shift from the acute-type inflammatory immune response to a chronic-type in mice infected with isolate 2003.

**Conclusions:** A significant reduction in the number of bacteria was observed when initiating treatment 1 h post-infection compared with initiating treatment after 24 h, although the latest isolate avoided complete clearance. Early antibiotic treatment directed at the mucoid phenotype in mice also reduced the inflammation and, thereby, the lung tissue damage.

Keywords: tobramycin, MIP-2, G-CSF, cystic fibrosis

**Introduction**

The majority of patients with cystic fibrosis (CF) acquire a chronic *Pseudomonas aeruginosa* lung infection, which results in premature death.1 One area of interest is the milieu of the CF lung, where *P. aeruginosa* continually adapts to the respiratory and conductive zones of the lungs, to the inflammatory host defence, and to the antibiotic treatments.2 The adaptive mechanisms of *P. aeruginosa* contribute to different genotypes3 and phenotypes, including the mucoid phenotype, which has been isolated from the CF lung.4 There is accumulating evidence that in young children with CF, early *P. aeruginosa* infection increases the risk of morbidity and mortality.5–7 It seems that children with CF typically acquire the non-mucoid phenotype of *P. aeruginosa* early after birth and that the non-mucoid *P. aeruginosa* gradually develops into the mucoid phenotype.5 Although different genes are involved in the conversion of the non-mucoid phenotype into
the mucoid one, the most common mutation causing overproduction of alginate, which results in the appearance of the mucoid phenotype, is in the mucA gene.\textsuperscript{3,9} The massive infiltration of phagocytic cells, such as macrophages and polymorphonuclear leucocytes (PMNs), which surround the biofilm-growing bacteria in the CF lung, is characteristic of the chronic state of infection. The PMNs also contribute to the mutations in mucA which leads to the appearance of mucoid biofilms.\textsuperscript{10} The ability to form biofilms is believed to play a prominent role in the persistence of the lung infection. Since mucoid P. aeruginosa apparently cannot be eradicated by current antibiotics,\textsuperscript{11,32} becomes predominant with age\textsuperscript{13} and predicts shortened CF survival,\textsuperscript{14} early prevention and detection of not only mucoid but also non-mucoid P. aeruginosa are critical.\textsuperscript{3,11,15}

In CF patients it is possible to eradicate initial infection with non-mucoid P. aeruginosa by applying early aggressive anti-

Pseudomonas treatment.\textsuperscript{16–21} Early aggressive therapy of intermittent colonization with oral ciprofloxacin and additional colistin inhalation has delayed the onset of chronic infection, and intensive anti-

Pseudomonas chemotherapy has greatly improved the prognosis for chronically infected CF patients.\textsuperscript{18,19,22} The usual antibiotic maintenance therapy of CF patients with chronic infections used in the Copenhagen CF Centre to suppress the chronic infection involves intravenous (iv) courses with an aminoglycoside, such as tobramycin, and a β-lactam every third month combined with daily colistin inhalations and oral azithromycin between iv courses.\textsuperscript{23,24} It is recognized that antibiotic therapy improves lung function, relieves the symptoms of possible acute pulmonary exacerbations and improves the quality of life of CF patients. Nonetheless, it does not eradicate the bacteria, but reduces the mortality.\textsuperscript{25–27}

A new version of a P. aeruginosa pulmonary infection model in mice has been established by Moser et al.,\textsuperscript{28} using six PFGE-identical isolates, consisting of three pairs of non-mucoid and mucoid isolates from 1988, 1997 and 2003. Two of the non-

mucoid strains were initially presented in the work by Lee et al.,\textsuperscript{29} showing that the ability to form biofilm in vitro decreased during the timeframe of isolation. When the strains were investigated in vivo in the mouse model used in this study, they showed a reduced ability to establish a lung infection and, thereby, persist in the lung.\textsuperscript{28} The mouse model experimentally reflects the time course and characteristics of several years of adaptation during chronic infection with P. aeruginosa in CF patients.\textsuperscript{28} Since the host response may also change during antibiotic treatment, the present study aimed to investigate the influence of treatment with the antibiotic tobramycin at different times during chronic P. aeruginosa infection with mucoid or non-mucoid isolates. Since early aggressive antibiotic treatment in most cases can prevent progression from intermittent to chronic P. aeruginosa lung infection, we also compared the course of infection when treatment was initiated 1 or 24 h after inoculation of bacteria in the lungs of the mice. At the dose chosen for inoculation in the initial pilot study, the non-mucoid bacteria were cleared independently of treatment, and no significant difference was observed between the treated and untreated groups. The study showed a clearance of 67% for non-mucoid isolates 1988 and 2003 at day 3 in the untreated groups. Non-mucoid isolate 1997 was cleared by day 2. This was in correlation with Moser et al.,\textsuperscript{28} where a clearance of 50% was seen for non-mucoid isolate 1988, and a clearance of 100% was seen for non-mucoid isolates 1997 and 2003 at day 5. Therefore, the present study focuses on the work done with the mucoid isolates.

Materials and methods

Bacterial strains

The clinical P. aeruginosa isolates were obtained from the sputum by expectoration or endolaryngeal suction of one CF patient attending the Copenhagen CF Center, Rigshospitalet, Copenhagen. Gram staining and microscopic examination were used to confirm the origin of P. aeruginosa from the lower airway and conventional biochemical tests were used to identify P. aeruginosa. All collected P. aeruginosa isolates were stored at −80°C in broth supplemented with 10% glycerol.\textsuperscript{29} The isolates were obtained in 1988, 1997 and 2003. In 1988 the patient had been chronically infected for 16 years. The relatedness of the isolates was determined by PFGE and the isolate from 2003 was obtained after a lung transplant.\textsuperscript{28,29}

Animals

Female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark) at 9–11 weeks of age, and were maintained on standard mouse chow and water ad libitum for a minimum of 1 week before challenge. All experiments were authorized by the National Animal Ethics Committee, Denmark.

MICs and pharmacokinetics of tobramycin

The MICs of tobramycin were determined by Etest (AB Biodisk, Sweden) for the non-mucoid and mucoid isolates. Mice eliminate antibiotics much faster than humans,\textsuperscript{30} therefore, in order to determine the dose of tobramycin, a preliminary drug dosage study was conducted with the aim of reaching a therapeutic success of 10–12 times above the MIC (\(f_{\text{Cmax}}/\text{MIC ratio}\)) when administrated once every 24 h. Tobramycin was administrated subcutaneously (sc) or intraperitoneally (ip) in non-infected mice. At 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 6 h post-dose, mice (\(n=4\) per timepoint) were euthanized with 10.0 mL/kg of body weight pentobarbital (DAK, Copenhagen, Denmark) injected ip, and blood was aseptically sampled from the heart with a syringe and transferred to a BD Microtainer SST gold tube (ref. 365956). After centrifugation, serum was stored at −20°C until determination of the concentration using a fluorescence polarization immunoassay (TDx, Abbott Diagnostics, USA). Both routes of administration were well tolerated, had comparable pharmacokinetics and a therapeutic success of 10–12 times above the MIC (\(f_{\text{Cmax}}/\text{MIC ratio}\)). However, due to more accessible administration and to avoid any, although minor, first-pass hepatic metabolism, sc administration was chosen.

Pulmonary infection model

Immobilization of P. aeruginosa in seaweed alginate beads was performed as described by Pedersen et al.,\textsuperscript{31} with the following modifications: bacterial overnight cultures were centrifuged for 10 min, and the supernatants were discarded and the bacterial precipitate was resuspended in 5 mL of ox bouillon (SSI, Copenhagen, Denmark). Directly before challenge, the suspension was adjusted to 1–10\textsuperscript{8} CFU/mL in 0.9% NaCl. Animals were challenged according to the methods described by Moser et al.,\textsuperscript{32} with the following modification: mice were anaesthetized by sc injections in the groin area with Hypnorm®/midazolam (Roche) [one part Hypnorm®...
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(0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone), one part midazolam (5 mg/mL) and two parts sterile water. To euthanize the mice at the termination of the experiments, 10.0 mL/kg of body weight pentobarbital was injected ip. For every isolate there were three groups: one where treatment was initiated 1 h post-infection, one where treatment was initiated 24 h post-infection and an untreated group.

**Quantitative bacteriology**

At termination of the experiments, isolated lungs were placed in 5 mL of 0.9% NaCl and kept on ice until homogenization for 15–20 s (SilentCrusher M, Heidolph, Germany). Serial dilutions were plated onto blue agar plates (modified Conradi–Drigalski medium selective for Gram-negative bacilli; State Serum Institute, Denmark) for colony counting. The plates were incubated at 37°C for 1 or 2 days and the cfu/lung values were determined.

**Histopathology**

The macroscopically affected parts of the left lungs were prepared for histopathological examination by fixation in 4% (w/v) formaldehyde solution (Bie & Berntsen, Denmark), embedding in paraffin wax and cutting into 5 μm thick sections, followed by haematoxylin and eosin staining. All the histopathological examinations were done by the blind method.

**Pulmonary cytokines**

Determination of granulocyte colony-stimulating factor (G-CSF) and macrophage inflammatory protein 2 (MIP-2) in lung homogenates was achieved by ELISA following the manufacturer’s directions (R&D, Minneapolis, USA). All determinations were performed in duplicate.

**Statistical analysis**

The number of mice in each group was calculated to provide a power of 0.8 or higher for continuous data. For analysing quantitative data, the Mann–Whitney *U*-test was used for calculating *P* values in the statistical program GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, USA). For qualitative data, the χ² test was used for calculating *P* values. *P* values of ≤0.05 were considered significant.

**Table 1.** MICs (mg/L) of tobramycin determined by Etest for the non-mucoid and mucoid isolates from years 1988, 1997 and 2003

<table>
<thead>
<tr>
<th></th>
<th>Non-mucoid</th>
<th>Mucoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate 1988</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Isolate 1997</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Isolate 2003</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

**Results**

**Determination of MICs and pharmacokinetics of tobramycin**

MICs for both the non-mucoid and mucoid isolates were determined by Etest, and are displayed in Table 1. The MICs ranged from 3 to 24 mg/L. The pharmacokinetics of 120 mg/kg of body weight tobramycin in non-infected BALB/c mice was determined prior to the treatment experiment, and had a therapeutic success of >45 times above the MIC (*fC*ₘₐₓ/MIC ratio) for all the mucoid isolates and from 9 up to 57 times above the MIC for the non-mucoid isolates. The pharmacokinetic parameters are displayed in Table 2.

**Bacterial assessment of infected lungs**

Female BALB/c mice were infected intratracheally with alginate beads containing isolate 1988 (n=94), 1997 (n=84) or 2003 (n=122). Eight mice infected with each isolate were euthanized directly after challenge to estimate the content of bacteria in the lungs (Figure 1). For every isolate there were three groups: one where treatment was initiated 1 h post-infection; one where treatment was initiated 24 h post-infection; and an untreated group that received isotonic saline initiated after 1 h. Mice were treated once every 24 h.

Mice were euthanized at day 1, 2 or 3 post-infection to estimate the bacterial content of the lungs. For isolates 1997 and 2003, treatment initiated 1 h post-infection significantly reduced the number of bacteria compared with treatment initiated after 24 h (*P*<0.0004) (Figure 1). Both treatment regimens significantly reduced the number of bacteria when compared with the untreated group for all the isolates (*P*<0.03) (Figure 1). Even though the estimated infection inoculum was nearly the same for all the isolates at day 0 (Figure 1), the untreated group infected with isolate 1988 presented a decline from day 1 in cultivated bacteria from the lungs compared with isolates 1997 and 2003, resulting in a difference of between 2 and 5 log during the 3 days. Furthermore, treatment with tobramycin not only cleared isolate 1988 when initiated 1 h post-infection, but also when initiated after 24 h. This was not observed for isolates 1997 and 2003 when treatment was initiated after 24 h.

**Histopathological examinations**

On day 3, the macroscopically affected parts of the lungs were prepared for histopathological examination, and thereafter grouped by blinded research according to the degree and type of inflammation (Tables 3 and 4). The importance of initiating treatment 1 h post-infection compared with after 24 h was seen with isolates 1997 and 2003, where treatment initiated 1 h post-infection resulted in a significant reduction in the degree of inflammation (*P*<0.002) (Table 3). Treatment initiated 1 h post-infection also resulted in less inflammation compared with no treatment for all of the isolates (*P*<0.01). Only isolates 1988 and 2003 showed a significant reduction in the degree of inflammation between the 24 h treatment groups and the untreated groups (*P*<0.02).

With regard to the type of inflammation, a significant difference was not observed for any of the isolates when treatment was initiated 1 h post-infection compared with after 24 h. However, in the untreated group a more severe inflammatory
response dominated by PMNs when comparing isolate 2003 with isolate 1988 or 1997 ($P<0.0001$) (Table 4). A significant difference in the type of inflammation was observed for isolates 1988 and 2003, between the 1 h treatment groups and the untreated groups ($P<0.001$). This was also observed when comparing treatment initiated 24 h post-infection with the untreated groups ($P<0.005$).

Production of pulmonary cytokines, G-CSF and MIP-2

The detection of the pulmonary cytokines G-CSF and MIP-2 in lung homogenate was achieved using ELISA. The results showed that treatment initiated 1 h post-infection significantly reduced the concentration of G-CSF for all three isolates ($P<0.05$) when compared with treatment initiated 24 h post-infection. A corresponding reduction in the concentration of MIP-2 was also observed for isolates 1997 and 2003 ($P<0.05$) (Figure 2). When comparing the concentration of both cytokines in the groups where treatment was initiated 1 h post-infection with the untreated groups, a significant decrease was observed for all three isolates ($P<0.004$).

Treatment initiated 24 h post-infection showed a significant decrease in G-CSF on day 2 for both isolates 1988 and 2003, and on day 3 for all three isolates compared with the untreated group ($P<0.02$). Also, when comparing treatment initiated 24 h post-infection with no treatment, a significant decrease in MIP-2 was observed for isolates 1988 and 1997 on day 2, and for all three isolates on day 3 ($P<0.02$).

Isolates 1988 and 1997 induced lower concentrations of G-CSF and MIP-2 compared with isolate 2003 on day 1 when treatment was initiated 1 h post-infection (Figure 2). For both cytokines there was a reduction in the detected concentration during the 3 days for isolate 1988 when compared with the untreated group. In contrast, for isolates 1997 and 2003 there was either a slight increase or the concentration was kept at a steady state, except for G-CSF at day 3 in the group infected with isolate 1997.

Discussion

Establishment of a new version of a pulmonary infection mouse model using sequential P. aeruginosa isolates from one chronically infected CF patient revealed distinct courses of the chronic infection.28 So far, no other described in vivo model reflects the 20–30 years of bacterial–host interplay observed in CF patients, because it is not possible to maintain a lung infection in mice for >2–3 weeks (see Moser et al.28 for a more detailed discussion of in vivo models). We investigated whether the distinct course of chronic infection observed in the new version of the pulmonary infection mouse model would be reflected during an antibiotic treatment regimen with tobramycin, which is frequently used in the treatment of CF patients.

In the initial pilot study, the mice were able to clear the non-mucoid isolates despite treatment with tobramycin, which was why we decided to focus on the mucoid isolates. However, the clearance was consistent with the study by Moser et al.,28 since at the inoculum chosen ($10^8$ cfu/mL) the non-mucoid isolates were correlated to a reduction in virulence, resulting in a faster clearance from the lungs of the mice and, thereby, reduced pulmonary production of G-CSF and MIP-2.28 On the contrary, the virulence of the mucoid isolates was shown to increase with time.

In the present study, we showed a significant difference between treatment regimens initiated 1 or 24 h post-infection. At day 1 in our study, about half of the mice infected with isolate 1988 or 1997 were free of bacteria (58% and 42%, respectively) and by day 2 all were cleared when treatment was initiated 1 h post-infection. Isolate 2003 had a lower success rate following the 1 h treatment regime, since bacteria from the lungs were cultured on all days (92%, 31% and 50% on days 1, 2 and 3, respectively). When treatment was initiated 24 h post-infection, bacteria were cultured from all mice infected with isolate 1997 or 2003 on all days, indicating that eradication of bacterial infections with the mucoid phenotype of P. aeruginosa is best carried out as early as possible.

The failure of bacterial clearance when treatment was initiated 24 h post-infection could be a consequence of the ability to form biofilms. The mucoid P. aeruginosa has been found to play a role in the antibiotic resistance of in vitro biofilms, besides being an important virulence factor.36 In vitro studies have shown that a young biofilm of both mucoid and non-mucoid P. aeruginosa can be eradicated when using tobramycin and piperacillin, but older biofilms cannot.37 In addition, both blood and serum kill young mucoid biofilm of 2 days, but not old mucoid biofilm of 7 days, indicating that ageing biofilms may contribute to the persistence of the bacterial infection.28

In CF patients the host response is characterized by a persistent inflammation dominated by PMNs, which induces lung tissue damage since the infection cannot be eradicated. To

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Table 2. Pharmacokinetics of 120 mg/kg of body weight tobramycin administrated sc in non-infected BALB/c mice

<table>
<thead>
<tr>
<th>$f_{\text{C}_{\text{max}}}$ (mg/L)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$f_{1/2}$ (h)</th>
<th>$f_{\text{AUC}}$ (mg.h/L)</th>
<th>CL$^{\text{f}}$ [L/(kg.h)]</th>
<th>$V^{\text{g}}$ (L/kg)</th>
<th>$f_{\text{AUC}}$/MIC$^{\text{h}}$ ratio</th>
<th>$f_{\text{C}_{\text{max}}}$/MIC$^{\text{h}}$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>226</td>
<td>0.25</td>
<td>0.33</td>
<td>165</td>
<td>0.73</td>
<td>21</td>
<td>33</td>
<td>45</td>
</tr>
</tbody>
</table>

Notes:
- $f_{\text{C}_{\text{max}}}$: Maximum concentration of drug in serum ($C_{\text{max}}$).
- $T_{\text{max}}$: Time to maximum concentration of drug in serum ($T_{\text{max}}$).
- $f_{1/2}$: Half-life ($f_{1/2}$) determined using linear regression analysis on the first seven points of the serum concentration–time curve.
- $f_{\text{AUC}}$: Area under the 24 h concentration–time curves ($f_{\text{AUC}}$) calculated by the trapezoidal-rule integral.
- CL: Clearance (CL) is the total clearance of the drug/kg of body weight.
- $V$: Volume of distribution ($V$) in volume/kg of body weight.
- MIC: Minimum inhibitory concentration.

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evaluate the effect of treatment on the host response, we investigated the degree and type of inflammation at day 3. For isolate 2003, treatment initiated 1 h post-infection resulted in the best

Table 3. Histopathological evaluation of the degree of inflammation in the lungs of mice infected with mucoid isolates 1988, 1997 and 2003 at day 3 post-infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Degree of inflammation</th>
<th>isolate 1988</th>
<th>isolate 1997</th>
<th>isolate 2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1 h</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>24 h</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*By blinded research, the degree of inflammation was microscopically grouped as: 0, normal; 1, mild focal inflammation; 2, moderate to severe focal inflammation with areas of normal lung tissue; and 3, severe inflammation to necrosis or severe inflammation throughout the lung with microabscesses.

Table 4. Histopathological evaluation of the types of leucocytes in the lungs of mice infected with mucoid isolates 1988, 1997 and 2003 at day 3 post-infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of inflammation</th>
<th>isolate 1988</th>
<th>isolate 1997</th>
<th>isolate 2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>NAD</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1 h</td>
<td>NAD</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>24 h</td>
<td>NAD</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Using a scoring system based on the percentage of PMNs and mononuclear cells (MNs) present, the inflammatory response was scored as an acute type of inflammation, which is defined as inflammatory infiltrates dominated by PMNs (≥90% and ≤10% MNs), or as a chronic type of inflammation, which is characterized by a predominance of MNs (≥90% and ≤10% PMNs). A mixed type of inflammation is when both types are present (MN/PMN), but neither dominating. NAD indicates that nothing abnormal was detected.
outcome, causing a shift from the acute, severe inflammatory response dominated by PMNs to the chronic-type inflammatory response that also involves mononuclear cells (MNs).

Surprisingly, the mice infected with isolate 2003 showed a higher degree of inflammation in the group where treatment was initiated 24 h post-infection compared with the untreated group. However, the inflammation type in the untreated group was dominated by PMNs, whereas the inflammation type in the treatment group was a mixture of PMNs and MNs, reflecting the fact that the bacteria were being cleared and that the inflammation was proceeding to the resolution state where macrophages dominate.39

Since epithelial cells and alveolar macrophages are important components of innate immunity, providing the initial response to inhaled pathogens that elude mechanical and mucociliary clearance by the expression of cytokines, the

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Figure 2. Pulmonary cytokine concentrations of G-CSF and MIP-2 induced by mucoid isolates 1988 (a and d), 1997 (b and e) and 2003 (c and f) in mice treated with tobramycin. Circles, squares and triangles represent the amount of G-CSF or MIP-2 per lung in individual mice; bars represent the medians. Circles represent the untreated groups (treated with 0.9% saline) [day 1: \( n = 13 \) (1988), 11 (1997) and 19 (2003); day 2: \( n = 12, 14 \) and 19; and day 3: \( n = 11, 4 \) and 23, respectively], squares represent the 1 h post-infection treatment groups (day 1: \( n = 12, 12 \) and 13; day 2: \( n = 12, 12 \) and 13; and day 3: \( n = 11, 11 \) and 12, respectively) and triangles represent the 24 h post-infection treatment groups (day 2: \( n = 12, 15 \) and 11; and day 3: \( n = 11, 5 \) and 12, respectively). At days 2 and 3, G-CSF was significantly reduced in the 1 h post-infection treatment groups of all three isolates compared with the 24 h post-infection treatment groups (\( P < 0.05 \)). For MIP-2, a significant decrease was observed for isolates 1997 and 2003 on days 2 and 3 in the 1 h post-infection treatment groups when compared with the 24 h post-infection treatment groups (\( P < 0.005 \)). For both cytokines, treatment initiated 1 h post-infection resulted in a significant decrease for all three isolates when compared with the untreated groups on all days (\( P < 0.004 \)). When treatment was initiated 24 h post-infection, a significant decrease in G-CSF was observed on day 2 for both isolates 1988 and 2003, and on day 3 for all three isolates when compared with the untreated group (\( P < 0.02 \)). A significant decrease in MIP-2 was observed for isolates 1988 and 1997 on day 2, and for all three isolates on day 3 when comparing treatment initiated after 24 h with no treatment (\( P < 0.02 \)).
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concentrations of G-CSF and MIP-2 in lung homogenates were also investigated.40 G-CSF is the major mobilizer of PMNs from the bone marrow, and MIP-2 is a PMN chemoattractant and murine equivalent to human interleukin-8. Both are involved in the inflammatory response in lungs exposed to bacteria.41,42 In the present study, treatment resulted in reduced G-CSF and MIP-2 concentrations, which correlated to a reduced number of bacteria. These observations are in accordance with Jensen et al.,43 who found that CF patients infected with P. aeruginosa had elevated concentrations of G-CSF and PMNs, which predicted poor lung function. However, after antibiotic treatment the lung function was improved, and a reduction of PMNs and G-CSF was seen, supporting the clinical relevance of our results.

In recent years, adaptation of P. aeruginosa to the host response and different niches of the lung environment in CF patients have attracted intense interest. One antibiotic resistance mechanism of P. aeruginosa isolated from the CF lung has been attributed to the adaptation of the mucoid phenotype. This has lead to comparative studies on the susceptibility of the mucoid and non-mucoid isolates to antibiotics, suggesting that mucoid isolates are more resistant than non-mucoid isolates44–46 or vice versa.47,48 These investigations have been done in vitro and often on planktonic cells. However, Irvin et al.49 have presented data with both resistant and susceptible mucoid isolates in a sputum sample, and Markowitz et al.50 concluded that the mucoid P. aeruginosa are not more resistant than their non-mucoid revertants. The MICs for the mucoid isolates in this study were lower than those for the non-mucoid isolates, except for both non-mucoid and mucoid isolate 1988, which had similar MIC values. Our experience is that when isolating P. aeruginosa from a chronically infected CF patient, even different phenotypes with the same PFGE profile from, for example, the same sputum sample display different resistance patterns. However, as concluded by Moset et al.,28 the mucoid phenotype seems to have an adaptive advantage for establishment and persistence in the mouse lung. In contrast, the role of the non-mucoid isolates might be another story, since their ability to establish an infection in vivo and persist decreases with time. This could indicate the evolution of other survival strategies where the increased antibiotic resistance in vitro plays a role. Regardless of equal susceptibility to antibiotics tested in vitro, the non-mucoid strains are eradicated with anti-Pseudomonas treatment, leaving the mucoid strains in the lungs.12

Besides the mucoid phenotype, the mutator phenotypes, which have an increased rate of mutation, are also commonly found in chronically infected CF patients for 5–10 years of chronic lung infection.51 The role of hypermutability has been linked to antimicrobial resistance,52 and the mutation in mutS (a DNA mismatch repair gene) seems beneficial when there is a need for rapid accumulation of genetic adaptations and is therefore assumed to play a crucial role in the adaptation required for long-term establishment in the heterogeneous and changing CF lung environment.53 Mutator phenotypes enhance the process of genetic adaptation to the CF lung and have been shown in vitro to favour the emergence of other phenotypic variants, leading to the mucoid53 variant and mutation in lasR.54 The inactivation of the transcriptional regulator LasR in P. aeruginosa, which is part of the quorum sensing (QS) cascade, has been attributed as an adaptive mutation. The lasR mutation may arise earlier than the mucoid phenotype and, thereby, represent a marker of an early stage of chronic infection in the CF lung.55 QS-deficient lasR mutants are believed to have a selective advantage in the presence of QS wild-type bacteria in vitro, because they benefit from the extracellular products (public goods) produced by QS-proficient bacteria in the vicinity without paying the metabolic costs themselves.56 Indeed, in the study by Smith et al.,57 a high degree of mutation was observed in isolates from one CF patient cultured during a 7.5 year interval. In general, there was a loss of virulence factors, including mutations in the QS systems as well as an increase in antibiotic resistance. Although mucA mutations were reported, the paper did not clarify the mucoid phenotype of the two isolates investigated. With the reduced virulence and increased antibiotic resistance, the two isolates were presumed to be non-mucoid. This was confirmed by personal communication at the 106th ASM General Meeting, Orlando, Florida (E. E. Smith, University of Washington, Seattle, personal communication).

Although the mucoid isolates used in this study have the same genotype, we saw an evolution in the ability to establish an infection and persist in the lungs. Also, an evolution in the response to treatment with tobramycin was observed. The mice infected with isolate 1988 did not show the same significant difference between treatments initiated 1 or 24 h post-infection as the two other isolates, because isolate 1988 was cleared from the lungs. Isolate 1997 showed the same response as seen with isolate 1988 with respect to the treatment regimen initiated after 1 h. The response to treatment initiated after 24 h for isolate 1997 did not resemble that of isolate 1988, but rather that of isolate 2003. The response to treatment initiated after 1 h for isolate 2003 did not resemble either of the other two isolates. In addition, since the treatment regimen initiated 1 h post-infection led to a clearance of isolates 1988 and 1997, but not of isolate 2003, this could indicate that the time span of 15 years between isolates isolates 1988 and 2003 has resulted in an adaptation leading to a more successful strain, capable of both establishment and persistence in the mouse lung despite antibiotic treatment.

The concentrations of G-CSF and MIP-2 in the lung homogenates from mice infected with isolates 1988 and 1997 were also shown to be lower compared with isolate 2003 on day 1 when treatment was initiated 1 h post-infection. In the previous study, when mice were infected using a lower inoculum (2×10^7 cfu/mL), the concentrations of both G-CSF and MIP-2 at day 5 were significantly increased for isolate 2003 compared with isolates 1988 and 1997, illustrating the induced virulence of isolate 2003.28 Also, the domination of PMNs in the untreated group infected with isolate 2003 was supported by the cytokine measurements, since the level of G-CSF was increased at day 3 for isolate 2003 compared with isolates 1988 and 1997.

Increased virulence of isolate 2003, reflected as the ability to establish and persist in the mouse lung despite treatment with tobramycin, was observed. Since isolate 2003 was isolated after the patient underwent lung transplantation it was considered to be a reason for the induced virulence. However, it has been reported that there is no change in the genotype of P. aeruginosa before and after a lung transplant, indicating that the sinuses, together with the trachea, act as the bacterial reservoir causing the reinfection of the transplanted lung with the same bacterial genotype.58,59 Moreover, the finding of increasing virulence of the mucoid isolates was confirmed by two sets of
sequentially isolated mucoid \textit{P. aeruginosa} from two other individual CF patients.\textsuperscript{28}

Together with the findings by Moser \textit{et al.},\textsuperscript{28} the results of this study show that the adaptation of \textit{P. aeruginosa} results in a distinct host response, not only to mucoid and non-mucoid phenotypes, but also to isolates from different time periods of a chronic lung infection in a CF patient undergoing antibiotic treatment. The induction of completely diverse host responses may indicate a need for different treatment strategies. Through the years it has been illustrated that early aggressive treatment with antibiotics does improve lung function and postpones the chronic state of infection.\textsuperscript{11,17,18,22,60,61} It has also been shown that treatment of chronically infected CF patients is able to reduce, but not eradicate, the bacterial load in the lungs.\textsuperscript{12,26} In the early eradication strategy, it has been hypothesized that addition of anti-inflammatory treatment could prevent the generation of mucoid strains, since the mutations leading to the mucoid phenotype are believed to be caused by the reactive oxygen species of the immune system.\textsuperscript{10} A clinical trial on this subject is currently being performed in Scandinavia. For chronic \textit{P. aeruginosa} lung infection, consideration should be given to whether more intense anti-inflammatory treatments should be added to the antibiotic treatment. Actually, this is necessary in the late stages of some infections in CF patients, where inflammation is ongoing and the antibiotic treatments are no longer possible due to resistance. Taking into account the induction of completely diverse host responses and the genetic markers of the \textit{P. aeruginosa} colonizing the individual CF patient, it might be possible to predict the response to antimicrobial therapy and offer new opportunities for treatment.

In conclusion, the data presented in this animal study emphasize the importance of early aggressive antibiotic treatment of \textit{P. aeruginosa}, both in order to clear the bacteria, but also for reducing the inflammation. Treatment of chronic \textit{P. aeruginosa} lung infection should be aimed at the mucoid phenotype, since non-mucoid isolates were eliminated independently of antibiotic treatment.

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