**Pseudomonas aeruginosa** mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection

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**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic pathogen; *P. aeruginosa* lung infection is commonly found in patients with cystic fibrosis (CF) and immunocompromised persons. The complications associated with *P. aeruginosa* pulmonary infection are the main cause of death in CF patients (Govan & Deretic, 1996; Høiby, 1993). The reason for this is partly because of the fact that once chronic *P. aeruginosa* infection becomes established in the CF lung, it cannot be eradicated with antibiotic treatment mainly due to development of resistance to the host defence systems, and the production of alginate and β-lactamase (Ciofu *et al.*, 1994; May *et al.*, 1991; Pedersen, 1992). It is therefore urgent to find a new approach to the treatment of chronic *P. aeruginosa* lung infection in CF patients (Finch *et al.*, 1998; Hartman & Wise, 1998).

*P. aeruginosa* can produce a number of cell-associated and extracellular virulence factors which contribute to its pathogenesis (Doring *et al.*, 1984, 1985, 1987; Jaeger, 1994; Van Delden & Iglewski, 1998). The extracellular virulence factors include proteases (elastase, Staphylocytic protease and alkaline protease), pigments (pyocyanin, pyoverdine), haemolysins, exoenzyme S and exotoxin A (Pollack, 1984; Van Delden & Iglewski, 1998). In *P. aeruginosa*, it has been demonstrated that the production of most of the extracellular virulence factors is controlled by quorum sensing systems *in vitro* (Pesci *et al.*, 1997; Pesci & Iglewski, 1997; Van Delden...
& Iglewski, 1998). Quorum sensing systems exert their action by small diffusible signal molecules called N-acylhomoserine lactones (AHLs) (Salmond et al., 1995; Fuqua et al., 1996; Fuqua & Greenberg, 1998). The signal molecules are synthesized from precursors by a synthetase (a LuxI homologue) and they interact with a transcriptional activator (a LuxR homologue) to induce the expression of target genes (Fuqua et al., 1996). In P. aeruginosa there are at least two different quorum sensing systems: las and rhl (Gambello & Iglewski, 1991; Seed et al., 1995; Ochsner & Reiser, 1995), which code for synthetases (Las/I/Rhl) and cognate transcriptional regulators (LasR/RhlR). The las–las system has been shown to modulate expression of lasR itself (Seed et al., 1995), lasB (elastase) (Passador et al., 1993; Pearson et al., 1997), lasA (Staphyloptlytic protease) (Gambello et al., 1993), apr (alkaline protease) (Gambello et al., 1993), the xcp secretion pathway (Chapon-Herve et al., 1997), twitching motility (Glessner et al., 1999) and rhlR (Gambello & Iglewski, 1997). The rhl–rhl system modulates expression of rhlR itself (Latifi et al., 1996), rhlAB (rhamnolipid biosynthesis) (Ochsner & Reiser, 1995; Pearson et al., 1997), lasB (elastase) (Brint & Ohman, 1995; Pearson et al., 1995, 1997), twitching motility (Glessner et al., 1999) and rpoS (Latifi et al., 1996). The lasI and rhl products are N-oxododecanoyl homoserine lactone (OdDHL), 3OC12-HSL or PAI-1 (Pearson et al., 1994) and N-butyryl homoserine lactone (BHL, C4-HSL or PAI-2) (Pearson et al., 1995; Winson et al., 1995), respectively. Knowledge of how quorum sensing systems of P. aeruginosa operate during infection may help us to find a new approach to the treatment of chronic P. aeruginosa lung infections (Finch et al., 1998; Hartman & Wise, 1998). A few in vivo studies have demonstrated that the virulence of P. aeruginosa is associated with quorum sensing. For instance, the importance of a functional lasR gene has been shown in a neonatal mouse model of pneumonia (Tang et al., 1996). The virulence of lasI and rhl mutants has been reported in a burned-mouse model (Rumbaugh et al., 1999), and we have previously demonstrated that the production of AHLs from P. aeruginosa can be directly detected in the lung tissues of mice with P. aeruginosa infection (Wu et al., 2000). OdDHL has been shown to induce an imbalance of the Th1/Th2 (T helper cells) response in vitro, i.e. suppressing interleukin (IL)-12 synthesis, enhancing antibody and IgG1 responses, and promoting IgE production from blood cells stimulated by IL-4 (Telford et al., 1998). All these data indicate that the quorum sensing systems of P. aeruginosa play an important role during the infectious process.

We wished to further elucidate the involvement of quorum sensing in chronic P. aeruginosa lung infection. In this study, we used a P. aeruginosa PAO1 las rhl double mutant (Pearson et al., 1997) to infect rats intratracheally and compared the severity of the resulting lung infection with that caused by its wild-type counterpart PAO1. The bacteria were embedded in alginate beads and the resultant infection mimics the P. aeruginosa infection found in the CF lung (Pedersen et al., 1990). Immune parameters, lung bacteriology and lung pathology were evaluated at four different time points on days 3, 7, 14 and 28 post-inoculation.

**METHODS**

**Bacterial strains, culture media and experimental animals.** The P. aeruginosa strains used were wild-type PAO1 and its double mutant, PAO1 las rhl, which is also called PAO1-JP2 (PAO1 Δsrnl::Tn501 lasI::Tc, Hg) (Brint & Ohman, 1995; Pearson et al., 1997). Both PAO1 and its double mutant were grown in Luria–Bertani (LB) liquid medium or agar plates. Tetracycline (100 µg ml⁻¹) and mercuric chloride (50 µg ml⁻¹) (Sigma) were added to LB medium to maintain the mutations in PAO1 (las rhl). A total of 170 female Lewis rats, each 7 weeks old with a body weight of about 150 g (Charles River) were used; details of animal numbers used in the study are shown in Table 1.

**Immobilization of bacteria in seaweed alginate beads.** Alginate is commonly produced by the mucoid P. aeruginosa, which is frequently found in the lungs of CF patients. The importance of alginate in the pathogenesis of P. aeruginosa has been demonstrated by the ability of intratracheally inoculated P. aeruginosa, embedded in minute seaweed alginate beads, to establish chronic P. aeruginosa lung infection. The two P. aeruginosa strains were each immobilized in seaweed alginate beads, to establish chronic P. aeruginosa lung infection. The two P. aeruginosa strains were each immobilized in seaweed alginate beads as described by Pedersen et al. (1990). First, 1 ml bacteria cultured at 37 °C for 18 h was mixed with 9 ml sterile seaweed alginate (Protanal 60, Protan A) immobilized in seaweed alginate beads as described by Pedersen et al. (1990). First, 1 ml bacteria cultured at 37 °C for 18 h was mixed with 9 ml sterile seaweed alginate (Protanal 10/60, Protan A/S). Next, the mixture was forced with air through a channel into a solution of 0.1 M CaCl₂ in 0.1 M Tris/HCl buffer (pH 7.0). The suspension of PAO1 or PAO1 las rhl was then adjusted to 5 × 10⁸ c.f.u. ml⁻¹ and the yield was confirmed by colony counts.

**Challenge procedures and blood sample collection.** Before challenge, all rats were anaesthetized by subcutaneous injection of a 1:1 mixture of etomidate (Janssen) and midazolam (Roche) at a dose of 1·5 ml (kg body weight)⁻¹ and tracheoctomized (Johansen et al., 1993). Intratracheal challenge with alginate beads was performed as described by Johansen et al. (1993). Each rat received 0·1 ml alginate beads containing 5 × 10⁸ c.f.u. PAO1 or PAO1 las rhl. The incision was sutured with silk and healed without any complications. The animals were killed by using 20% (w/v) pentobarbital (DAB) at a dosage of 2 ml (kg body weight)⁻¹ and blood samples were obtained by cardiac puncture.

**Macroscopic pathology of the lungs.** Eighteen to nineteen lungs from each time point in each group were macroscopically described in situ and after removal from the thoracic cavities. The macroscopic lung pathology was expressed as the lung index of macroscopic pathology (LIMP) as described by Song et al. (1998) according to the modified formula: LIMP = lung area with pathological changes divided by the area of the whole lung. The macroscopic lung pathology included lung abscess, consolidation, atelectasis and haemorrhage.

**Lung histopathology.** Lung histopathology was carried out with eight lungs selected randomly from each group of rats at each time point. The following parameters were evaluated.

**Size of lung abscesses.** This was expressed as diameter (mm). Lung sections were made at the middle of a lung abscess to get its maximal diameter, which was measured with a micro-ruler under the microscope.
IgM, IgA, IgG, IgG1 and IgG2a against inflammation was defined as a predominance of MNs (MNs cells and the presence of granulomas (Johansen by PMNs (PMNs 90%, MNs 10%). Whereas chronic inflammation was defined as a predominance of MNs (MNs > 90%, PMNs < 10%), which included lymphocytes, plasma cells, mast cells, and the presence of granulomas (Johansen et al., 1994).

Mast cell count. Toluidine blue staining was performed to detect mast cells in the lung tissues (Kierman, 1981). Ten representative fields (viewed at 500 × magnification) were selected along the inflammatory foci to count the number of mast cell as described previously (Song et al., 1997).

Preparation of PMNs. PMNs were isolated from 9 to 12 citrated peripheral blood specimens in each group of rats by dextran sedimentation and sodium metrizoate/Ficoll (lymphoprep; Nyegaard) separation (Kharaizi et al., 1984a). The remaining erythrocytes were removed by hypotonic lysis. PMNs were then counted and the concentration was adjusted to 10^6 cells ml^{-1} in Krebs Ringer’s solution with 5 mM glucose. The purity and cell viability were both >97%.

Blood PMN chemiluminescence. A luminol-enhanced assay was performed with a luminometer (model 1251; LKB-Wallac), which was placed in an air-conditioned thermostat-controlled environment at 21 ± 1 °C. Zymosan and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were purchased from Sigma. A total volume of 1 ml of a mixture containing 0.1 ml PMN suspension, 0.2 ml serum-opsonized zymosan at 10 mg ml^{-1} and 0.7 ml luminol at 10^{-4} mol l^{-1} was used. The peak chemiluminescence (in mV) and the time taken to peak chemiluminescence were measured.

Lung bacteriology. Ten to eleven random lung samples from each group of rats at each time point were prepared for quantitative bacteriological examination as described by Johansen et al. (1993). Each lung was mixed with 3 ml cold sterile PBS and the mixture was homogenized in a blender. Appropriately diluted samples were plated on ‘Blue agar plates’ (a modified Conradi–Drigalsky medium selective for Gram-negative rods and containing lactose, pH 7.0; State Serum Institute, Copenhagen) to determine the number of bacterial c.f.u. after 20–24 h incubation at 37 °C.

ELISA. Serum antibody responses. The concentrations of serum IgM, IgA, IgG, IgG1 and IgG2a against P. aeruginosa standard antigen (St-Ag) in 15 serum samples from each group of rats at each time point were determined by ELISA as reported previously (Johansen & Høiby, 1992; Johansen et al., 1993). The serum antibody titres expressed as ELISA units were obtained by dividing the mean absorbance of the samples by the mean absorbance of an internal standard expressing between 0.30 and 0.40 absorbance units.

Cytokine production The concentrations of IL-4 and IFN-γ in 10–11 supernatants of the lung homogenate (from the detection of lung bacteriology) from each group of rats at each time point were determined by ELISA kits (Nordic BioSite AB). Standard curves for IL-4, ranging from 8 to 500 pg ml^{-1} (lower detection limit 2 pg ml^{-1}), and IFN-γ, ranging from 10 to 2000 pg ml^{-1} (lower detection limit 10 pg ml^{-1}), were constructed.

Statistical analyses. The categorical data were analysed by the chi-squared test. The Mann–Whitney U test was used to compare the data between two groups.

RESULTS

Mortality

Mortality of the infected rats was followed in both groups from 1 to 28 d post-infection (p.i.). Dead animals were mostly found on days 2 and 3 p.i. In the group of PAO1-infected rats, the mortality was 7.5% (6/80), while in the lasI rhlI group, it was 16.7% (15/90). The difference in mortality between the two groups was not statistically significant.

Macroscopic lung pathology

To calculate the LIMP, which was used as an indicator of the severity of the lung pathology, we measured the area of the lungs exhibiting pathological changes. The major pathological changes observed were lung consolidation, abscesses, adhesion, haemorrhage and atelectasis. On days 3 and 7 p.i., lung adhesion was rarely found, while lung consolidation with haemorrhage and abscesses were the main pathological changes. However, from day 14 p.i., lung adhesion and single huge or multiple lung abscesses became the important charac-

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Group</th>
<th>Gross pathology</th>
<th>Bacteriology and cytokines</th>
<th>Histopathology</th>
<th>Chemiluminescence</th>
<th>Antibody response</th>
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<tr>
<td>3</td>
<td>PAO1</td>
<td>19</td>
<td>11</td>
<td>8</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>lasI rhlI</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>PAO1</td>
<td>19</td>
<td>11</td>
<td>8</td>
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<td>15</td>
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<tr>
<td></td>
<td>lasI rhlI</td>
<td>11</td>
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<td>8</td>
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<tr>
<td>14</td>
<td>PAO1</td>
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<td>10</td>
<td>8</td>
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<td></td>
<td>lasI rhlI</td>
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<td>8</td>
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<tr>
<td>28</td>
<td>PAO1</td>
<td>18</td>
<td>10</td>
<td>8</td>
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<td>15</td>
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<tr>
<td></td>
<td>lasI rhlI</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>15</td>
</tr>
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Table 1. Number of animals used in each group for evaluation of different parameters on different days p.i.

The actual total number of animals used in the study was 74 rats in the PAO1 group and 75 rats in the lasI rhlI group since some rats died from infection.

Pseudomonas mutations result in milder infection
characteristics, particularly in the PAO1 group. A few lung samples with atelectasis were found on day 28 p.i. in both groups of rats. The LIMP in the last rhlI group was higher (P < 0.007) on day 3 p.i. but declined significantly on days 14 and 28 p.i. (P < 0.003 and P < 0.008, respectively) compared to the PAO1 group. On day 7 p.i., no difference was observed between the two groups (Fig. 1). In the last rhlI group, each of the differences in the LIMP between days 3 and 7 p.i. (P < 0.04), days 7 and 14 p.i. (P < 0.001), and days 14 and 28 p.i. (P < 0.02) was significant. In contrast, each of the differences in the LIMP between days 3 and 7 p.i. and days 7 and 14 p.i. in the PAO1 group was not significant; only the difference between days 14 and 28 p.i. was significant (P < 0.005). These results indicated that PAO1 caused more persistent and severe lung pathological changes than PAO1 last rhlI.

Lung histopathology
To classify the lung inflammation and the severity of lung damage, we measured the diameters of lung abscesses, determined the proportions of PMNs and MNs, and counted the number of mast cells in lung foci. Acute lung inflammation with significant infiltration of PMNs, single huge or multiple lung abscesses with significant tissue damage, oedema, haemorrhage and consolidation were seen in both groups of rats from days 3 to 14 p.i. Chronic lung inflammation could be seen in only three out of eight lungs from both groups on day 28 p.i. There was no difference in the inflammatory classification between the two groups on days 3, 7, 14 and 28 p.i. However from days 14 to 28 p.i., the size of lung abscesses in the last rhlI group was reduced compared to the PAO1 group (P < 0.05) (Table 2). On day 7 p.i. similarly sized lung abscesses were found in the two groups. On day 3 p.i. we were unable to determine the size of the lung abscesses because the border of the abscess was not well defined. Mast cells were found along the lung foci. Significant degranulation was observed in the mast cells closer to the centre of an inflammatory focus. The mast cell count in the inflammatory area.

Table 2. Size of lung abscesses and number of lung mast cells

Values are expressed as the median with the range in parentheses. ns, Not significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter of abscess (mm)</th>
<th>No. mast cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lasI rhlI</td>
<td>5.8 (2.8–15.2)</td>
<td>12.4 (5.8–18.8)</td>
</tr>
<tr>
<td>PAO1 vs. lasI rhlI</td>
<td>ns</td>
<td>P &lt; 0.04</td>
</tr>
</tbody>
</table>

*The mast cells were counted in 10 microscopic fields (magnification, 400 x) along the inflammatory area.
†Five animals were used in each group; abscesses were only seen in 5 of 8 rats in each group.
**Table 3.** Peripheral blood PMN chemiluminescence on different days p.i.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Group</th>
<th>PAO1 vs. lasI rhlI</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PAO1</td>
<td>lasI rhlI</td>
</tr>
<tr>
<td>3</td>
<td>6.51</td>
<td>9.75</td>
</tr>
<tr>
<td>7</td>
<td>28.12</td>
<td>32.57</td>
</tr>
<tr>
<td>14</td>
<td>4.65</td>
<td>4.50</td>
</tr>
</tbody>
</table>

The results are expressed as the median with the range given in parentheses. The unit is mV. NS, Not significant.

**Fig. 3.** Amount of cytokines in lung tissues on different days p.i. in the PAO1 and lasI rhlI groups. A higher level of IFN-γ on days 3 and 14 p.i. was found in the lasI rhlI group, but both higher IFN-γ and IL-4 levels on day 7 were detected in the PAO1 group. Values are expressed as the mean ± standard errors of the pg ml⁻¹. Significant differences between groups are indicated by asterisks: *P < 0.05; **P < 0.01. ○, PAO1 IFN-γ; □, PAO1 IL-4; ■, lasI rhlI IFN-γ; ■, lasI rhlI IL-4.

**Fig. 4.** Serum antibody responses against St-Ags on different days p.i. in the PAO1 and lasI rhlI groups. Stronger IgM and IgG responses on day 7 but weaker IgG production on days 14 and 28 are seen in the lasI rhlI group, compared with the PAO1 group. The levels of antibodies are expressed as the means ± standard errors of ELISA units. Significant differences between groups are indicated by asterisks: *P < 0.05; **P < 0.01. ○, lasI rhlI IgG; □, lasI rhlI IgM; ●, PAO1 IgG; ■, PAO1 IgM.

bacteria in the lungs on day 3 p.i. was higher than that on day 7 p.i. (P < 0.006), day 7 p.i. was higher than day 14 p.i. (P < 0.006) and day 14 p.i. was higher than day 28 p.i. (P < 0.0005). The lung bacteriology in the lasI rhlI group correlated positively with the LIMP (P < 0.025, r = 0.976). In contrast, there was no significant difference between the numbers of bacteria in the lungs on days 3 and 7 p.i., and days 7 and 14 p.i. in the PAO1 group, except on day 28 p.i., which was significantly lower than that on day 14 p.i. (P < 0.001). These results suggested that the signal-generation-defective bacteria were cleared away more quickly than the wild-type P. aeruginosa PAO1.

**Lung bacteriology**

Bacteria from the lung tissues of both groups of rats were enumerated and the results enabled us to evaluate the ability of PAO1 and PAO1 lasI rhlI to resist host immune clearance. On day 3 p.i., the bacterial counts from both groups of rats did not differ significantly. The bacterial counts in the lasI rhlI group dropped significantly on days 7, 14 and 28 p.i. (P < 0.05, P < 0.01 and P < 0.04, respectively, Fig. 2) compared to the PAO1 group. In the lasI rhlI group, the lung bacteriology decreased progressively with time, i.e. the number of bacteria in the lungs on day 3 p.i. was higher than that on day 7 p.i. (P < 0.006), day 7 p.i. was higher than day 14 p.i. (P < 0.006) and day 14 p.i. was higher than day 28 p.i. (P < 0.0005). The lung bacteriology in the lasI rhlI group correlated positively with the LIMP (P < 0.025, r = 0.976). In contrast, there was no significant difference between the numbers of bacteria in the lungs on days 3 and 7 p.i., and days 7 and 14 p.i. in the PAO1 group, except on day 28 p.i., which was significantly lower than that on day 14 p.i. (P < 0.001). These results suggested that the signal-generation-defective bacteria were cleared away more quickly than the wild-type P. aeruginosa PAO1.

**Blood PMN chemiluminescence**

Myeloperoxidase-mediated chemiluminescence is one of the major antimicrobial systems manifested by PMNs (Kharazmi et al., 1984a). The results showed that the PMN chemiluminescence response in the lasI rhlI group was higher on day 3 p.i. than that in the corresponding PAO1 group (P < 0.05). No difference in the PMN chemiluminescence response was found between the two groups of rats on days 7 and 14 p.i. (Table 3). The PMN chemiluminescence on day 7 p.i. in both groups of rats was significantly higher compared with that on day 3 p.i. (P < 0.0002). On day 14 p.i., the PMN chemiluminescence in both groups decreased markedly compared to day 7 p.i. (Table 3).
Cytokine production from the lungs

The type of immune response depends on the profile of cytokines. On day 3 p.i., severe lung pathological changes occurred in both groups of rats. The amount of the lung IFN-\(\gamma\) in the \(\text{lai} \ \text{rhi} \) group was significantly higher than that in the PAO1 group (\(P < 0.02\)). On day 14 p.i., a higher IFN-\(\gamma\) level could still be detected in the \(\text{lai} \ \text{rhi} \) group (\(P < 0.05\)). The production of IL-4 and IFN-\(\gamma\) in the PAO1 group were higher than those in the \(\text{lai} \ \text{rhi} \) group on day 7 p.i. (\(P < 0.03\)) (Fig. 3), indicating a delay in the immune response in rats infected by PAO1.

Serum antibody responses

On days 7, 14 and 28 p.i., the serum antibody levels against \(P. \ aeruginosa\) St-Ag and alginate were measured by ELISA.

Anti-\(P. \ aeruginosa\) St-Ag antibody responses. During the early immune response, serum IgM and IgG levels in the \(\text{lai} \ \text{rhi} \) group were significantly higher than those in the PAO1 group (\(P < 0.007\)). However, from day 14 p.i., the situation changed. The IgG titre found in the \(\text{lai} \ \text{rhi} \) group was much lower than that in the PAO1 group (\(P < 0.03\)), and the IgM response in both groups dropped markedly (Fig. 4). The production of IgA in the two groups of rats did not differ significantly (data not shown).

Anti-\(P. \ aeruginosa\) St-Ag IgG subclass response. In the \(\text{lai} \ \text{rhi} \) group, the IgG2a level was notably higher on day 7 p.i. (\(P < 0.0004\)) and the IgG1 level was lower on day 28 p.i. when compared with the levels in the corresponding PAO1 group (\(P < 0.05\)). On day 14 p.i., the response of IgG2a did not differ between the two groups of rats. However, in both groups the IgG2a titres on days 14 and 28 p.i. were much higher than the IgG2a titre on day 7 p.i., and the increase in IgG2a paralleled that in IgG. The IgG1 response was similar in the two groups of rats on days 7 and 14 p.i. (Fig. 5).

DISCUSSION

Our results revealed that infection with \(P. \ aeruginosa\) \(\text{lai} \ \text{rhi}\), a strain with defective quorum sensing signal-generating systems, resulted in a faster and stronger immune response against the bacterial infection in the early phase as judged from the severity of lung pathology (Fig. 1), higher lung IFN-\(\gamma\) production (Fig. 3), stronger oxidative burst of blood PMNs (Table 3) and faster antibody response (Figs 4 and 5), compared to the infection with wild-type \(P. \ aeruginosa\) PAO1. Subsequently, quicker bacterial clearance from the lungs (Fig. 2), milder lung pathology (Fig. 1 and Table 2) and a more pronounced early humoral immune response (Fig. 4 and 5) were also detected in the rats infected with PAO1 \(\text{lai} \ \text{rhi}\) in comparison with the PAO1-infected rats.

A stronger oxidative burst response in peripheral blood PMNs is indirect evidence of PMN activation in the lung. During the initial infection process host phagocytes commonly play a major role in the defence against invading micro-organisms. Among the phagocytes, blood PMNs are the most active and important. Previous studies have shown that some \(P. \ aeruginosa\) virulence factors, such as alkaline protease and elastase, could interfere with phagocytosis of neutrophils (Khazaemiz et al., 1984a, b, 1986), especially inhibiting the oxidative burst response of neutrophils to opsonized zymosan. Our results suggest that PAO1 \(\text{lai} \ \text{rhi}\) produces less virulence factors and lessens the suppression of the PMN oxidative burst in early stages of lung infection, which would be helpful for the lung phagocytes to kill the bacteria. On the other hand, wild-type \(P. \ aeruginosa\) PAO1 can be induced to produce extracellular products which inhibit PMN chemiluminescence (Table 3), one of the major antimicrobial systems of PMNs. The increased chemiluminescence is required for the bactericidal activity of PMNs but it might also, in some
circumstances, worsen the tissue damage (Kharazmi et al., 1989) (Fig. 1). The release of elastase and other lysosomal enzymes from neutrophils are other likely candidates participating in the process of tissue damage (Doring & Dauner, 1988; Doring, 1994). IFN-γ is known to enhance the oxidative burst of PMNs in response to lipopolysaccharide, to increase neutrophil-mediated antibody-dependent cytotoxicity, and to facilitate phagocytosis and bactericidal activity by increasing the expression of high affinity IgG receptors and C3b receptors on PMNs (Hokland & Berg, 1981; Petroni et al., 1988; Roilides et al., 1992). The results of lung bacteriology showed that the lung bacterial clearance in the lasI rhlI group was significantly faster than that in the PAO1 group, suggesting that it may be associated with an early stronger PMN chemiluminescence response in the lasI rhlI group. On day 7 p.i., PMN chemiluminescence in both groups increased markedly compared with that found on day 3 p.i., suggesting the suppression of PMN activity on day 3 p.i., which might be associated with the septicaemia found at the same time (Solberg et al., 1982). Spleen culture showed that P. aeruginosa could be detected in 60–70% of the rats in both groups on day 3 p.i., indicating septicaemia. On day 7 p.i., the pathogen was no longer found in the spleen (data not shown). However, the difference of PMN chemiluminescence between the two groups was not significant on days 7 and 14 p.i. This might be due to the formation of serum IgM directed against virulence factors in the PAO1 group, thereby removing the inhibition of PMN chemiluminescence by virulence factors.

On day 7 p.i., higher lung IFN-γ and IL-4 production as well as lower serum IgM, IgG and IgG2a responses were detected in the PAO1 group than the lasI rhlI group. This may reflect a delayed immune response due to the cleaving effects on immunoglobulins by the virulence factors produced by wild-type PAO1 (Doring et al., 1981, 1984; Bainbridge & Fick, 1989) (Figs 3, 4 and 5). The lower lung IFN-γ on day 3 p.i. may be partly due to the inactivation of IFN-γ by the alkaline protease and elastase of PAO1 (Horvat et al., 1989). The higher production of lung IFN-γ on day 7 p.i. in the PAO1 group could be explained by the formation of neutralizing antibody (IgM) in the serum. Cytokines are released from macrophages, lymphocytes and appropriately stimulated cells during the infection. In lung foci produced by P. aeruginosa infection, the accumulation of different cytokines would lead to local induction of the Th1 cell response towards the Th1/Th2 type. High-level production of IFN-γ activates macrophages and facilitates the production of the IgG2a subclass, which correlates with the Th1 response. The Th1 response favours cell-mediated immunity (Mosmann & Coffman, 1989; Mosmann & Sad, 1996). High-level production of IL-4 would stimulate the humoral immunity and promote the production of the IgG1 subclass, which associates with the Th2 response (Haczku et al., 1996; Mosmann & Sad, 1996). The Th1 response would benefit the host fighting against chronic P. aeruginosa lung infection and the Th2 response correlates with a poor prognosis (Johansen et al., 1996; Moser et al., 1997; Song et al., 1997). Telford et al. (1998) suggested on the basis of an in vitro study that N-oxododecanoyl homoserine lactone, a major signal molecule produced by LasI, inhibits the production of IL-12, a Th1-supportive cytokine, and induces a Th2-like response (increases IgG1 level and stimulates the production of IgE with IL-4). Our results provide in vivo evidence regarding production of cytokines during P. aeruginosa lung infection and supports the view that AHLs originating from the quorum sensing systems of P. aeruginosa are important in the pathogenesis of chronic pulmonary infection.

From days 14 to 28 p.i., milder pathology, lower mast cell count, higher IFN-γ production in lungs, and lower serum IgG and IgG1 responses were found in the PAO1 lasI rhlI infected group when compared with the PAO1 infected group, indicating a Th1-like immune response. Mast cells and serum IgG and IgG1 are involved in Th2 responses (Johansen et al., 1996; Krishnan et al., 1996; Mosmann & Sad, 1996; Moser et al., 1997). Chronic P. aeruginosa lung infection in CF patients is characterized by the persistent and significant antibody response in serum and the remarkable infiltration of PMNs in the lung (Høiby et al., 1990). In the present study, a higher serum IgG level was found in the PAO1 infected rats than the PAO1 lasI rhlI infected rats during the chronic lung infection. The increased level of serum antibodies could lead to the formation of a larger quantity of immune complexes in the lung foci and this is thought to play an important role in the immunopathology of CF. In addition, the larger number of mast cells in the lung foci leading to the release of PMN chemoattractants, together with the activation of complement by the immune complexes would result in significant infiltration of PMNs into the lung foci and damage of the lung tissues (Abraham & Malaviya, 1996). Moreover, a high antibody titre in CF patients with chronic P. aeruginosa lung infection has been correlated with a poor prognosis (Høiby et al., 1986, 1990). The results of antibody responses revealed that P. aeruginosa PAO1 with the ability of producing AHLs inhibited the immune response during the early phase of infection but stimulated the humoral immune reaction during the chronic infection.

Our results suggest that functional quorum sensing systems significantly affect the severity of P. aeruginosa lung infection in both acute and chronic phases. AHL signal molecules as well as receptors (LasR and RhLR) are therefore promising new targets in the quest for a new type of therapy of CF patients with chronic P. aeruginosa lung infection.

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