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

Hong Wu,[1] Zhijun Song,[1,4] Michael Givskov,[2] Gerd Doring,[3] Dieter Worlitzsch,[3] Kalai Mathee,[4] Jørgen Rygaard[5] and Niels Høiby[1]

Author for correspondence: Hong Wu. Tel: +45 35456427. Fax: +45 35456412. e-mail: wuhong@get2net.dk

[1] Department of Clinical Microbiology, 9301 Rigshospitalet, Juliane Maries Vej 22, DK-2100, Copenhagen Ø, Denmark

[2] Institute of Microbiology, Technical University of Denmark, DK-2800, Lyngby, Denmark

[3] Hygiene Institute, University of Tübingen, D-72074, Tübingen, Germany

[4] Department of Biological Sciences, Florida International University, University Park, Miami, FL 33199, USA

[5] Bartholin Institute, Kommunehospitalet, Copenhagen, Denmark

To understand the importance of quorum sensing in chronic *Pseudomonas aeruginosa* lung infection, the *in vivo* pathogenic effects of the wild-type *P. aeruginosa* PAO1 and its double mutant, PAO1 *lasI rhlI*, in which the signal-generating parts of the quorum sensing systems are defective were compared. The rat model of *P. aeruginosa* lung infection was used in the present study. The rats were killed on days 3, 7, 14 and 28 after infection with the *P. aeruginosa* strains. The results showed that during the early stages of infection, the PAO1 double mutant induced a stronger serum antibody response, higher production of pulmonary interferon γ, and more powerful blood polymorphonuclear leukocyte (PMN) chemiluminescence compared to its wild-type counterpart. On days 14 and 28 post-infection, significantly milder lung pathology, a reduction in the number of mast cells present in the lung foci, a reduced number of lung bacteria, and minor serum IgG and IgG1 responses but increased lung interferon γ production were detected in the group infected with the PAO1 double mutant when compared with the PAO1-infected group. Delayed immune responses were observed in the PAO1-infected group and they might be associated with the production of virulence factors that are controlled by the quorum sensing systems. The conclusion of this study is that functional *lasI* and *rhlI* genes of *P. aeruginosa* PAO1 play a significant role during lung infection.

Keywords: *lasI* and *rhlI*, *N*-acylhomoserine lactones, chronic lung infection, cystic fibrosis, rat model

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, Staphylolytic 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

Abbreviations: AHL, *N*-acylhomoserine lactone; CF, cystic fibrosis; IFN-γ, interferon γ; IL, interleukin; LIMP, lung index of macroscopic pathology; MN, mononuclear leukocyte; p.i., post-infection; PMN, polymorphonuclear leukocyte; St-Ag, *P. aeruginosa* standard antigen.

& 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 (LasI/RhlI) and cognate transcriptional regulators (LasR/RhlR). The *lasI–lasR* system has been shown to modulate expression of *lasI* itself (Seed *et al*., 1995), *lasB* (elastase) (Passador *et al*., 1993; Pearson *et al*., 1997), *lasA* (Staphylolytic 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* (Latifi *et al*., 1996; Pesci *et al*., 1997). The *rhlI–rhlR* system modulates expression of *rhlI* 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 *rhlI* products are *N*-oxododecanoyl homoserine lactone (OdDHL, $3OC_{12}$-HSL or PAI-1) (Pearson *et al*., 1994) and *N*-butyryl homoserine lactone (BHL, C_4-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 *rhlI* 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 *lasI rhlI* 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 *lasI rhlI*, which is also called PAO1-JP2 (PAO1 Δ*rhlI*::Tn*501 lasI*::Tc^r, Hg^r) (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 (*lasI rhlI*). 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 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 $CaCl_2$ in 0·1 M Tris/HCl buffer (pH 7·0). The suspension of PAO1 or PAO1 *lasI rhlI* was then adjusted to 5×10^8 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 tracheotomized (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 5×10^7 c.f.u. PAO1 or PAO1 *lasI rhlI*. The incision was sutured with silk and healed without any complications. The animals were killed by using 20% (w/v) pentobarbital (DAK) 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.

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.

Days p.i.	Group	Gross pathology	Bacteriology and cytokines	Histopathology	Chemiluminescence	Antibody response
3	PAO1	19	11	8	9	15
	lasI rhlI	19	11	8	12	15
7	PAO1	19	11	8	11	15
	lasI rhlI	19	11	8	12	15
14	PAO1	18	10	8	11	15
	lasI rhlI	19	11	8	12	15
28	PAO1	18	10	8	—	15
	lasI rhlI	18	10	8	—	15

Classification of acute or chronic inflammation. This was assigned by a scoring system based on the proportion of polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes (MNs) in the inflammatory foci. Acute inflammation was defined as an inflammatory infiltration dominated 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 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 (Kiernan, 1981). Ten representative fields (viewed at $500\times$ 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 (Kharazmi *et al*., 1984a). The remaining erythrocytes were removed by hypotonic lysis. PMNs were then counted and the concentration was adjusted to 10^7 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 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-

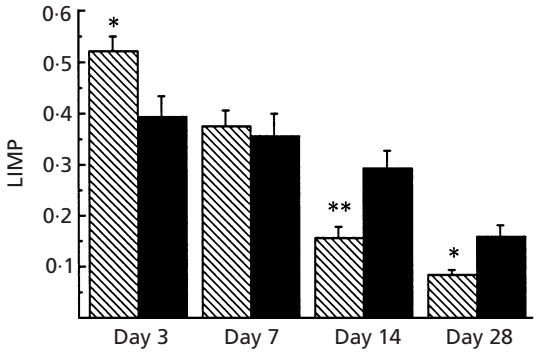

Fig. 1. Comparison of the LIMP of the PAO1 and the *lasI rhlI* groups on different days p.i. A sharply reduced LIMP value with respect to time is observed in the *lasI rhlI* group compared with the PAO1 group. The results are represented as the means ± standard errors. Significant differences between groups are indicated by asterisks: * $P < 0{\cdot}01$; ** $P < 0{\cdot}005$. Black bars, PAO1; hatched bars *lasI rhlI*.

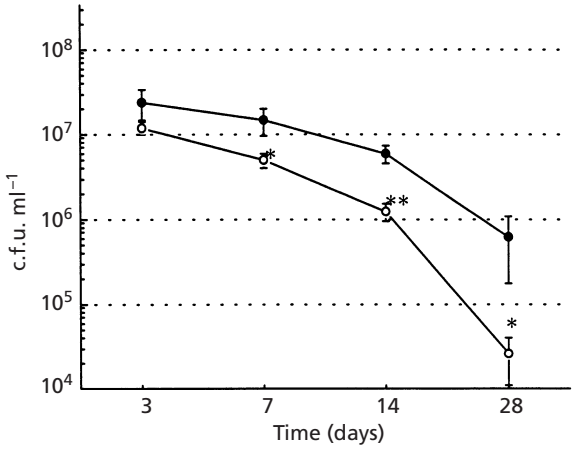

Fig. 2. Number of lung bacteria in the PAO1 and the *lasI rhlI* groups. The bacterial number decreases more quickly in the *lasI rhlI* group than the PAO1 group. The results are expressed as the means ± standard errors of the c.f.u. ml^{-1}. Significant differences between groups are indicated by asterisks: * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$. ●, PAO1; ○, *lasI rhlI*.

teristics, 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 *lasI rhlI* group was higher ($P < 0{\cdot}007$) on day 3 p.i. but declined significantly on days 14 and 28 p.i. ($P < 0{\cdot}003$ and $P < 0{\cdot}008$, respectively) compared to the PAO1 group. On day 7 p.i., no difference was observed between the two groups (Fig. 1). In the *lasI rhlI* group, each of the differences in the LIMP between days 3 and 7 p.i. ($P < 0{\cdot}04$), days 7 and 14 p.i. ($P < 0{\cdot}001$), and days 14 and 28 p.i. ($P < 0{\cdot}02$) 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 ($P < 0{\cdot}005$). These results indicated that PAO1 caused more persistent and severe lung pathological changes than PAO1 *lasI 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 *lasI rhlI* group was reduced compared to the PAO1 group ($P < 0{\cdot}05$) (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 *lasI rhlI*

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.

Group	Diameter of abscess (mm)			No. mast cells*	
	Day 7	Day 14	Day 28†	Day 14	Day 28
PAO1	5·8 (2·8–15·2)	12·4 (5·8–18·8)	10·0 (1·6–16·4)	76 (15–140)	133 (46–215)
lasI rhlI	11·2 (6·8–16·4)	8·2 (0·0–14·4)	5·0 (0·5–8·0)	45 (36–97)	46 (7–94)
PAO1 vs. *lasI rhlI*	NS	$P < 0{\cdot}04$	$P < 0{\cdot}05$	NS	$P < 0{\cdot}03$

* The mast cells were counted in 10 microscopic fields (magnification, 400×) 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.

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

Days p.i.	Group		PAO1 vs. *lasI rhlI*
	PAO1	*lasI rhlI*	
3	6·51 (2·81–14·95)	9·75 (4·91–19·45)	$P < 0·05$
7	28·12 (17·95–33·85)	32·57 (15·10–48·12)	NS
14	4·65 (3·57–9·97)	4·50 (2·85–9·50)	NS

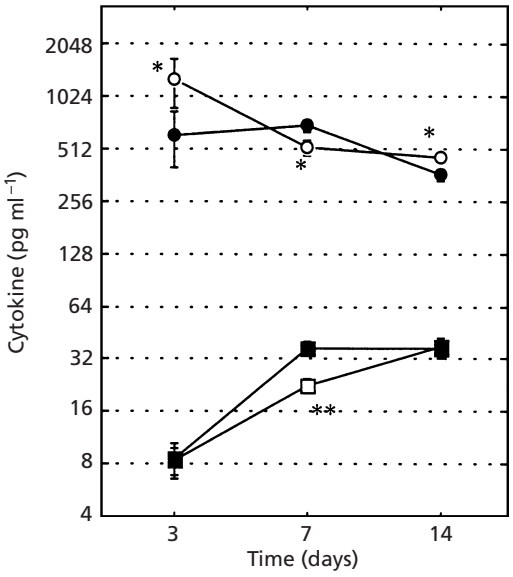

Fig. 3. Amount of cytokines in lung tissues on different days p.i. in the PAO1 and *lasI rhlI* groups. A higher level of IFN-γ on days 3 and 14 p.i. was found in the *lasI rhlI* group, but both higher IFN-γ and IL-4 levels on day 7 were detected in the PAO1 group. Values are expressed as the mean $\pm$ standard errors of the pg ml^{-1}. Significant differences between groups are indicated by asterisks: * $P < 0·05$; ** $P < 0·01$. ●, PAO1 IFN-γ; ○, *lasI rhlI* IFN-γ; ■, PAO1 IL-4; □, *lasI rhlI* IL-4.

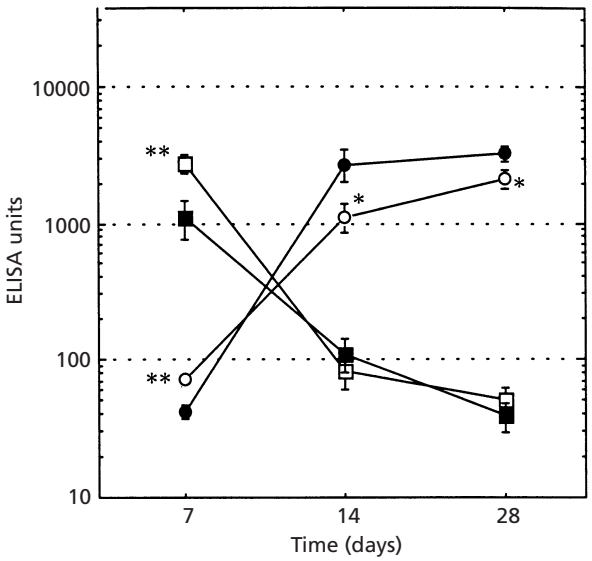

Fig. 4. Serum antibody responses against St-Ags on different days p.i. in the PAO1 and *lasI rhlI* groups. Stronger IgM and IgG responses on day 7 but weaker IgG production on days 14 and 28 are seen in the *lasI rhlI* group, compared with the PAO1 group. The levels of antibodies are expressed as the means $\pm$ 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.

group was notably lower than in the PAO1 group on day 28 p.i. ($P < 0·03$) (Table 2).

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).

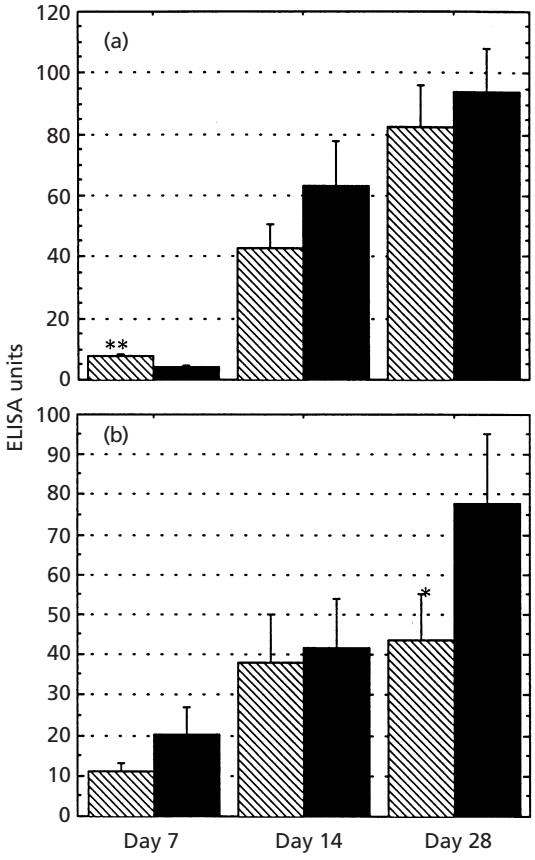

Fig. 5. Serum IgG subclasses response against St-Ags on different days after challenge in the PAO1 and *lasI rhlI* groups. The results are expressed as the means ± standard errors of ELISA units. (a) Higher IgG2a production on day 7 is seen in the *lasI rhlI* group. Hatched bars, *lasI rhlI* Ig2a; black bars, PAO1 Ig2a. (b) Significantly increased IgG1 level on day 28 is found in the PAO1 group. Hatched bars, *lasI rhlI*, IgG1; black bars, PAO1 IgG1. Significant differences between groups are indicated by asterisks: * $P < 0{\cdot}05$; ** $P < 0{\cdot}0005$.

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-γ in the *lasI rhlI* group was significantly higher than that in the PAO1 group ($P < 0{\cdot}02$). On day 14 p.i., a higher IFN-γ level could still be detected in the *lasI rhlI* group ($P < 0{\cdot}05$). The production of IL-4 and IFN-γ in the PAO1 group were higher than those in the *lasI rhlI* group on day 7 p.i. ($P < 0{\cdot}03$) (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 *lasI rhlI* group were significantly higher than those in the PAO1 group ($P < 0{\cdot}007$). However, from day 14 p.i., the situation changed. The IgG titre found in the *lasI rhlI* group was much lower than that in the PAO1 group ($P < 0{\cdot}05$), 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 *lasI rhlI* group, the IgG2a level was notably higher on day 7 p.i. ($P < 0{\cdot}0004$) and the IgG1 level was lower on day 28 p.i. when compared with the levels in the corresponding PAO1 group ($P < 0{\cdot}05$). 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 lasI rhlI*, 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-γ 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 *lasI rhlI* 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 (Kharazmi *et al.*, 1984a, b, 1986), especially inhibiting the oxidative burst response of neutrophils to opsonized zymosan. Our results suggest that PAO1 *lasI rhlI* 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 Th 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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