Pseudomonas aeruginosa alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection

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Pseudomonas aeruginosa is an opportunistic respiratory pathogen that accounts for most of the morbidity and mortality in cystic fibrosis (CF) patients. In CF-affected lungs, the bacteria undergo conversion from a non-mucoid to a non-tractable mucoid phenotype, due to overproduction of alginate. The effect of alginate production on pathogenicity was investigated by using an acute lung infection mouse model that compared a non-mucoid *P. aeruginosa* strain, PAO1, to its constitutive alginate-overproducing derivative, Alg⁺ PAO^{mucA22}, and an alginate-defective strain, Alg⁻ PAO^{algD}. Bacterial suspensions were instilled into the left bronchus and examined 24 and 48 h post-infection. The highest bacterial loads and the most severe lung pathology were observed with strain Alg⁻ PAO^{algD} at 24 h post-infection, which may have been due to an increase in expression of bacterial elastase by the mutant. Significantly lower lung and spleen bacterial loads were found in the two non-mucoid (PAO1 and Alg⁻ PAO^{algD}) groups, compared to the mucoid Alg⁺ PAO^{mucA22} group, between 24 and 48 h post-infection. The positive correlation between lung bacteriology and lung macroscopic pathology in the Alg⁺ PAO^{mucA22} group suggests that alginate production not only impedes pulmonary clearing, but also results in severe lung damage. Positive correlations between IL12 levels and lung macroscopic pathology, and between IL12 and IFN- γ levels in the Alg⁺ PAO^{mucA22} group, suggested a possible contribution of these pro-inflammatory cytokines to tissue damage. No significant differences were found between the three groups in lung cytokine responses at 24 or 48 h post-infection. However, on comparison within each group at 24 and 48 h post-infection, a significant increase in the pro-inflammatory cytokine IFN- γ was observed. Higher ratios of IFN- γ /IL4 and IFN- γ /IL10, but lower IL10 levels, were also found in all three groups. These results indicate a Th1-predominated immune response in these animals. Such cytokine responses could have aided the clearance of non-mucoid *P. aeruginosa*, but were not sufficient to alleviate infection by the mucoid variants. Alginate production may promote survival and persistence of this pathogenic micro-organism in the lung.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen of immunocompromised patients and a common cause of chronic lung infection in patients with cystic fibrosis (CF) (Høiby, 1975). The majority (60–90%) of CF patients are colonized initially by non-mucoid *P. aeruginosa*, but most strains isolated from patients with advanced lung disease have mucoid colony morphology (Høiby *et al.*, 2001). This phenotype is rare among strains that cause other infections

(Høiby, 1975). Mucoid *P. aeruginosa* strains appear to have a distinct survival advantage in the CF-affected lung. The mucoid phenotype is due to overproduction of the exopolysaccharide alginate, an *O*-acetylated linear polymer of D-mannuronate and L-guluronate residues (Davidson *et al.*, 1977). Alginate aids biofilm growth (Lam *et al.*, 1980) and bacterial adherence to human cells and protects bacteria from host defences (Lyczak *et al.*, 2002). The biofilm mode of growth improves bacterial survival in the lung environment, increases resistance to antibiotics and biocides and decreases susceptibility to host defences (Høiby *et al.*, 2001).

Alginate production *in vitro* can be triggered by nutrient

Abbreviations: CF, cystic fibrosis; LIMP, lung index of macroscopic pathology; MN, mononuclear leukocyte; PMN, polymorphonuclear cell.

starvation, antibiotic treatment, slow growth rate, ethanol dehydration, high osmotic pressure or high ionic strength (Evans & Linker, 1973; Govan & Fyfe, 1978; DeVault *et al.*, 1990; Terry *et al.*, 1991). We have demonstrated that local accumulation of activated polymorphonuclear cells (PMNs) exerts selective pressures on the bacteria from phagocytosis and oxygen free radicals, resulting in conversion from the non-mucoid to the mucoid phenotype (Mathee *et al.*, 1999). Approximately 84 % of mucoid CF isolates have mutations in the *mucA* gene, which encodes a negative regulator of an alternative sigma factor, AlgT/U (Boucher *et al.*, 1997). MucA interacts directly with AlgT/U, inhibiting its activity (Hughes & Mathee, 1998). On inactivation of MucA, AlgT/U triggers biosynthesis of alginate via a complex regulatory circuit (Mathee *et al.*, 2002). This culminates in activation of the 18 kb alginate biosynthesis (*algD*) operon (Mathee *et al.*, 2002).

There is a strong humoral immune response against chronic *P. aeruginosa* infection in most CF patients; this is dominated by a T helper type 2 (Th2) immune response (Høiby *et al.*, 2001). This is characterized by production of cytokines IL4, IL5, IL6 and IL10 and antibodies IgG1 and IgE. The Th1 response is associated with an increase in levels of IFN- γ , IL2, IL12 and IgG2a (Banchereau, 1995; Germann *et al.*, 1995). The pronounced antibody response in CF patients leads to the formation of immune complexes in the lung that activate complement and result in an infiltration of PMNs, which is a hallmark of chronic infection in CF-affected lungs (Baltimore *et al.*, 1989; Høiby *et al.*, 2001). Bronchoalveolar lavage fluid of young CF patients has normal levels of IL10 (Noah *et al.*, 1997), whereas patients with chronic infections have significantly lower levels of this cytokine (Bonfield *et al.*, 1995b). Furthermore, normal epithelial cells produce IL10 constitutively, but epithelial cells from chronically infected CF patients are defective in IL10 production (Bonfield *et al.*, 1995a).

Many animal models have been used successfully to recreate both acute and chronic *P. aeruginosa* infections (Stotland *et al.*, 2000). However, only a few studies have addressed the role of alginate in pulmonary clearance and these have produced conflicting data. The earliest reported study, which used guinea pigs as an animal model of chronic infection, a CF *P. aeruginosa* isolate and genetically undefined non-mucoid strains, concluded that the Alg⁺ phenotype did not selectively impair pulmonary clearing (Blackwood & Pennington, 1981). In a more recent study that used a neutropenic mouse model, no difference was observed between the wild-type strain PAO1 and the mutant strain *algT/U::Tc^R* (Yu *et al.*, 1996). Interestingly, the null mutant showed a reduced mean time of death in the normal C57BL/6 mouse and the endotoxin-resistant C3H/HeJ mouse. The authors concluded that inactivation of *algT/U* resulted in increased virulence (Yu *et al.*, 1996). Another study looked at clearance of CF isolates from the murine lung in an aerosol infection model with C57BL/6J, BALB/c and DBA/2NHsd mice, using a wild-type strain and a number of isogenic derivatives (Martin *et al.*, 1993; Boucher *et al.*, 1997). In these experi-

ments, the Alg⁺ strain survived better in the lungs than the parent non-mucoid strain and the *algD* mutant (Boucher *et al.*, 1997). Similar results were observed following repeated aerosol exposure of C57BL/6J mice to other PAO1 derivatives (Yu *et al.*, 1998). The increased host survival could not be attributed to an increase in the pro-inflammatory cytokine TNF- α (Boucher *et al.*, 1997; Yu *et al.*, 1998). There was no difference in lung histopathology or in MIP-2 (the equivalent of IL8) between the two groups of animals at either the 4 or 18 h time-points (Yu *et al.*, 1998).

Although we know much about the organism, regulation of the alginate genes and changes in the patient's immune response from onset of infection to death, no advances have been made in using this information to control *P. aeruginosa* successfully in CF patients. Animal studies clearly support the view that alginate production impedes bacterial clearance and favours their survival in the lungs; however, survival could not be correlated with host immune response. In the current study, we present a detailed analysis of the role of alginate in the pathogenesis of lung infection by using a mouse model, in which we compare the prototype strain of *P. aeruginosa*, PAO1, with a constitutive alginate-overproducing derivative and an alginate-defective strain.

METHODS

Animals. Female, 12-week-old NMRI mice (provided by the Panum Institute, University of Copenhagen, Denmark) were used. The animals were divided randomly into three 'bacterial groups' with 30 animals in each group. They were killed 24 and 48 h after intra-tracheal bacterial infection. All animal experiments were performed under authorization from the National Animal Ethics Committee of Denmark.

Bacterial strains. The prototypic non-mucoid *P. aeruginosa* strain PAO1 (Holloway & Morgan, 1986) and its isogenic derivatives were used. These derivatives were the mucoid strain Alg⁺ PAO*mucA22* (PDO300), which carries the *mucA22* allele that results in constitutive production of alginate (Mathee *et al.*, 1999), and PAO*algD*, a non-mucoid strain that carries a deletion in *algD* (Garrett *et al.*, 1999). Strains were cultured in LB broth (1⁻¹: 10 g tryptone, 5 g yeast extract and 5 g NaCl) overnight at 37 °C and bacterial cells were harvested by centrifugation and resuspended in saline to approximately 1.5×10^8 c.f.u. ml⁻¹.

Animal model. Before challenge, all mice were anaesthetized subcutaneously with a 1:1 (v/v) mixture of etomidate (Janssen) and midazolam (Roche) at a dose of 10 ml (kg body wt)⁻¹ and then tracheotomized (Moser *et al.*, 1997). Each mouse was challenged intra-tracheally with 0.04 ml bacterial suspension as described previously (Moser *et al.*, 1997). Animals were killed by administering 2 ml 20 % pentobarbital (DAK) kg⁻¹.

Macroscopic pathology. Lung index of macroscopic pathology (LIMP) was calculated by using the following modified formula: LIMP = lung area with pathological changes divided by area of the whole lung (Song *et al.*, 1998). Changes in lung pathology are consolidation, haemorrhage and oedema. Gross pathological changes were grouped according to severity of inflammation as previously described (Song *et al.*, 1998): I, normal; II, swollen lungs, hyperaemia and small atelectasis (< 10 mm²); III, pleural adhesions and atelectasis (< 40 mm²); IV, abscesses, large atelectasis and haemorrhages.

Histopathological scores. Cellular alterations in the lungs were classified as acute or chronic inflammation by a scoring system based on the proportion of neutrophils or PMNs and mononuclear leukocytes (MNs) in the inflammatory foci (Johansen *et al.*, 1993). Acute inflammation was defined as cellular infiltration in which PMNs were predominant ($\geq 90\%$) with $\leq 10\%$ MNs, whereas chronic inflammation was defined as a prevalence of MNs (MNs $\geq 90\%$; PMNs $\leq 10\%$), which included lymphocytes, plasma cells and the presence of granulomas (Johansen *et al.*, 1993). Both macroscopic and histopathological evaluations were performed as a double-blind study.

Lung and spleen bacteriology. Quantitative bacteriological examination was done as described previously (Johansen *et al.*, 1993). In brief, lungs or spleens were removed aseptically from mice and homogenized in 5 ml PBS. Serially diluted samples were plated onto LB agar plates to determine bacterial count (c.f.u.). The remaining lung homogenate was centrifuged and the supernatant was retained for cytokine determination.

Cytokine determination. Concentrations of IFN- γ , IL4, IL10 and IL12 in lung homogenate supernatants were determined by using ELISA kits (Nordic BioSite). Standard curves were constructed for IL4, range 7.8–500 pg ml⁻¹ (lower detection limit, 0.96 pg ml⁻¹); IFN- γ , 40–5000 pg ml⁻¹ (10 pg ml⁻¹); IL10, 1.2–80 pg ml⁻¹ (1 pg ml⁻¹); and IL12, 5–1000 pg ml⁻¹ (< 5 pg ml⁻¹). Optical density of each sample was plotted on the standard curve of the respective cytokine ELISA plate to obtain cytokine concentrations.

Protease activity and alginate determination. Bacteria were incubated at 37 °C in LB broth with rapid shaking for 18 h. Cells were collected by centrifugation and LasB elastase activity was determined spectrophotometrically by using elastin–Congo red (Sigma) as substrate (Ohman *et al.*, 1980). Protease activity was expressed as A_{595} units min⁻¹ (g protein)⁻¹. Concentrations of alginate, expressed as mg (l culture supernatant)⁻¹, were measured by the carbazole–borate method (Knutson & Jeanes, 1968).

Statistical analysis. Data were analysed by using the statistical software package SPSS (version 10.0 for Windows; SPSS, Chicago, IL, USA; <http://www.spss.com>). Unpaired differences in continuous data were analysed by the Mann–Whitney U-test and categorical data were compared by using a χ^2 -squared test. Simple regression tests were used for correlation analysis between parameters.

RESULTS AND DISCUSSION

This study was undertaken to investigate the effect of alginate production on the virulence of three genetically defined *P. aeruginosa* strains in an animal model of acute infection. The prototypic non-mucoid strain PAO1 is capable of producing alginate (Holloway & Morgan, 1986). The isogenic derivatives of this strain were Alg⁺ PAO μ ucA22 (PDO300), which is constitutive for alginate production (Mathee *et al.*, 1999), and Alg⁻ PAO μ algD, which has a mutation in the key alginate biosynthetic gene (*algD*, which encodes GDP-mannose dehydrogenase), rendering it incapable of producing alginate (Garrett *et al.*, 1999). These strains were instilled into mouse lungs to create acute infection; animals were killed 24 or 48 h post-infection.

Alginate production results in severe lung pathology

Twenty-four hours post-infection, pulmonary histopatho-

logical changes in the PAO1 group were milder and less PMN infiltration was seen than in the other strains (Fig. 1). Significant pulmonary oedema with milder haemorrhage was seen in the Alg⁻ PAO μ algD group, but marked PMN infiltration was observed in the Alg⁺ PAO μ ucA22 group (Fig. 1). All three groups showed clear infiltration of PMNs in the lung foci at 48 h post-infection, although it was more profound in the Alg⁺ PAO μ ucA22 group (Fig. 1).

Judging by the lung scoring, significantly milder changes in lung pathology were found in the PAO1 group ($P < 0.05$) at 24 h post-infection than in the Alg⁻ PAO μ algD group (Table 1). LIMP analysis of all three groups at 24 h post-infection showed that the scores of the Alg⁻ PAO μ algD and Alg⁺ PAO μ ucA22 groups were significantly higher than the PAO1 group ($P \leq 0.001$ and $P \leq 0.05$, respectively; Table 1). Furthermore, the Alg⁻ PAO μ algD group also had significantly higher LIMP scores than the Alg⁺ PAO μ ucA22 group ($P < 0.05$; Table 1). At 48 h post-infection, the lung pathology of the Alg⁺ PAO μ ucA22 group was significantly more severe than that of the PAO1 group ($P \leq 0.02$, Table 1). In the Alg⁻ PAO μ algD group, LIMP score at 48 h post-infection was obviously lower than that at 24 h ($P < 0.01$).

Both micro- and macroscopic pathological analyses revealed that wild-type PAO1 infection resulted in the mildest pathology (Fig. 1). In terms of infective severity at 24 h, the order of the strains was PAO1 < Alg⁺ PAO μ ucA22 < Alg⁻ PAO μ algD, whereas at 48 h post-infection it was PAO1/Alg⁻ PAO μ algD < Alg⁺ PAO μ ucA22. Of the three strains used, only the Alg⁺ strain produced copious amounts of alginate [255 mg (l culture supernatant)⁻¹]. No alginate was detected in broth culture supernatants of the non-mucoid strains. Alginate production has been inversely correlated with protease activity (Mohr *et al.*, 1990); elastase activity in Alg⁺ PAO μ ucA22 (17.8 U) was reduced approximately four- and sixfold compared to its parent strain (PAO1; 69.6 U) and Alg⁻ PAO μ algD (107.9 U), respectively. The Alg⁻ PAO μ algD strain produced 1.5-fold more elastase than PAO1. The severity of Alg⁻ PAO μ algD at 24 h post-infection may be explained by the ability of this strain to produce higher amounts of elastase.

Similarly, in another study that used a neutropenic mouse model of fatal *P. aeruginosa* sepsis, the authors concluded that loss of ability to make alginate (inactivation of *algT/U*) resulted in increased virulence (Yu *et al.*, 1996). They did not observe any differences in LD₅₀ between wild-type PAO1 and the *algT/U*::Tc^R mutant. However, the null mutant showed a reduced mean time of death in two of the animal strains tested: the normal C57BL/6 mouse and the endotoxin-resistant C3H/HeJ mouse (Yu *et al.*, 1996). Although it appeared initially that the loss of alginate production resulted in increased virulence, it was temporary. At 48 h post-infection, we did not observe any difference between the non-mucoid strains.

Despite significant PMN infiltration in the lung foci at 48 h post-infection in Alg⁺ PAO μ ucA22-infected lungs, this did not appear to influence bacterial clearance. This supports *in*

Fig. 1. Lung histopathology showing inflammatory response against *P. aeruginosa* (magnification, $\times 250$). Panels (a), (b) and (c) show pathology at 24 h post-infection; panels (d), (e) and (f), at 48 h post-infection. Panels (a) and (d): PAO1 strain shows less PMN infiltration and milder lung haemorrhage at 24 h and increased PMN infiltration at 48 h post-infection. Panels (b) and (e) (Alg⁺ PAOmucA22) show significant PMN infiltration at 24 h (b) and 48 h (e) post-infection. Panels (c) and (f) (Alg⁻ PAOalgD) show PMN infiltration, milder lung haemorrhage and significant lung oedema at 24 h (c) and extensive PMN infiltration in both bronchi and lung tissues at 48 h (f) post-infection.

Fig. 2. Lung (a) and spleen (b) bacteriology: bacterial count (c.f.u.) from homogenized lung and spleen tissues from the PAO1 (●), Alg⁻ PAOalgD (■) and Alg⁺ PAOmucA22 (▲) groups. Thirteen animals per bacterial strain were used.

Table 1. Lung pathology analysis

Strain		LIMP*	P value‡	Lung score*	P value‡
Wild-type PAO1	24 h	0.38 (0.03–0.55)		4.00 (2.00–4.00)	
	48 h	0.40 (0.15–0.52)		4.00 (3.00–4.00)	
	P value†				
Alg ⁺ PAO _{mucA22}	24 h	0.50 (0.25–0.62)	< 0.05 (PAO1)	4.00 (3.00–4.00)	
	48 h	0.50 (0.40–0.80)	< 0.02 (PAO1)	4.00 (4.00–4.00)	
	P value†				
Alg ⁻ PAO _{algD}	24 h	0.52 (0.45–0.75)	< 0.001 (PAO1); < 0.05 (<i>mucA</i>)	4.00 (4.00–4.00)	< 0.05 (PAO1)
	48 h	0.49 (0.26–0.51)		4.00 (3.00–4.00)	
	P value†	< 0.002			

*Thirteen animals per bacterial strain were used; values are medians with range in parentheses.

†Significant *P* values between 24 and 48 h analysis.

‡Significant values compared to strain groups indicated in parentheses; PAO1 and *mucA* refer to wild-type PAO1 and Alg⁺ PAO_{mucA22}, respectively.

in vitro studies that demonstrated that alginate protected bacteria from phagocytosis, opsonization, antibodies, complement and PMN infiltration (Laharrague *et al.*, 1984; Oliver & Weir, 1985; Eftekhari & Speert, 1988; Krieg *et al.*, 1988; Stiver *et al.*, 1988; Jensen *et al.*, 1990; Pedersen *et al.*, 1990; Mathee *et al.*, 1999). Conversion of bacteria to the non-tractable, alginate-producing phenotype is partly due to PMN infiltration (Mathee *et al.*, 1999). Alginate has been demonstrated to interfere with PMN functions such as adherence, oxygen metabolism, degranulation and bactericidal activity (Pedersen *et al.*, 1990; Mai *et al.*, 1993). At the molecular level, alginate also alters the signal transduction pathway by modulating PMN protein kinase C and G-protein activity (König *et al.*, 1992).

Excessive infiltration of PMNs contributes to lung tissue damage, due to the release of reactive oxygen species, and the severity of histopathological changes correlates with the ability of the bacteria to persist in the lung and evade host clearance. We observed a positive correlation ($r = 0.66$) between quantitative lung bacteriology and score of lung pathology in the Alg⁺ PAO_{mucA22} group at 24 h post-infection (Table 2). Thus, alginate production not only impedes pulmonary clearance, but also results in severe tissue damage. This is reminiscent of a late-stage chronic infection that exhibits marked local destruction of lung tissues of CF patients with Alg⁺ *P. aeruginosa* strains; for some such patients, lung transplantation is the only hope for survival (Pedersen *et al.*, 1990).

Alginate production and bacterial persistence in the lung

Persistence of invading bacteria and ability of the host immune system to clear bacteria can be inferred from lung bacteriology. At 24 h post-infection, significantly higher bacterial counts were found in the Alg⁻ PAO_{algD} group than in the PAO1 ($P \leq 0.05$) and the Alg⁺ PAO_{mucA22}

($P \leq 0.05$) groups (Fig. 2). Bacterial load at 48 h post-infection in the Alg⁺ PAO_{mucA22} group was 57-fold higher than that in the Alg⁻ PAO_{algD} group ($P < 0.02$) and 158 times higher than that in the PAO1 group ($P < 0.04$) (Fig. 2). Comparing the 24 and 48 h time-points, the wild-type PAO1 and Alg⁻ PAO_{algD} groups showed significantly reduced bacterial loads ($P < 0.002$ and $P = 0.0002$, respectively), but there was no significant change in the Alg⁺ PAO_{mucA22} group ($P > 0.4$) (Fig. 2).

The order of these three strains in terms of their lung bacterial load at 24 h post-infection was Alg⁺ PAO_{mucA22} < PAO1 < Alg⁻ PAO_{algD}, and at 48 h post-infection it was PAO1 < Alg⁻ PAO_{algD} < Alg⁺ PAO_{mucA22} (Table 1). At 24 h post-infection, the Alg⁻ PAO_{algD} group had the highest bacterial load; however, at 48 h post-infection, the Alg⁺ PAO_{mucA22} strain prevailed, suggesting significant microbial protection that was probably due to the over-production of alginate. These data contradict an earlier reported animal study that used guinea pigs, which concluded that the mucoid phenotype had no selective effect to impede pulmonary clearing (Blackwood & Pennington, 1981). The negative control non-mucoid strain used in the guinea pig study was isolated by repeated passages of the mucoid CF strain that is likely to have a defective *mucA* allele, encoding the anti-sigma factor (Martin *et al.*, 1993; Boucher *et al.*, 1997). Based on our current understanding of alginate genes, it is likely that these non-mucoid strains have second site suppressor mutations, possibly in *algT/U*, which encodes the sigma factor (DeVries & Ohman, 1994; Schurr *et al.*, 1994). The use of two strains with undefined mutations may have affected the outcome of their experiment.

The most detailed study to date looked at the effect of alginate on bacterial clearance by using an aerosol infection model in which C57BL/6J, BALB/c and DBA/2Nhsd mice (Boucher *et al.*, 1997) were infected with *P. aeruginosa* PAO381 (Fyfe & Govan, 1980) and its derivatives, PAO578I (*mucA22*),

Table 2. Correlation analysis with various parameters

Parameter	Time (h)	Wild-type PAO1	Alg ⁺ PAO <i>mucA22</i>	Alg ⁻ PAO <i>algD</i>
Lung bacteriology				
Spleen bacteriology	24			$r = 0.75, P = 0.01$
	48	$r = 1, P = 0.0001$		$r = 0.99, P = 0.0001$
LIMP	24		$r = 0.66, P < 0.04$	
IL4				
Spleen bacteriology	24			$r = -0.67, P < 0.04$
	48	$r = -0.71, P = 0.02$		
Lung bacteriology	24			$r = -0.63, P = 0.05$
	48	$r = -0.72, P < 0.02$		
Lung score	48	$r = 0.72, P < 0.02$		
IL10				
Lung score	24			$r = -0.68, P < 0.032$
Lung bacteriology	48		$r = -0.78, P < 0.009$	
Spleen bacteriology	48		$r = -0.7, P < 0.025$	
IL12				
LIMP	24		$r = 0.83, P < 0.003$	$r = 0.8, P < 0.006$
IFN- γ	24	$r = 0.97, P = 0.0001$	$r = 0.64, P < 0.05$	
	48	$r = 0.86, P < 0.002$		$r = 0.76, P = 0.01$
Spleen bacteriology	24		$r = 0.83, P < 0.003$	

PAO578II (*mucA22*, *sup-2*; salt-dependent Alg⁺ phenotype) and PAO578II*algD*::Gm (Martin *et al.*, 1993; Boucher *et al.*, 1997). In this 4 h post-infection study, the alginate-producing strain had greater survival in the lungs than the parent strain and its *algD* mutant (Boucher *et al.*, 1997). Similar results were observed in a repeated aerosol exposure study on C57BL/6J mice that used PAO381 and PAO578I (Yu *et al.*, 1998). Although the experimental strains and animal infection models used here are different, the parallels in our conclusions are clear: alginate production promotes bacterial survival and persistence in lung infection.

Alginate production and persistence in the spleen

The presence of bacteria in the spleen reflects the ability of invading bacteria to spread from the initial point of inoculation. At 24 h post-infection, the spleen bacterial count for the Alg⁺ PAO*mucA22* group was significantly lower than that for the PAO1 group ($P < 0.002$) (Fig. 2). However, at 48 h post-infection, higher counts were found for the Alg⁺ PAO*mucA22* group than for the Alg⁻ PAO*algD* group ($P < 0.006$), with bacterial counts approximately 5.6- and 40-fold higher for the groups with the non-mucoid strains PAO1 and Alg⁻ PAO*algD*, respectively. Comparing the two time points at 24 and 48 h post-infection, significant differences were found for the PAO1 group ($P = 0.01$, Fig. 2) and the Alg⁻ PAO*algD* group ($P < 0.0008$), but no significant difference was confirmed for the mucoid Alg⁺ PAO*mucA22* group ($P > 0.6$), indicating that the Alg⁺ strain is more resistant to immune clearance associated with its mucoid phenotype (Fig. 2).

The order of strains in terms of spleen bacterial load at

24 h post-infection was Alg⁺ PAO*mucA22* < PAO1/Alg⁻ PAO*algD*; whereas at 48 h, it was PAO1/Alg⁻ PAO*algD* < Alg⁺ PAO*mucA22* (Fig. 2). Each strain produced significant septicaemia, but at 24 h post-infection, a significantly lower spleen bacterial count for the Alg⁺ PAO*mucA22* group compared to the parent stain was evident. This difference may be partly associated with the reduced motility of the strain, compared to the non-mucoid strain PAO1 (data not shown). In addition, alginate production has been negatively correlated with synthesis of flagella, which may reduce the number of bacteria entering the bloodstream (Garrett *et al.*, 1999). At 24 h post-infection, a positive correlation ($r = 0.75$) between lung and spleen bacteriology was evident for the Alg⁻ PAO*algD* group (Table 2). Both the PAO1 ($r = 1.00$) and Alg⁻ PAO*algD* ($r = 0.99$) groups showed positive correlation at 48 h post-infection, whereas no significant correlation was seen in the Alg⁺ PAO*mucA22* group. The lower spleen bacterial count in the non-mucoid groups at 48 h post-infection suggests that the host was able to clear these non-mucoid organisms more efficiently than Alg⁺ PAO*mucA22*.

Th1 cytokines are up-regulated at 48 h post-infection

The immune response is modulated by T helper cells via changes in cytokine levels. In the present study, at 24 and 48 h post-infection, no significant differences were found in IFN- γ , IL4, IL10 or IL12 between the three infection groups (Table 3). However, a comparison of the 24 and 48 h post-infection data indicated a significant increase in IFN- γ and decrease in IL10 in all three groups (Table 3). Higher IL12 production, however, was detected in the PAO1 group compared to the

Table 3. Cytokine evaluation in mice 24 and 48 h after intra-tracheal challenge with *P. aeruginosa* suspension
Cytokine levels in lung tissues were measured as described in the text; values represent median and range (units, pg ml⁻¹).

Strain*		IFN- γ	IL4	IL10	IL12	IFN- γ /IL4 ratio	IFN- γ /IL10 ratio
Wild-type PAO1	24 h	549 (148–4597)	104 (11–341)	132 (91–191)	151 (64–859)	5.07 (1.26–48.39)	3.8 (1.63–28.20)
	48 h	2075 (314–4912)	56 (6–76)	99 (60–112)	190 (57–740)‡	31.26 (6.04–86.18)	20.45 (3.08–51.71)
	<i>P</i> value†	< 0.02	< 0.02	< 0.007		< 0.02	< 0.006
Alg ⁺ PAO <i>mucA</i> 22	24 h	313 (119–2122)	81 (1–148)	122 (90–143)	148 (106–319)	5.19 (1.14–25.57)	2.84 (1.12–15.60)
	48 h	1577 (285–2692)	59 (41–147)	97 (52–121)	130 (11–565)	16.61 (5.09–46.90)	15.93 (2.94–22.97)
	<i>P</i> value†	< 0.02		< 0.01		< 0.01	< 0.03
Alg ⁻ PAO <i>algD</i>	24 h	430 (134–2117)	68 (14–120)	122 (101–174)	98 (65–713)	10.21 (1.26–20.50)	3.74 (1.09–19.60)
	48 h	1399 (141–3328)	44 (27–108)	92 (85–116)	101 (51–217)	24.21 (1.81–75.64)	16.34 (1.22–29.45)
	<i>P</i> value‡	< 0.03		< 0.004		< 0.01	< 0.03

*Ten animals per bacterial group were used.

†Significant *P* values between 24 and 48 h analysis.

‡*P* < 0.05, compared to the Alg⁻ PAO*algD* group.

Alg⁻ PAO*algD* group at 48 h post-infection (*P* < 0.05; Table 3). Significant reduction of IL4 levels was seen only in the PAO1 group (*P* < 0.02; Table 3) at 48 h post-infection.

Positive correlations between IFN- γ and IL12 were found in the PAO1 (*r* = 0.97, *P* < 0.0001) and Alg⁺ PAO*mucA*22 (*r* = 0.64, *P* < 0.05) groups at 24 h post-infection, and in the PAO1 (*r* = 0.86, *P* < 0.002) and Alg⁻ PAO*algD* (*r* = 0.76, *P* = 0.01) groups at 48 h post-infection (Table 2). This was not unexpected, as both of these cytokines are Th1 indicators. IL12 stimulates production of IFN- γ by NK and T cells (Belardelli, 1995). The increase of IFN- γ and decrease of IL10 in lung tissues that were seen at 48 h post-infection, compared to the results at 24 h post-infection, are predicted as IL10, if produced, suppresses the synthesis of pro-inflammatory cytokines by inhibiting IL12 and IFN- γ production (Belardelli, 1995). In addition, levels of the pleiotropic cytokine IL4, a product of Th2 lymphocytes that inhibits the differentiation of Th1 cells (Hart *et al.*, 1989; Belardelli, 1995), were also reduced in all three bacterial groups at 48 h post-infection, even though this difference was not statistically significant. These results suggest that the immune response to acute infection with *P. aeruginosa* at 48 h post-infection was predominantly a Th1-type response, compared to the results at 24 h post-infection. This was further demonstrated by analysing the ratios of IFN- γ /IL4 and IFN- γ /IL10 in all three groups. These ratios can be used as an indicator of Th1 versus Th2 responses; a higher ratio is an indicator of a Th1-dominated response and a lower ratio suggests a Th2-dominated response. The significant increase in these ratios between the 24 and 48 h time-points is indicative of a Th1-based immune response (Table 3). The immune response in CF patients with chronic *P. aeruginosa* infections is predominantly a Th2 response and is correlated with a poor prognosis (Moser *et al.*, 2000). It has been proposed that shifting the immune response to Th1 may help to combat *P. aeruginosa* infection (Høiby *et al.*, 2001). However, our results suggest that although the animals mount a Th1 response, it is not sufficient to alleviate mucoid *P. aeruginosa* infection.

Diagnostic value of IL4 levels

Early intermittent colonization of CF-affected lungs by non-mucoid *P. aeruginosa*, often treatable with minimal clinical symptoms, precedes obdurate chronic infection with mucoid variants. In our study, infection by the prototypic, non-mucoid strain PAO1 exhibited the mildest pathology and was cleared more quickly than the other strains. A negative correlation was found between the level of IL4 and lung (*r* = -0.72, *P* < 0.02) and spleen (*r* = -0.71, *P* = 0.02) bacteriology in the PAO1 group at 48 h post-infection. IL4 levels were positively correlated with lung pathological scoring in the PAO1 group at 48 h post-infection (*r* = 0.72, *P* < 0.02). In addition, the Alg⁻ PAO*algD* group showed a negative correlation between IL4 levels and lung (*r* = -0.63, *P* = 0.05) and spleen (*r* = -0.67, *P* < 0.04) bacteriology at 24 h post-infection (Table 2). It appears that the immune response mounted was successful in lowering bacterial load,

but the inflammatory defence mechanisms did lead to tissue damage. It is possible that the outcome in the non-mucoid groups was determined by the significant reduction in IL4 levels and relatively higher IFN- γ levels. However, Jain-Vora *et al.* (1998) reported enhanced pulmonary clearance of *P. aeruginosa* in acute infection in transgenic mice by IL4. Thus, the role of IL4 levels in infection remains unclear with non-mucoid *P. aeruginosa*.

Severity of infection is correlated with the imbalance between pro- and anti-inflammatory cytokine levels

The Alg⁻ PAOalgD group had the highest bacterial load, the most severe pathology and showed a negative correlation between lung scores and IL10 levels ($r = -0.68$, $P < 0.032$) and a positive correlation between IL12 levels and LIMP ($r = 0.8$, $P < 0.006$) at 24 h post-infection. At 48 h post-infection, the Alg⁺ PAOmucA22 strain prevailed, suggesting significant microbial protection that is probably due to overproduction of alginate. The severity of lung pathology and PMN infiltration in the Alg⁺ PAOmucA22 group may be due to an increase in pro-inflammatory cytokines IL12 and IFN- γ . This is supported by positive correlations between IL12 levels and lung pathology ($r = 0.83$, $P < 0.003$) and between IL12 and IFN- γ levels ($r = 0.64$, $P < 0.05$), and negative correlations between IL10 levels and bacterial counts in the lung ($r = -0.78$, $P < 0.009$) and spleen ($r = -0.7$, $P < 0.025$) for the Alg⁺ PAOmucA22 group at 48 h post-infection (Table 2).

The imbalance of pro- versus anti-inflammatory cytokines and the correlation with the severity of infection are similar to those reported in CF patients (Kronborg *et al.*, 1993). It has been shown that bronchoalveolar lavage fluid of CF patients with chronic infection by mucoid strains has significantly lower levels of IL10 (Bonfield *et al.*, 1995b). These authors further demonstrated that normal epithelial cells produce IL10 constitutively, but epithelial cells from chronically infected CF patients are defective for production of IL10 (Bonfield *et al.*, 1995a). The IL10 knockout mice studies of Yu *et al.* (1998) provided supportive evidence for the role of IL10 in CF infection: following repeated aerosol exposure, they observed higher mortality and severe lung pathology in the IL10-deficient mice compared to controls (Yu *et al.*, 1998). In another study, using a chronic endobronchial infection by *P. aeruginosa*, it was demonstrated that IL10 knockout mice had severe weight loss and an increased area of lung inflammation, but no alterations in bacterial burden, compared to wild-type mice (Chmiel *et al.*, 1999, 2002). Interestingly, in the current study, no correlation was seen between the PAO1 group and IL10 levels. This is in agreement with Noah *et al.* (1997), who showed that bronchoalveolar lavage fluid of young CF patients, whose early onset of infection was caused by non-mucoid *P. aeruginosa*, had normal levels of IL10.

Constitutive production of IL10 may help to prevent local tissue destruction by pro-inflammatory cytokines (Moore *et*

al., 1993). It is possible that severity of lung pathology could be reduced by increasing IL10 levels, which would suppress synthesis of the pro-inflammatory cytokines IL12 and IFN- γ (Belardelli, 1995). This idea is also supported by studies in which IL10 treatment increased the survival rate of mice infected with *P. aeruginosa*. The treatment enhanced bacterial clearance (Sawa *et al.*, 1997; Matsumoto *et al.*, 1998), improved survival, lessened severe weight loss, lowered the number of bronchoalveolar lavage neutrophils and decreased the area of lung inflammation (Chmiel *et al.*, 1999, 2002).

Currently, most of the knowledge that concerns mucoid strains of *P. aeruginosa*, especially alginate production and its gene regulation, has been achieved from studies performed *in vitro*. Investigations *in vivo* are still quite limited and have focused on pulmonary clearance of the pathogen. As far as we know, the present study is the most comprehensive analysis to date of the role of alginate in the pathogenicity of *P. aeruginosa*, in terms of its bacterial survival in the lung and spleen and the host cytokine response during an early acute infection. Overproduction of alginate could be an important contributory factor for Alg⁺ PAOmucA22 persistence and virulence. This study supports previous inferences that alginate is a virulence factor that contributes to bacterial adherence and persistence (Marcus & Baker, 1985; Ramphal & Pier, 1985; Doig *et al.*, 1987), formation of microcolony and biofilm growth (Høiby, 1975; Lam *et al.*, 1980; Pedersen, 1992), inhibition of PMN chemotaxis (Stiver *et al.*, 1988; Pedersen *et al.*, 1990), suppression of lymphocyte and PMN function (Pedersen *et al.*, 1990; Mai *et al.*, 1993), formation of a physical barrier to the immune system and antibiotics (Nichols *et al.*, 1989; Evans *et al.*, 1991; Hoyle & Costerton, 1991; Mathee *et al.*, 1994) and resistance to opsonic killing by PMNs and macrophages (Meshulam *et al.*, 1984; Cabral *et al.*, 1987; Jensen *et al.*, 1990; Pedersen *et al.*, 1992).

Patients with chronic infection should benefit from consistent use of anti-inflammatory treatment. However, to better understand the role of alginate in chronic infection and the host immune response, we need to look at the expression of pro- and anti-inflammatory cytokines at both the molecular and cellular levels. This will allow novel therapeutic measures against chronic *P. aeruginosa* infection in CF patients to be developed.

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REFERENCES

- Baltimore, R. S., Christie, C. D. & Smith, G. J. (1989). Immunohisto-pathologic localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis. Implications for the pathogenesis of progressive lung deterioration. *Am Rev Respir Dis* **140**, 1650–1661.
- Banchereau, J. (1995). Converging and diverging properties of human interleukin-4 and interleukin-10. *Behring Inst Mitt* **96**, 58–77.
- Belardelli, F. (1995). Role of interferons and other cytokines in the regulation of the immune response. *Acta Pathol Microbiol Immunol Scand* **103**, 161–179.
- Blackwood, L. L. & Pennington, J. E. (1981). Influence of mucoid coating on clearance of *Pseudomonas aeruginosa* from lungs. *Infect Immun* **32**, 443–448.
- Bonfield, T. L., Konstan, M. W., Burfeind, P., Panuska, J. R., Hilliard, J. B. & Berger, M. (1995a). Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol* **13**, 257–261.
- Bonfield, T. L., Panuska, J. R., Konstan, M. W., Hilliard, K. A., Hilliard, J. B., Ghnaim, H. & Berger, M. (1995b). Inflammatory cytokines in cystic fibrosis lungs. *Am J Respir Crit Care Med* **152**, 2111–2118.
- Boucher, J. C., Yu, H., Mudd, M. H. & Deretic, V. (1997). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun* **65**, 3838–3846.
- Cabral, D. A., Loh, B. A. & Speert, D. P. (1987). Mucoid *Pseudomonas aeruginosa* resists nonopsonic phagocytosis by human neutrophils and macrophages. *Pediatr Res* **22**, 429–431.
- Chmiel, J. F., Konstan, M. W., Knesebeck, J. E., Hilliard, J. B., Bonfield, T. L., Dawson, D. V. & Berger, M. (1999). IL-10 attenuates excessive inflammation in chronic *Pseudomonas* infection in mice. *Am J Respir Crit Care Med* **160**, 2040–2047.
- Chmiel, J. F., Konstan, M. W., Saadane, A., Krenicky, J. E., Lester Kirchner, H. & Berger, M. (2002). Prolonged inflammatory response to acute *Pseudomonas* challenge in interleukin-10 knockout mice. *Am J Respir Crit Care Med* **165**, 1176–1181.
- Davidson, I. W., Sutherland, I. W. & Lawson, C. J. (1977). Localization of O-acetyl groups of bacterial alginate. *J Gen Microbiol* **98**, 603–606.
- DeVault, J. D., Kimbara, K. & Chakrabarty, A. M. (1990). Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates alginate gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. *Mol Microbiol* **4**, 737–745.
- DeVries, C. A. & Ohman, D. E. (1994). Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. *J Bacteriol* **176**, 6677–6687.
- Doig, P., Smith, N. R., Todd, T. & Irvin, R. T. (1987). Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. *Infect Immun* **55**, 1517–1522.
- Eftekhari, F. & Speert, D. P. (1988). Alginase treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocyte-derived macrophages. *Infect Immun* **56**, 2788–2793.
- Evans, L. R. & Linker, A. (1973). Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol* **116**, 915–924.
- Evans, D. J., Allison, D. G., Brown, M. R. & Gilbert, P. (1991). Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *J Antimicrob Chemother* **27**, 177–184.
- Fyfe, J. A. M. & Govan, J. R. W. (1980). Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a chromosomal locus involved in control. *J Gen Microbiol* **119**, 443–450.
- Garrett, E. S., Perlegas, D. & Wozniak, D. J. (1999). Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). *J Bacteriol* **181**, 7401–7404.
- Germann, T., Bongartz, M., Dlugonska, H. & 7 other authors (1995). Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses *in vivo*. *Eur J Immunol* **25**, 823–829.
- Govan, J. R. W. & Fyfe, J. A. M. (1978). Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the mucoid form to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants *in vitro*. *J Antimicrob Chemother* **4**, 233–240.
- Hart, P. H., Vitti, G. F., Burgess, D. R., Whitty, G. A., Piccoli, D. S. & Hamilton, J. A. (1989). Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci U S A* **86**, 3803–3807.
- Høiby, N. (1975). Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. *Acta Pathol Microbiol Scand Suppl* **83**, 549–552.
- Høiby, N., Krogh Johansen, H., Moser, C., Song, Z., Ciofu, O. & Kharazmi, A. (2001). *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. *Microbes Infect* **3**, 23–35.
- Holloway, B. W. & Morgan, A. F. (1986). Genome organization in *Pseudomonas*. *Annu Rev Microbiol* **40**, 79–105.
- Hoyle, B. D. & Costerton, J. W. (1991). Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res* **37**, 91–105.
- Hughes, K. T. & Mathee, K. (1998). The anti-sigma factors. *Annu Rev Microbiol* **52**, 231–286.
- Jain-Vora, S., LeVine, A. M., Chroneos, Z., Ross, G. F., Hull, W. M. & Whitsett, J. A. (1998). Interleukin-4 enhances pulmonary clearance of *Pseudomonas aeruginosa*. *Infect Immun* **66**, 4229–4236.
- Jensen, E. T., Kharazmi, A., Lam, K., Costerton, J. W. & Høiby, N. (1990). Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. *Infect Immun* **58**, 2383–2385.
- Johansen, H. K., Espersen, F., Pedersen, S. S., Hougen, H. P., Rygaard, J. & Høiby, N. (1993). Chronic *Pseudomonas aeruginosa* lung infection in normal and athymic rats. *Acta Pathol Microbiol Immunol Scand* **101**, 207–225.
- Knutson, C. A. & Jeanes, A. (1968). A new modification of the carbazole analysis: application to heteropolysaccharides. *Anal Biochem* **24**, 470–481.
- König, B., Friedl, P., Pedersen, S. S. & König, W. (1992). Alginate – its role in neutrophil responses and signal transduction towards mucoid *Pseudomonas aeruginosa* bacteria. *Int Arch Allergy Immunol* **99**, 98–106.
- Krieg, D. P., Helmke, R. J., German, V. F. & Mangos, J. A. (1988). Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. *Infect Immun* **56**, 3173–3179.
- Kronborg, G., Hansen, M. B., Svenson, M., Fomsgaard, A., Høiby, N. & Bendtsen, K. (1993). Cytokines in sputum and serum from patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection as markers of destructive inflammation in the lungs. *Pediatr Pulmonol* **15**, 292–297.
- Laharrague, P. F., Corberand, J. X., Fillola, G., Gleizes, B. J., Fontanilles, A. M. & Gyrard, E. (1984). *In vitro* effect of the slime of *Pseudomonas aeruginosa* on the function of human polymorphonuclear neutrophils. *Infect Immun* **44**, 760–762.

- Lam, J., Chan, R., Lam, K. & Costerton, J. W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun* **28**, 546–556.
- Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**, 194–222.
- Mai, G. T., Seow, W. K., Pier, G. B., McCormack, J. G. & Thong, Y. H. (1993). Suppression of lymphocyte and neutrophil functions by *Pseudomonas aeruginosa* mucoid exopolysaccharide (alginate): reversal by physicochemical, alginase, and specific monoclonal antibody treatments. *Infect Immun* **61**, 559–564.
- Marcus, H. & Baker, N. R. (1985). Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infect Immun* **47**, 723–729.
- Martin, D. W., Schurr, M. J., Mudd, M. H., Govan, J. R. W., Holloway, B. W. & Deretic, V. (1993). Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc Natl Acad Sci U S A* **90**, 8377–8381.
- Mathee, K., Ciofu, O., Sternberg, C. & 9 other authors (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* **145**, 1349–1357.
- Mathee, K., Kharazmi, A. & Høiby, N. (2002). Role of exopolysaccharide in biofilm matrix formation, the alginate paradigm. In *Molecular Ecology of Biofilms*. Edited by R. J. C. McLean & A. W. Decho. Wymondham, UK: Horizon.
- Matsumoto, T., Tateda, K., Miyazaki, S., Furuya, N., Ohno, A., Ishii, Y., Hirakata, Y. & Yamaguchi, K. (1998). Effect of interleukin-10 on gut-derived sepsis caused by *Pseudomonas aeruginosa* in mice. *Antimicrob Agents Chemother* **42**, 2853–2857.
- Meshulam, T., Obedeau, N., Merzbach, D. & Sobel, J. D. (1984). Phagocytosis of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. *Clin Immunol Immunopathol* **32**, 151–165.
- Mohr, C. D., Rust, L., Albus, A. M., Iglewski, B. H. & Deretic, V. (1990). Expression patterns of genes encoding elastase and controlling mucoidy: co-ordinate regulation of two virulence factors in *Pseudomonas aeruginosa* isolates from cystic fibrosis. *Mol Microbiol* **4**, 2103–2110.
- Moore, K. W., O'Garra, A., de Waal Malefyt, R., Vieira, P. & Mosmann, T. R. (1993). Interleukin-10. *Annu Rev Immunol* **11**, 165–190.
- Moser, C., Johansen, H. K., Song, Z., Hougen, H. P., Rygaard, J. & Høiby, N. (1997). Chronic *Pseudomonas aeruginosa* lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice. *Acta Pathol Microbiol Immunol Scand* **105**, 838–842.
- Moser, C., Kjaergaard, S., Pressler, T., Kharazmi, A., Koch, C. & Høiby, N. (2000). The immune response to chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is predominantly of the Th2 type. *Acta Pathol Microbiol Immunol Scand* **108**, 329–335.
- Nichols, W. W., Evans, M. J., Slack, M. P. E. & Walmsley, H. L. (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *J Gen Microbiol* **135**, 1291–1303.
- Noah, T. L., Black, H. R., Cheng, P. W., Wood, R. E. & Leigh, M. W. (1997). Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. *J Infect Dis* **175**, 638–647.
- Ohman, D. E., Cryz, S. J. & Iglewski, B. H. (1980). Isolation and characterization of a *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. *J Bacteriol* **142**, 836–842.
- Oliver, A. M. & Weir, D. M. (1985). The effect of *Pseudomonas* alginate on rat alveolar macrophage phagocytosis and bacterial opsonization. *Clin Exp Immunol* **59**, 190–196.
- Pedersen, S. S. (1992). Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Acta Pathol Microbiol Immunol Scand Suppl* **28**, 1–79.
- Pedersen, S. S., Kharazmi, A., Espersen, F. & Høiby, N. (1990). *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. *Infect Immun* **58**, 3363–3368.
- Pedersen, S. S., Høiby, N., Espersen, F. & Koch, C. (1992). Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* **47**, 6–13.
- Ramphal, R. & Pier, G. B. (1985). Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. *Infect Immun* **47**, 1–4.
- Sawa, T., Corry, D. B., Gropper, M. A., Ohara, M., Kurahashi, K. & Wiener-Kronish, J. P. (1997). IL-10 improves lung injury and survival in *Pseudomonas aeruginosa* pneumonia. *J Immunol* **159**, 2858–2866.
- Schurr, M. J., Martin, D. W., Mudd, M. H. & Deretic, V. (1994). Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J Bacteriol* **176**, 3375–3382.
- Song, Z., Kharazmi, A., Wu, H., Faber, V., Moser, C., Krogh, H. K., Rygaard, J. & Høiby, N. (1998). Effects of ginseng treatment on neutrophil chemiluminescence and immunoglobulin G subclasses in a rat model of chronic *Pseudomonas aeruginosa* pneumonia. *Clin Diagn Lab Immunol* **5**, 882–887.
- Stiver, H. G., Zachidniak, K. & Speert, D. P. (1988). Inhibition of polymorphonuclear leukocyte chemotaxis by the mucoid exopolysaccharide of *Pseudomonas aeruginosa*. *Clin Invest Med* **11**, 247–252.
- Stotland, P. K., Radzioch, D. & Stevenson, M. M. (2000). Mouse models of chronic lung infection with *Pseudomonas aeruginosa*: models for the study of cystic fibrosis. *Pediatr Pulmonol* **30**, 413–424.
- Terry, J. M., Pina, S. E. & Mattingly, S. J. (1991). Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. *Infect Immun* **59**, 471–477.
- Yu, H., Boucher, J. C., Hibler, N. S. & Deretic, V. (1996). Virulence properties of *Pseudomonas aeruginosa* lacking the extreme-stress sigma factor AlgU (sigmaE). *Infect Immun* **64**, 2774–2781.
- Yu, H., Hanes, M., Chrisp, C. E., Boucher, J. C. & Deretic, V. (1998). Microbial pathogenesis in cystic fibrosis: pulmonary clearance of mucoid *Pseudomonas aeruginosa* and inflammation in a mouse model of repeated respiratory challenge. *Infect Immun* **66**, 280–288.