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Role of Neutrophils in Internalisation and Early Clearance of *Pseudomonas aeruginosa* in the Mouse Lung

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ABSTRACT

This study aimed to evaluate the role of neutrophils (polymorphonuclear leukocytes [PMN]) in internalisation and early clearance of *Pseudomonas aeruginosa* in the lung. Mice were rendered leukopenic by intraperitoneal injection of cyclophosphamide (CP) and challenged with *P. aeruginosa* at day four of treatment. At 24 h post-infection with a sublethal dose (1×10^4 colony forming units [cfu]), CP-treated mice showed a bacterial burden almost four orders of magnitude higher than that of control mice ($P<0.05$). Evaluation of the spleens showed higher numbers of bacteria in CP-treated mice than in the control mice ($P=0.0004$). At 4 h post-infection with a high-dose of *Pseudomonas* (1×10^7 cfu), although the total bacterial number was not different between the two groups, leukopenic mice showed significantly less internalised bacteria than control mice ($P<0.05$). Our study shows that *P. aeruginosa* internalisation and early clearance are greatly impaired in leukopenic mice, indicating an important role for PMN in the early defense of the lung against *P. aeruginosa*.

Keywords: clearance, cyclophosphamide, internalisation, lung, neutrophils
Abbreviations: AM, alveolar macrophages; **cfu**, colony forming units; **CF**, cystic fibrosis; **CP**, cyclophosphamide; **PMN**, polymorphonuclear leukocytes; **WBC**, white blood cell

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and bacteremia. It also causes a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. Chronic endobronchial infections due to *P. aeruginosa* are acquired early by cystic fibrosis (CF) patients (Hoiby 1995) and are the major cause of morbidity and mortality in these patients. CF *P. aeruginosa* strains soon become resistant to antibiotic therapy, due to the embedding of microcolonies in a mucoid exopolysaccharide called alginate (Hoiby *et al*. 2001).

Host defense mechanisms of the airways against bacteria are comprised of innate and acquired immune system responses and of an epithelial cell barrier (reviewed in (Diamond *et al*. 2000; Zhang *et al*. 2000). Innate defenses consist mainly of the phagocytic defenses provided by the resident alveolar macrophages (AM) and the polymorphonuclear leukocytes (PMN) that are recruited into the lung in response to pulmonary infection. The role of AM in defending the lung against *P. aeruginosa* and in regulating inflammatory reactions has been assessed in studies in which AM were depleted. In rodent models, AM can be depleted to 5 to 22% of normal numbers by liposome-encapsulated clodronate disodium delivered to the pulmonary tract (Hashimoto *et al*. 1996; Kooguchi *et al.* 1998; Cheung *et al*. 2000).

PMNs influx is necessary for clearance of infectious agents in the airspaces of the lungs. Mice rendered leukopenic either with the use of specific antigranulocyte antibody (Rehm *et al*. 1980) or by cyclophosphamide (CP) treatment (Mayer and Walzl 1983; Rowatt *et al*. 1983; Shirai *et al*. 1997; Babalola *et al*. 2004) showed reduced clearance of *P. aeruginosa* from the lung with pneumonia development.

Epithelial respiratory cells function as part of the innate immune responses and not merely only as a physical barrier. Multiple substances with pro- and anti-inflammatory as well as antimicrobial activities are secreted by epithelial cells (Diamond *et al*. 2000; Bals and Hiemstra 2004). Moreover, it has been recently shown that lung epithelial cells can directly interact with bacteria and therefore are involved in the colonisation of the airways by *P. aeruginosa* (Allewelt *et al.* 2000; Schroeder *et al.* 2001). *In vitro* and *in vivo* studies have shown that human respiratory epithelial cells can uptake *P. aeruginosa* through a specific interaction between the core of bacterial LPS and the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) receptor (Pier *et al*. 1996, 1997).

The main objective of this study was to further elucidate the *in vivo* role of PMN, AM and epithelial cells in early defense of the lung against *P. aeruginosa* challenge. Leukopeny was induced by means of intraperitoneal CP treatment. After mice were dosed with *P. aeruginosa* by intratracheal inoculation, the effects of PMN depletion on bacterial clearance and internalisation by lung cells were assessed. Moreover, the spread of bacteria in blood circulation was studied by evaluating the presence of bacteria in the spleen.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The non-mucoid laboratory strain *P. aeruginosa* PAO1 (Stover *et al*. 2000) (kindly provided by Dr. Gerd Döring, University of Tübingen, Germany) was grown on 5% sheep blood Columbia agar plates (Heipha, Heidelberg, Germany) and then cultured in Trypticase Soy Broth (TSB) (Difco, Becton Dickinson, Maryland, USA) overnight at 37°C and the culture adjusted to an optical density at 600 nm (OD_{600}) of 0.05 with TSB. Bacteria were grown for 2 h to reach the log-phase and then diluted to the desired concentration for inoculation.

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Animals

Specific pathogen-free C57Bl/6 male mice of 8-10 weeks and weighing 24 to 26 g were obtained from Charles River (Calco, Italy). All mice were housed under SPF conditions in the animal care facility at the H.S. Raffaele Scientific Institute, Milano, Italy. The animal procedures were reviewed and approved by the H.S. Raffaele Animal Care and Use Committee, Milano, Italy.

CP-treatment

Animals were intraperitoneally injected with 150 mg of CP (Endoxan-Asta, Asta Medica, Milano, Italy) per kg of body weight 4 successive days before the bacterial challenge. Control animals were treated with PBS.

Intratracheal administration of *P. aeruginosa*

Mice were anesthetized by an intraperitoneal injection of a solution of 2.5% Avertin (2,2,2-tribromoethanol) (Sigma-Aldrich, Italy) in 0.9% NaCl and administered at a volume of 0.015 ml/g body weight. After mice were placed in dorsal recumbency, the trachea was directly visualised by a ventral midline incision, exposed and intubated with a sterile, flexible 22-G cannula (Becton Dickinson, Heidelberg, Germany) attached to a 1 ml syringe. A 50 µl inoculum of a bacterial suspension was implanted via the cannula into the lung and all lobes were inoculated. After inoculation, the incisions were closed by sutures. The mice were maintained under specific pathogen-free conditions in sterile cages which were put into a ventilated isolator (Charles River, Calco, Italy). After 4 and 24 h, mice were euthanised. For quantitative bacteriology, mouse lungs and spleens were excised aseptically and homogenised in 1 ml PBS using the DIAX 900 homogenizer (Heidolph GmbH, Schwabach, Germany). One-hundred µl of the homogenates and 10-fold serial dilutions were spotted onto TSB plates and colony forming unit (cfu) determined after 24 h growth at 37°C.

For determination of intracellular *P. aeruginosa*, mice were injected intratracheally with 1×10^7 cfu. Lungs were aseptically removed and single cell suspensions were made by forcing the tissue through first a 100- and then an 80-mesh sterile screen into DMEM/F12, 10% fetal bovine serum tissue culture medium (Euroclone Life Sciences Division, Milano, Italy). For determination of the total amount of bacteria, including both extracellular and intracellular organisms, 0.5% saponin was added to a measured portion of the single-cell suspension. The amount of intracellular bacteria was determined by centrifuging another measured portion, resuspending the cells in DMEM/F12 medium containing 300 mg gentamicin/ml (Gentalyn, Schering-Plough, Milano, Italy), and incubating the suspension for 1 h at 37°C. The cells were then washed three times in DMEM/F12 medium to remove the antibiotic and then suspended in DMEM/F12 medium with 0.5% saponin to release intracellular bacteria, which were quantified by serial dilution and plating as described above.

Statistical analysis

Statistical significance was analysed using the StatView program (Abacus Concepts Inc., Berkeley, CA) on a Power Macintosh iBook. ANOVA tests, with *post hoc* Fisher PLSD or Scheffe F-test, or Mann-Whitney tests were run to determine *P* values. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chronic models of lung infection might be more appropriate to mimick certain pathological conditions, like CF (Bragonzi *et al*. 2005; Montanari *et al*. 2007). However, in these models, mucoid or non mucoid *P. aeruginosa* is entrapped in agar beads (van Heeckeren *et al*. 1997; Bragonzi *et al*. 2005), making unlikely direct interaction of bacteria with macrophages and airway epithelial cells. PMN are recruited in the airways of animals infected with *P. aeruginosa*-laden beads correlating with high levels of cytokines and chemokines in the bronchoalveolar lavage fluid (van Heeckeren *et al*. 1997). However, in this model, mortality is ascribed to bronchopneumonia (due to hyperinflammation) and not to sepsis, indicating that there is no breach of the epithelial barrier. Conversely, an acute model of infection might allow us to dissect the interaction of *P. aeruginosa* with resident airway cells, i.e. macrophages and epithelial cells. We undertook this study to investigate the relative role of PMN, AM and epithelial cells in the early clearance and bacterial internalisation of *P. aeruginosa* in an acute model of pulmonary infection.

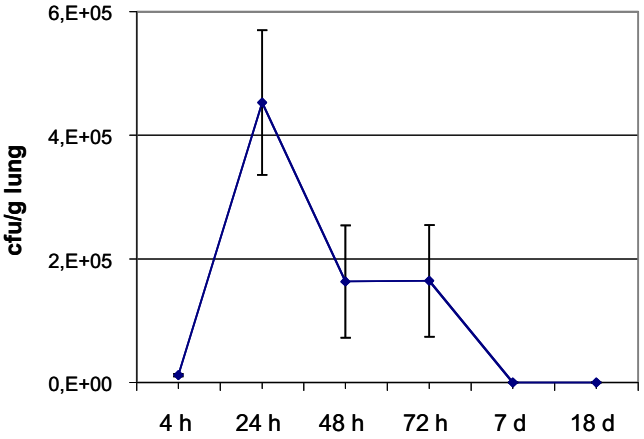

Fig. 1 Clearance of *P. aeruginosa* from the lung. Mice were injected intratracheally with 1×10^4 cfu and lung was harvested at different time points post-injection (n=3-6 for each time point). Values represent cfu/g lung tissue. Data are expressed as the means ± SD of two experiments.

Preliminary studies from our laboratory showed that *P. aeruginosa* PAO-1 strain (inocolum: 1×10^4 cfu) proliferated in the C57Bl/6 murine lung for 24-72 h and then it was cleared from the airways (**Fig. 1**). *P. aeruginosa* internalisation by lung epithelial cells has been shown to peak 4 h post-infection when the inocolum contained 1 X 10^7 cfu (Schroeder *et al*. 2001). Based on these data, bacterial internalisation and clearance and blood spreading were evaluated with different inocula at 4 and 24 h post-infection, respectively.

Leukopenic mice show reduced bacterial clearance and internalisation

C57Bl/6 mice were rendered leukopenic by i.p. injection of CP (Jakab and Warr 1981). **Table 1** shows the WBC counts in blood of control and CP-treated mice. The WBC counts in CP-treated mice were 41.6%, 25% and 4.87% of those of control mice at 1 d, 3 d and 4 d, respectively. The following experiments were performed after 4 d of CP treatment.

At 4 h postinfection, CP-treated mice challenged with 1×10^4 cfu presented in their lungs the same amount of colonies as compared to control mice; at 24 h, treated mice showed a bacterial burden higher almost four orders of magnitude than that found in control mice (**Fig. 2A**).

Evaluation of bacteria in the spleens at 24 h resulted in higher cfu numbers in CP-treated mice than control mice (**Fig. 3**), suggesting that the integrity of the epithelial barrier was disrupted in leukopenic mice.

Following the injection of 1×10^7 cfu, although the total bacteria found in the lungs were not different between the two groups at 4 h, CP-treated mice showed significantly less internalised bacteria than control mice (**Fig. 2B**). In

Table 1 Effect of CP treatment on WBC blood counts.

Mouse group	WBC in blood x 10^3/ml		
	1 d	3 d	4 d
Control	6.91 ± 1.07	6.96 ± 0.54	6.16 ± 3.14
CP-treated	2.88 ± 0.92	1.74 ± 0.45	0.30 ± 0.28

The value before the treatment was 8.31 ± 1.87 cells × 10^3/ml.

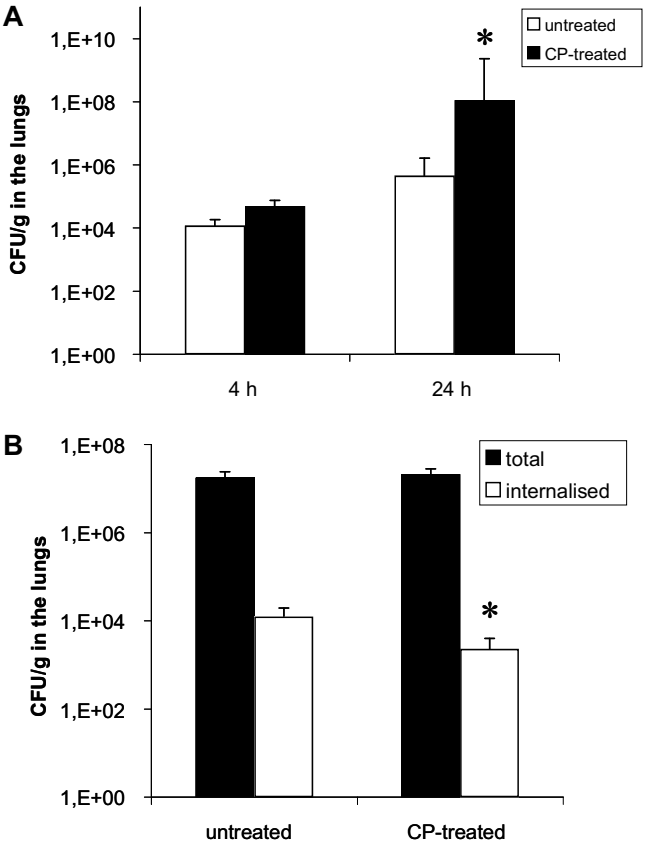

Fig. 2 Effect of leukopeny on bacterial clearance and internalisation. (**A**) Mice tretated with PBS (controls) (n=24) or CP (n=26) were challenged with *P. aeruginosa* PAO-1 (1×10^4 cfu). At 4 h (PBS, n=10; CP, n=10) and 24 h (PBS, n=14; CP, n=16), bacterial counts in lung homogenates were determined. Values represent cfu/g lung tissue. Data are expressed as the means ± SD of three experiments.* $P<0.05$. (**B**) Mice treated with PBS (controls) (n=10) or CP (n=9) were challenged with PAO-1 (1×10^7 cfu). At 4 h, single cell suspensions were obtained from lungs and number of internalised bacteria were determined by the gentamicin exclusion assay (see MATERIALS AND METHODS). Values represent cfu/g lung tissue. Data are expressed as the means ± SD of three experiments. * $P<0.05$.

percentage of the total burden, the internalisation in CP-treated mice was 6.9-fold less than in control mice.

Which cell type is responsible for *P. aeruginosa* internalisation in the lung? Alveolar macrophages (AM), located at the interphase between air and lung tissue, provide the first line of phagocytic defense against microbial invasion in the lower respiratory tract. Besides their phagocytic and microbicidal functions, AM also secrete numerous chemical

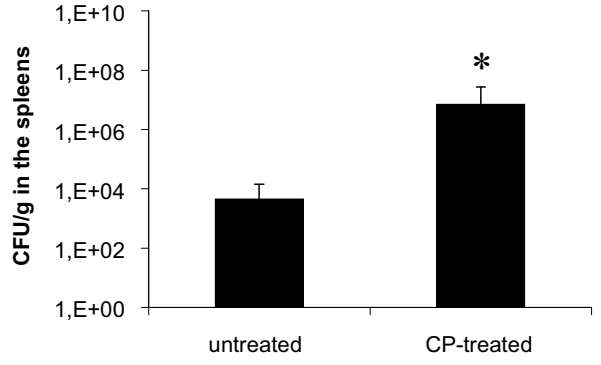

Fig. 3 Leukopeny determines an increased bacterial burden in the spleens. Mice were treated with PBS (controls) (n=14) or CP (n=16) were challenged with *P. aeruginosa* PAO-1 (1×10^4 cfu). At 24 h, bacterial counts in spleen homogenates were determined. Values represent cfu/g spleen tissue. Data are expressed as the means ± SD of three experiments. * $P=0.0004$.

mediators upon stimulation, thereby playing a role in regulating inflammatory reactions in the lung (Hauschildt and Kleine 1995). However, data published by Cheung and colleagues demonstrate that AM depletion of 78 to 88% by intranasal administration of liposome-encapsulated dichloromethylene diphosphonate did not affect the survival rate of infected mice or clearance of *P. aeruginosa* from the lung compared to the control group (Cheung *et al.* 2000). On the other hand, neutrophil recruitment was not impaired in AM-depleted mice. Expression of exogenous human cystic fibrosis transmembrane conductance regulator (CFTR) in lung epithelial cells corrected the bacterial clearance defect in cystic fibrosis mice, whereas expression of the same transgene in alveolar macrophages did not improve the bacterial clearance defect (Oceandy *et al.* 2002). Overall, these data suggest that AM do not play an important role in defense of the lung against *P. aeruginosa*.

In the last years, it has been recognised that airway epithelial cells are likely to play an important role in the pulmonary innate immunity. In the process of clearance of bacteria from the lungs, respiratory epithelial cells eventually come close into contact with pathogens. Previous studies have shown that human non-phagocytic epithelial cells can take up facultative intracellular bacteria (Fleiszig *et al.* 1995), like *P. aeruginosa*. Binding of *P. aeruginosa* to the respiratory epithelial cells is likely mediated by asialo-GM1, followed by a translocation of the CFTR into the cell membrane (Pier *et al.* 1997). The interaction of the first extracellular loop (aa 108-117) of CFTR with the outer core oligosaccharide portion of bacterial LPS (Pier *et al.* 1996, 1997) leads to bacterial internalization (reviewed in Goldberg and Pier 2000). *P. aeruginosa* interaction with epithelial cells activates acidic sphingomyelinase (ASM) and ceramide-mediated modification of membrane lipid rafts is required for bacteria cell internalization (Kowalski and Pier 2004). Contact of *P. aeruginosa* with CFTR localized to lipid rafts determines bacterial internalization, which leads to NF-κB activation and apoptosis, which are involved in the response of innate immunity and resolution of this response to infection (Schroeder *et al.* 2002; Grassmé *et al.* 2003).

However, only in one pathological condition, i.e. cystic fibrosis, this seems to be relevant. It was reported that CFT1 cells derived from a F508del homozygous CF patient were less efficient to internalise *P. aeruginosa* when compared with the isogenic cells transformed with cDNA encoding wild-type CFTR, which internalised 10-50 times more *P. aeruginosa* (Pier *et al.* 1996). Transgenic CF mice show a defect in the uptake of *P. aeruginosa* by respiratory epithelial cells and increased bacterial burden in the lung (Schroeder *et al.* 2001).

Our results indicate that bacterial uptake by respiratory epithelial cells does not play a major role in *P. aeruginosa* clearance in our model (using C57Bl/6 mice and evaluating internalisation at 4 h). In normal mice, the internalised bacteria were 0.07% of total bacteria in the lung, which is different from the 10% observed by others following intranasal application of bacteria (Allewelt *et al.* 2000). On the other hand, both internalisation at 4 h and clearance at 24 h were less in leukopenic mice as compared to control mice, and spreading in the blood circulation was higher. These data suggest that bacterial uptake by epithelial cells (or airway macrophages) might be involved in impeding high bacterial burden and bacterial spreading in blood circulation in the absence of neutrophils.

What is the role of neutrophils in *P. aeruginosa* internalisation in the lung? Under normal conditions, thre is a sizeable pool of marginated granulocytes in the lung circulation which is in dynamic equilibrium with the circulating granulocyte pool (Brigham and Meyrick 1984; Hogg 1987; Hogg and Doerschuk 1995). This marginated pool comprises 40% of total body neutrophils in mice (Nelson *et al.* 1995), with neutrophils in the pulmonary circulation outnumbering those in the peripheral circulation by two- or three-fold (Doerschuk *et al.* 1987). This marginated pool might be in-

volved in a fast fagocytic response to *P. aeruginosa*. In leukopenic mice, this marginated pool may be lowered, giving rise to bacterial survival and proliferation in the airways. Bacteria are then responsible for breaching the epithelial barrier and spread to the systemic circulation. Several virulence factors secreted by *Pseudomonas* have been shown to affect the structure and function of epithelial cells in such a way that bacterial passage through the epithelial monolayer is allowed. Elastase has been reported to decrease transepithelial resistance and permeability of polarized MDCK cells (Azghani 1996). *Pseudomonas* elastase also diminished ZO-1 staining in these cells but it did not change E cadherin staining (Azghani *et al*. 1993). Zulianello and co-workers demonstrated that only strains secreting rhamnolipids were able to diminish transepithelial resistance and increase permeability of an *in vitro* reconstituted respiratory epithelium, irrespective of their ability to release elastase and lipopolysaccharide (Zulianello *et al*. 2006).

Overall, these results indicate that bacterial internalisation by airway cells in the mouse is negligible and does not have a major role in the *P. aeruginosa* clearance. Bacterial uptake might be essential in the barrier function of respiratory epithelial cells impeding bacterial spread in the blood circulation.

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