Macrolide-treated *Pseudomonas aeruginosa* induces paradoxical host responses in the lungs of mice and a high mortality rate

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**Objective:** Accumulating data have demonstrated that macrolide antibiotics suppress *Pseudomonas aeruginosa* virulence, which may explain the efficacy of macrolides in clinical settings. We examined the virulence of macrolide-treated bacteria in vivo.

**Methods:** *P. aeruginosa* PAO-1 was grown for 24 h on agar containing sub-MIC antibiotics, and then mice were challenged intranasally with $10^7$ cfu of bacteria.

**Results and conclusions:** The mortality rate of mice inoculated with bacteria grown in the presence of clarithromycin (10 mg/L), erythromycin (10 mg/L) or azithromycin (5 mg/L) was 80%, 80% and 100%, respectively. In contrast, none of the mice inoculated with non-treated bacteria or bacteria treated with other antibiotics died. Lung weight and protein concentration in bronchoalveolar lavage fluid (BALF) were significantly higher in the clarithromycin group between 3 and 9 h. Moreover, we detected higher levels of tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and nitric oxide (NO) in the BALF of these mice. These data demonstrate that macrolide-treated *P. aeruginosa* induced paradoxically strong responses, such as elevation of TNF-$\alpha$, NO and permeability in the lungs.

**Introduction**

*Pseudomonas aeruginosa* is one of the most important bacterial pathogens in chronic pulmonary diseases, such as cystic fibrosis1–3 and diffuse panbronchiolitis.4 Once *P. aeruginosa* colonizes the airway mucosa in patients, it is difficult to eradicate it despite treatment with potent antibiotics. Since infection by *P. aeruginosa* usually leads to death due to respiratory failure or other consequences of respiratory infection, it is extremely important to inhibit colonization or attempt to eradicate the bacteria.

Certain macrolide antibiotics, such as erythromycin (ERY), clarithromycin (CLR) and azithromycin (AZM), have been reported to improve the clinical symptoms and prognosis of patients with chronic *P. aeruginosa* infections when these antibiotics are administered for prolonged periods, i.e. several months to years.5–9 Since at therapeutic doses these macrolides are not regarded as effective against *P. aeruginosa*, many investigators are interested in the mechanisms of the clinical efficacy of these antibiotics. On the one hand there is a large body of evidence that macrolide antibiotics modulate the host defence mechanisms;10–13 various groups have shown that macrolides suppress the virulence of some bacteria by inhibiting the production of toxins, for example.6,14–16 *P. aeruginosa* produces several exotoxins, including exotoxin A, protease and elastase, during the course of infection, which are of key importance in the pathogenesis of the disease caused by this bacterium.17,18 We, and others, have reported that some macrolide antibiotics at sub-MICs inhibit the production of *P. aeruginosa* exotoxins.6,14,15,19 In addition, we have reported that sub-MICs of macrolide antibiotics enhance the susceptibility of *P. aeruginosa* to serum bactericidal activity by altering cell surface structures, lipopolysaccharide (LPS) and outer membrane proteins.20,21 These data indicate that bacteria exposed to macrolide antibiotics may induce different responses.

In the present study, we examined host responses in the lungs of mice following exposure to antibiotic-treated *P. aeruginosa*. Paradoxically, our data demonstrated that...
CLR-, ERY- and AZM-treated bacteria were more virulent than non-treated bacteria or bacteria treated with other antibiotics. Lethality rate correlated well with the degree of host response, such as increase in tumour necrosis factor-α (TNF-α), nitric oxide (NO), elastase and permeability in the lungs.

### Materials and methods

**P. aeruginosa strains**

*P. aeruginosa* PAO-1 was kindly provided by Dr B. H. Iglewski (University of Rochester, School of Medicine and Dentistry, Rochester, NY, USA). Eight clinical isolates of *P. aeruginosa* (Nagasaki University Hospital, Nagasaki, Japan) were used in some experiments. Four strains were isolated from blood, and others were isolated from sputum.

**Animals**

Six-week-old male ICR mice were purchased from Charles River Japan (Kanagawa, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toho University School of Medicine.

**Antimicrobial agents**

The following antimicrobial agents were kindly provided by their manufacturers: ERY (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), CLR (Taisho Pharmaceutical Co., Ltd, Tokyo, Japan), AZM (Pfizer Laboratories, Groton, CT, USA), oleandomycin (OM) (Pfizer Laboratories), josamycin (JM) (Yamanouchi Pharmaceutical Co., Ltd, Tokyo, Japan), ceftazidime (CAZ) (Tanabe Pharmaceutical Co., Ltd, Osaka, Japan), minocycline (MIN) (Wyeth Lederle Japan, Ltd, Tokyo, Japan), tobramycin (TOB) (Shionogi Pharmaceutical Co., Ltd, Osaka, Japan), clindamycin (CLI) (Japan Upjohn Co., Ltd, Tokyo, Japan) and ofloxacin (OFX) (Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan).

**Pretreatment of *P. aeruginosa* with sub-MIC antibiotics**

The MICs of AZM, ERY, CLR, JM, OM, CAZ, TOB, MIN, OFX and CLI for *P. aeruginosa* PAO-1 were 128, 256, 128, >256, >256, 16, 0.5, 32, 0.5 and 256 mg/L, respectively. After pre-culture of *P. aeruginosa* for 24 h at 35°C on Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA), a single colony was plated on to Mueller–Hinton agar containing antibiotics at sub-MICs, and incubated for 24 h at 35°C. Considering the clinically achievable concentrations of antibiotics and results of previous reports, the following concentrations (1/53–1/4 × MIC, maximum 10 mg/L) were chosen as sub-MICs for *P. aeruginosa* PAO-1: CLR (10 mg/L), AZM (5 mg/L), ERY (10 mg/L), JM (10 mg/L), OM (10 mg/L), TOB (0.1 mg/L), MIN (2 mg/L), CAZ (0.3 mg/L), CLI (10 mg/L) and OFX (0.1 mg/L). To examine concentration dependence, AZM at concentrations of 1.25, 2.5 and 5 mg/L was selected. After incubation on agar containing sub-MICs of antibiotics, bacteria were suspended in 0.9% saline to the desired cell density, which was confirmed by plating on to Mueller–Hinton agar after serial 10-fold dilutions.

**Inoculation of *P. aeruginosa* into the lungs**

Mice were anaesthetized lightly by intramuscular injection of a mixture of 60 mg/kg ketamine (Sankyo Pharmaceutical, Tokyo, Japan) and 10 mg/kg xylazine (Bayer Japan, Tokyo, Japan) and challenged intranasally with a bacterial suspension containing c. 10⁷ cfu of organisms. Each experiment was repeated at least twice. Survival of mice was recorded 3, 6, 9, 24 and 48 h after challenge with the bacteria.

**Collection of bronchoalveolar lavage fluid (BALF)**

Mice were killed by inhalation of ether at the indicated time points. Bronchoalveolar lavage (BAL) was carried out by instilling 1 mL of 0.9% saline through the tracheal cannula, and BAL fluid (BALF) was centrifuged at 10 000 g for 5 min. Supernatants were stored at −80°C until assayed.

**Measurement of pulmonary permeability**

Total protein concentration in BALF was measured to examine changes in pulmonary permeability after inoculation of bacteria or bacterial toxins. BALF was mixed with Bio-Rad solution (Bio-Rad Laboratories, Richmond, CA, USA) and incubated at room temperature for 10 min. The absorbance of the fluid was then read at 595 nm on a spectrophotometer. The protein concentration was determined by comparison with the standard curve using bovine serum albumin.

**Histopathological examination of the lungs**

Mice challenged with antibiotic-treated *P. aeruginosa* were killed by inhalation of ether at the indicated time points, and the lungs removed. The lungs were fixed in 10% formalin, embedded in paraffin wax, sectioned, and then stained with haematoxylin–eosin. The wet weight of the lungs was determined before fixation in some experiments.

**TNF-α and NO assays**

TNF-α levels in BALF were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Inc., Camarillo, CA, USA), according to the manufacturer’s instructions. NO generated in BALF was determined by spectrophotometry using a commercially available kit (Assay Designs, Inc., Ann Arbor, MI, USA).
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![Graph showing mortality rate of mice challenged with antibiotic-treated *P. aeruginosa.* Bacteria were grown for 24 h on agar containing various antibiotics at the indicated concentrations. Mice were challenged intranasally with c. 10⁷ cfu of bacteria (n = 10).](image)

**Results**

*Mortality rate of mice challenged with *P. aeruginosa* grown in the presence of sub-MICs of antibiotics*

Interestingly, among the antibiotics examined, bacteria treated with macrolide antibiotics (CLR, ERY and AZM) induced the highest mortality rates (80%, 80% and 100%, respectively) (Figure 1). For AZM-treated bacteria, the mortality rate of mice increased from 10% (when challenged with bacteria treated with 1.25 mg/L antibiotic) to 100% (when challenged with bacteria treated with 5 mg/L). In contrast, none of the mice challenged with untreated bacteria (control bacteria) or bacteria treated with other antibiotics, namely OM, JM, CLI, TOB, OFX, CAZ and MIN, died. These data indicate that the increase in lethality of antibiotic-treated bacteria may be specifically induced by some macrolide antibiotics, such as CLR, ERY and AZM.

**Changes in the number of bacteria in the lungs**

Since in the lethality experiments the first mouse death was observed 6 h after inoculation and more than half of the mice died within 9 h, the results indicated acute toxic effects rather than multiplication of bacteria in the lungs. Thus, we examined the number of bacteria in the lungs of mice challenged with control bacteria and CLR-treated bacteria (Figure 2). More than a 10-fold reduction in pulmonary bacterial burden was demonstrated 6 h after challenge of control bacteria, whereas CLR-treated bacteria were not cleared at all. These data indicate that the macrolide-treated bacteria induce certain toxic host responses, which result in death of mice within 48 h after inoculation of bacteria.

**Histopathological examination**

Twenty-four hours after the challenge with CLR-treated bacteria the lungs of mice demonstrated influx of numerous inflammatory cells, intra-alveolar exudation and haemorrhagic changes in interstitial and alveolar spaces (Figure 3). The lungs of mice challenged with control bacteria also showed accumulation of many inflammatory cells, but only a few erythrocytes were observed in extra-vascular spaces.

**Wet lung weight and total protein in BALF of mice challenged with bacteria**

To characterize acute host responses induced by macrolide-treated bacteria in the lungs, we determined the wet weight of
lungs and the total protein content in BALF to measure pulmonary permeability. In both the control and CLR-treated bacteria, lung weight began to increase 3 h after inoculation of bacteria and reached a plateau at 6 h (Figure 4a). However, that of the lungs of mice inoculated with CLR-treated bacteria was significantly higher compared with the control group. Correlated with these data, Figure 4b shows higher protein levels in BALF of mice inoculated with CLR-treated bacteria compared with control bacteria, between 3 and 9 h. Taken together, these data demonstrate that CLR-treated *P. aeruginosa* induced a drastic increase in pulmonary permeability compared with control bacteria.

**Total protein in BALF of mice challenged with several clinical isolates**

To examine whether these phenomena were specific to *P. aeruginosa PAO-1* or were common to different clinical isolates, we investigated the total protein content in BALF from mice inoculated with eight clinical isolates, which were grown in the presence or absence of sub-MICs of CLR (Figure 5). Although the extent of the increase in protein concentration differed among the strains tested, it was usually higher in the case of CLR-treated bacteria, except for one strain. These data demonstrate that CLR, at sub-MICs, may enhance the pulmonary permeability-inducing activity of a large number of clinical isolates of *P. aeruginosa*. Although we examined eight strains that produce from large to small elastase concentrations (0.6–92.75 mg/L), the total protein content in BALF from mice did not have a certain relationship to elastase production in each strain of *P. aeruginosa* after inoculation of macrolide-treated organisms.

**TNF-α and NO in BALF**

To understand better the mechanisms involved in the increase in pulmonary permeability induced by CLR-treated bacteria, we examined the levels of TNF-α and NO in BALF of mice inoculated with non-treated and CLR-treated bacteria. We observed significantly higher levels of TNF-α at 3 h in BALF.
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Figure 4. Wet lung weight and total protein in BALF of mice challenged with bacteria. Wet lung weight (a) and total protein in BALF (b) were examined 0, 3, 6 and 9 h after challenge with $10^7$ cfu of control and CLR-treated bacteria. The results are expressed as means ± S.E.M. ($n = 6$). Open circles, control bacteria; closed circles, CLR-treated bacteria. *$P < 0.05$, compared with control groups.

of mice challenged with CLR-treated bacteria, although there was no difference at subsequent time points (Figure 6a). In contrast, significantly higher levels of NO were demonstrated in BALF of those mice during the whole experimental period (Figure 6b).

Elastase activity in BALF of mice

The data clearly showed significantly higher levels of elastase in BALF of mice challenged with CLR-treated bacteria during these experimental periods (Figure 7). In mice challenged with control bacteria, no detectable levels of elastase were demonstrated at 3 h, whereas >20 mg/L elastase was demonstrated in BALF from mice challenged with CLR-treated bacteria at this time point. Moreover, c. three-fold higher amounts of elastase were detected at 6 h in this group compared with mice challenged with control bacteria. Interestingly, the kinetic changes of elastase in the control and CLR-treated groups were quite similar to those in protein levels of BALF (Figure 4b). Taken together, these data indicate overproduction of elastase in mice lungs, which may be associated with elevation of pulmonary permeability in mice challenged with macrolide-treated bacteria.

Discussion

At the beginning of these experiments, we expected that virulence of macrolide-treated bacteria in this model would be attenuated, because data from other studies have demonstrated that macrolide antibiotics suppress the expression of substances that contribute to the virulence of *P. aeruginosa*, such as exoenzymes, exopolysaccharide and pigment. In *vitro* inhibition of *P. aeruginosa* exoenzymes by macrolides is a quite consistent observation, although the degrees of suppression depend on experimental conditions, such as antibiotic concentration, incubation time and strains used. Sakata *et al.* observed >50% reduction of *P. aeruginosa* elastase in 14 of 34 clinical isolates in the presence of 4 mg/L ERY. Clinically, some macrolide antibiotics, such as ERY, CLR and AZM, have been reported to improve the clinical symptoms and prognosis of patients with chronic *P. aeruginosa* infections. Our findings, however, demonstrate that macrolide-treated bacteria are more virulent and induce strong host responses in the lungs. Importantly, more than half of the mice died within 9 h after challenge with bacteria, suggesting involvement of acute toxic effects rather than multiplication of bacteria. In fact, no increase in bacterial burden was observed in the lungs of mice challenged with CLR-treated bacteria.

In addition, we observed higher levels of NO between 3 and 9 h and TNF-α at 3 h in BALF of mice inoculated with...
CLR-treated bacteria. Excessive production of these factors seems to be related directly to toxic reactions, such as hypotension and shock.\(^{26,27}\) Production of NO is up-regulated by a variety of stimuli, including pro-inflammatory cytokines (TNF-\(\alpha\), interleukin-1 and interferon-\(\gamma\)) and bacterial components (LPS and lipoteichoic acid).\(^{28,29}\) The larger amounts of NO and TNF-\(\alpha\) observed in this study may accelerate synergistically the changes associated with the inflammatory process, such as microvascular leakage and tissue damage in the lungs. Moreover, a large amount of elastase was detected in mice BALF. Unfortunately, we could not explain why higher amounts of elastase were induced in the lungs of mice challenged with CLR-treated bacteria. We assume that elastase is produced by mice neutrophils or \(P.\) aeruginosa.\(^{30}\) A number of authors have shown that human neutrophil elastase (HNE) is important in the pathophysiology of chronic pseudomonal lung infections.\(^{30,31}\) They have shown that both HNE and pseudomonal elastase can disrupt the respiratory epithelium\(^{30}\) and that HNE is associated with increased protein concentrations in sputum.\(^{31}\) This may, indeed, be the most likely explanation given the fact that the lung histology from mice infected with macrolide pre-treated organisms shows influx of numerous inflammatory cells. Another possibility is the production of elastase from \(P.\) aeruginosa. In the presence of sub-MICs of macrolide antibiotics, the virulence of bacteria may be suppressed, as reported previously. However, it is possible that after these bacteria are introduced into the lungs under antibiotic-free conditions, they might start to produce exoenzymes. Overproduction of elastase may be a reflection of the exoenzyme-suppressing activity of macrolides.

Increase of lethality was demonstrated only in mice challenged with CLR-, ERY- and AZM-treated bacteria, but not with bacteria exposed to the other antibiotics tested. CLR and ERY are 14-membered macrolides, and these share similar structures except for a difference of one side chain residue of the macrolide aglycone ring. In contrast, AZM is a 15-membered macrolide, which differs from ERY by the presence of a nitrogen at position 9a in the macrolide ring. Specific activities of ERY, CLR and AZM on \(P.\) aeruginosa were demonstrated under some experimental conditions. We have reported previously that incubation with ERY, CLR and AZM specifically sensitized the microorganism to serum bactericidal effects.\(^{20,21}\) Moreover, these three macrolides, at sub-MICs, were shown to suppress protein synthesis in \(P.\) aeruginosa and consequently induce loss of viability over longer incubation periods.\(^{23}\) On the other hand, we could not observe any change in lethality in mice challenged with bacteria treated with other macrolides (JM, 16-membered; OM, 14-membered) or other classes of antibiotics, including TOB, OFX, CAZ, MIN and CLI. These data indicate that the paradoxical effects on \(P.\) aeruginosa may be specific for some macrolide antibiotics. It is likely that the structures of these antibiotics, such as the substitutions on the lactone ring and/or sugar composition, in addition to their simple classification as being 14-, 15- or 16-membered, may be responsible for this activity.

![Figure 6.](image1.png) **Figure 6.** TNF-\(\alpha\) and NO in BALF of mice challenged with control and CLR-treated bacteria. TNF-\(\alpha\) (a) and NO (b) levels were examined 0, 3, 6 and 9 h after challenge with \(c.\) \(10^7\) cfu of control or CLR-treated bacteria. The results are expressed as means ± s.e.m. \((n = 6)\). Open circles, control bacteria; closed circles, CLR-treated bacteria. *\(P < 0.05\), compared with control groups.

![Figure 7.](image2.png) **Figure 7.** Production of elastase in the lungs of mice challenged with control and CLR-treated bacteria. Elastase activity in BALF of mice was examined 0, 3, 6 and 9 h after challenge with control and CLR-treated bacteria. The results are expressed as means ± s.e.m. \((n = 6)\). Open circles, control bacteria; closed circles, CLR-treated bacteria. *\(P < 0.05\), compared with control groups.
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In P. aeruginosa strains with various amounts of production of elastase (0.6–92.75 mg/L), the protein concentration in BALF of mice inoculated with CLR-treated bacteria was increased compared with that of mice inoculated with control bacteria (Figure 5). These data show that macrolide pre-treated strains other than P. aeruginosa PAO-1 also augment the host inflammatory response. When we administered macrolide antibiotics to mice after inoculation of P. aeruginosa PAO-1, as a model of treatment of pneumonia, the mortality of mice did not increase (data not shown). Our results are not consistent with current dogma, that macrolides reduce production of bacterial virulence factors. Although we speculate that the increase in virulence of macrolide pre-treated bacteria may be concerned with direct interaction between bacteria and antibiotics ex vivo, molecular analysis of the macrolide effects on elastase synthesis, such as transcription, translation and secretion processes, may be necessary to understand this phenomenon better. Moreover, how such phenomena are involved in macrolide therapy in a clinical setting, or whether it takes place in the lungs of patients, remains to be clarified in future investigations.

References


