Combination therapy for chronic Pseudomonas aeruginosa respiratory infection associated with biofilm formation

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There had been no reports of investigations into biofilms in chronic respiratory infection in vivo. Recently, we established a new murine model of chronic respiratory infection with Pseudomonas aeruginosa. In the present study, we examined the bacteriological effect of combined clarithromycin and levofloxacin against chronic respiratory infection with P. aeruginosa. Scanning electron micrograph of the surface of the catheter intubated in mouse bronchus for 7 days demonstrated in vivo formation of a biofilm containing blood cells, complex fibrous structures and bacteria. Treatment with either clarithromycin alone or levofloxacin alone had no statistical effect on the number of viable bacteria in lung. The combined use of both drugs resulted in a significant decrease in the number of viable bacteria. The present experiment demonstrates that the newly established murine model was useful to investigate the treatment of biofilm-associated chronic respiratory infection with P. aeruginosa, and combination therapy with clarithromycin and levofloxacin was effective in biofilm-associated chronic respiratory infection.

Introduction

Biofilm bacteria are a major concern for clinicians in the treatment of infectious diseases because of their resistance to a wide range of antibiotics.1 Biofilm has been found on the surface of biomaterials and tissues2 in chronic bacterial disease that is resistant to chemotherapy3 and to clearance by humoral or cellular host defence mechanisms.5 Some efforts have been made to eradicate biofilm bacteria. The combination of tobramycin and piperacillin5 and the combination of antibiotics and dextranase6 have been reported to be effective in the eradication of biofilm bacteria. The clinical usefulness of these experimentally effective strategies, however, is not yet established.

Recently, low-dose and long-term administration of macrolide antibiotics was reported to be clinically effective against diffuse panbronchiolitis, a condition that is associated with bacterial biofilms,7 although the maximum serum and sputum concentrations of macrolide antibiotics are below the MICs for major pathogens such as Haemophilus influenzae and Pseudomonas aeruginosa strains.

In order to identify the therapeutic mechanism of macrolide antibiotics, the potential effect of erythromycin on host defence systems and on the virulence of P. aeruginosa have been investigated.8–10 With regard to bacterial biofilms, Yasuda et al.11 showed the interaction between clarithromycin and biofilms formed by P. aeruginosa and also showed the effectiveness of combination therapy of clarithromycin and ofloxacin by using experimental infection subcutaneously in rats in the presence of biofilm formed by P. aeruginosa.

Study of the biofilm in respiratory tract infection has not yet been reported, because no adequate animal model has been available. Recently we established a new murine model of chronic P. aeruginosa respiratory infection which was produced by placing in the bronchus a plastic tube precoated with a biofilm-forming organism.12 Using this model, we reported previously that the effect of clarithromycin on lymphocyte infiltration in chronic P. aeruginosa respiratory infection is probably a consequence of its anti-inflammatory properties rather than antimicrobial activity.12

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In this study, we evaluated the bacteriological efficacy of the combination of clarithromycin and levofloxacin on the bacterial biofilm persisting in chronic respiratory *P. aeruginosa* infection.

**Materials and methods**

**Laboratory animals**

Male, ddY, 7-week-old, 30–35 g bodyweight, specific-pathogen-free mice were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at our institution.

**Bacterial strain**

Animals were infected with mucoid *P. aeruginosa* NUS10, a clinical isolate from the sputum of patients at Nagasaki University Hospital. The bacteria were stored at −70°C in brain–heart infusion broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% (v/v) glycerol and 5% (w/v) skimmed milk (Yukijirushi Co., Tokyo, Japan) until use.

**Intubation tube**

Disposable sterile plastic cut-down intravenous catheters (3 Fr, 1.0 mm diameter, Atom Co., Tokyo, Japan) were used for intubation. The tube was 3.0 mm long and four slits were made at the proximal end to prevent clogging by oral secretions.

**Coating tubes with bacteria**

*P. aeruginosa* was cultured on Trypticase Soy agar (BBL Microbiology System) for 24 h. The bacteria were suspended in saline, harvested by centrifugation (3000g, 4°C, 10 min), resuspended in sterile saline and adjusted to 1 × 10^8 cfu/mL as estimated by turbidimetry. The intubation tube was then immersed in the bacterial suspension for 3 days at 37°C. The number of bacteria on tubes after 3 days' incubation, before intubation, was 6.19 ± 0.35 (log 10 cfu/mL, mean ± s.d., n = 10). Following immersion, the tubes were subjected to scanning electron microscopy. The specimens were fixed for 2 h at 4°C with 1% glutaraldehyde in 0.1 M PBS. This was followed by re-fixation for 2 h at 4°C in 1% osmic acid in the same buffer, dehydration in a series of aqueous ethanol solutions (50–100%) and freeze drying. Samples were then coated with platinum–palladium using an ion sputter and observed with a JSC-35C scanning electron microscope (Nihon Dennshi Kogyo, Tokyo, Japan).

**Experimental model of infection**

The methods used have been described previously in detail. Infection was produced by placement of a plastic tube precoated with *P. aeruginosa*. The intubation procedure was performed under pentobarbital anaesthesia. Briefly, the blunted end of the inner needle of an intravenous catheter (Angiocath; Becton Dickinson Vascular Access, Sandy, UT, USA) was inserted through the mouth with the outer sheath and the attached tube at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was pulled out, then the outer sheath was gently pushed to place the precoated tube into the main bronchus. No animals died and the infection was restricted to the lungs. The animals were killed by cervical dislocation after the treatment. The tube was removed, and the lungs were excised under aseptic conditions. For bacteriological analysis, both lungs were homogenized and cultured quantitatively by serial dilution on Trypticase Soy agar.

**MIC determinations**

Clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan) and levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan) were dissolved in sterile water immediately before use. The MIC of each agent was determined by the agar dilution technique using Muller–Hinton agar (Becton Dickinson Microbiology Systems), with an inoculum of 10^3 cfu per spot. The MIC was defined as the lowest concentration of agent that inhibited visible growth of *P. aeruginosa* after 18 h of incubation at 37°C. The MICs of clarithromycin and levofloxacin were 250 and 0.78 mg/L, respectively.

**Drug administration**

Treatment commenced 7 days after intubation with oral administration of the antimicrobial agents. This time was chosen because it had been shown previously that the total number of lymphocytes in the lung increased significantly on day 7 after inoculation compared with the control period. Twenty animals were allocated into four treatment groups: clarithromycin (10 mg/kg/day), levofloxacin (10 mg/kg/day), clarithromycin plus levofloxacin, or saline for control. The dose of clarithromycin was as described in the previous report, where clarithromycin (10 mg/kg/day) was shown to have an anti-inflammatory effect. The selected dose for levofloxacin was equivalent to the therapeutic dose producing effective serum concentrations in humans. Each drug was administered once a day for 10 days.
Combination therapy against *Pseudomonas* biofilms

**Statistical analysis**

Data are expressed as mean ± S.E.M. The unpaired Student’s t-test was used to analyse the data. A P value of <0.05 was considered statistically significant.

**Results**

**Scanning electron microscopy of the precoated tube and in vivo intubated tube**

Panel (a) of the Figure shows a scanning electron micrograph of the surface of the intubated tube following incubation in a *P. aeruginosa* saline suspension at 37°C for 3 days. Dense colonization of bacteria and a thick membranous structure covering the colonies were observed. Scanning electron microscopy of the surface of the catheter intubated for 7 days in mouse bronchus demonstrated *in vivo* formation of a biofilm containing blood cells, complex fibrous structures and bacteria (Figure, panel b).

**Therapeutic effect of clarithromycin plus levofloxacin against chronic respiratory *P. aeruginosa* infection in mice**

Significant numbers of viable bacteria were found in the lungs of control animals (5.50 ± 0.46 log10 cfu/lung, mean ± S.E.M., n = 5).

Treatment with clarithromycin alone or levofloxacin alone had no statistically significant effect on the number of viable bacteria in the lungs (5.18 ± 0.37 and 4.67 ± 0.28 log10 cfu/lung, respectively). Use of the two drugs together resulted in a significant decrease in the number of viable bacteria compared with the other three groups (3.20 ± 0.62 log10 cfu/lung; P < 0.05) (Table).

![Figure](image_url)

**Table.** The number of viable bacteria in lungs after treatment with clarithromycin and/or levofloxacin in intubated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log10 cfu/lung of <em>P. aeruginosa</em></th>
<th>S10 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>5.50 ± 0.46</td>
<td></td>
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<tr>
<td>Clarithromycin</td>
<td>5.18 ± 0.37</td>
<td></td>
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<tr>
<td>Levofloxacin</td>
<td>4.66 ± 0.28</td>
<td></td>
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<tr>
<td>Clarithromycin + levofloxacin</td>
<td>3.20 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

*Values expressed as means ± S.E.M.

<sup>b</sup>P < 0.05 compared with the other three groups.

**Discussion**

Recently, bacterial biofilms have been detected on a number of living and inert surfaces within the human body. Establishment of a biofilm is a prelude to the development of various chronic, refractory infections, such as bio-material-associated infection and pulmonary infection in intubated patients and patients with cystic fibrosis or diffuse panbronchiolitis.<sup>14,15</sup> In chronic lower respiratory infection caused by *P. aeruginosa*, the bacteria in micro-colonies are embedded in a biofilm. *P. aeruginosa* is a major pathogen in biofilm-associated infections, and eradication with antibiotics of organisms in a biofilm is difficult to achieve. Therefore, there is a clinical demand to clarify the mechanism of biofilms in chronic *P. aeruginosa* respiratory infection.

From our experimental results, we demonstrated that the combined use of clarithromycin and levofloxacin resulted in an enhanced therapeutic efficacy of levofloxacin in biofilm-associated chronic respiratory *P. aeruginosa* infection.
infection. Taking into consideration that clarithromycin has no antibacterial activity against *P. aeruginosa*, the synergic therapeutic effect of clarithromycin and levofloxacin in this animal model of chronic respiratory infection is very interesting. The synergy of clarithromycin and levofloxacin may originate from the activity of clarithromycin in removing the polysaccharide glycocalyx in or on bacterial biofilms. It is not certain whether the ability of clarithromycin to remove the glycocalyx is independent of its antibacterial activity.

In this study, we demonstrate that the combination of clarithromycin and levofloxacin is effective in treating biofilm-associated chronic respiratory infection in an animal model. This may be a new strategy for the treatment of such infections in humans.

References


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