Bacterial Biofilm in Chronic Airway Infection

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Summary

We hypothesized that bacterial biofilm formation could be an important factor that makes some infections intractable, and conducted the following study to confirm the role of bacterial biofilm in airway infection.

We first microscopically examined airway surface in patients with an intractable airway infection and detected bacterial biofilms adhering to the airway surface. Most of the airway biofilm diseases were diffuse panbronchiolitis and bronchiectasia due to *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*.

*In vivo* examination revealed that chemotaxis of neutrophils in patients with biofilm bacteria was less than that in patients with floating type bacteria. Interaction of *Pseudomonas* biofilm with antibacterial agents was examined *in vitro*. The rate of survival of biofilm bacteria was higher than that of floating bacteria in contact with twice the minimal bactericidal concentration of ciprofloxacin (CPFX) or two to 10 times the minimal inhibitory concentration of cefclidin and meropenem which are highly potent antibacterial agents against *P. aeruginosa*.

Additionally, the effects of clarithromycin (CAM) on biofilm bacteria were studied in order to investigate new therapeutic maneuvers against a bacterial biofilm. Also, the combination of CPFX and CAM was more effective in decreasing the bacterial survival rate than CPFX alone. The results suggest that administration CAM can be one of the therapeutic maneuvers against biofilm bacteria.

Introduction

When bacteria are placed in a natural environment undesirable for their own growth, they produce a glycocalyx consisting primarily of polysaccharides on their surface. Subsequently, the bacteria aggregated by means of the glycocalyx form a bacterial biofilm on the surface of the substance to which they are attached. The phenomenon in natural systems was first described by Costerton et al.1) Peters et al.2) clinically observed bacterial colonies covered by slimy material in catheters inserted into blood vessels. Nickel et al.3) observed bacterial colonization in a urethral catheter (Foley catheter) and a penile prosthesis4). In addition Mills et al.5) reported that the vegetation nuclei in bacterial endocarditis were bacterial biofilms. Kobayashi6) detected a *Pseudomonas* biofilm on the airway surface of patients with chronic intractable airway infection. These reports suggest that bacterial biofilms are formed not only on foreign substances, but also on living tissues under appropriate conditions.

We have been finding that biofilm-associated diseases often make an infection intractable. Chronic airway infection caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) may be given as one of the notable
examples. These patients cannot be completely cured when treated with drugs to which the bacteria were highly sensitive in previous *in vitro* tests. Only 20-30\% of the bacteria are eradicated by such effective drugs.\footnote{2}

On the basis of these phenomena, this study was performed to explain the discrepancy between the high activity of the antibacterial agents *in vitro* and the low eradication in clinical patients, based on the possible association of bacterial biofilm with chronic airway infection with *P. aeruginosa*.

**Materials and Methods**

1. **Electron Microscopic Observation**
   1) **Observation of Clinical Specimens**

   Tissue specimens were fixed after immersion in 0.1 M phosphate-buffered solution containing 2.5\% glutaraldehyde for 24 hours. After the glutaraldehyde was rinsed off, the specimens were immersed in 1\% osmic acid fixative for one hour, and doubly fixed. After the osmic acid was rinsed off the specimens were

![Fig. 2 Scanning electron micrograph of *P. aeruginosa* on the teflon surface. (×5000)](image)

After overnight incubation, a many *P. aeruginosa* organisms adhered to the teflon surface. The surface of these adhering bacteria was as smooth as the bacteria that were just cultured in broth.

![Fig. 3 Scanning electron micrograph of *P. aeruginosa* after 6 days of incubation. (×20,000)](image)

The bacteria had produced a glycocalyx around their bodies and were aggregated with each other through the glycocalyx, forming a biofilm on the teflon surface.
immersed in 30% dimethyl sulfoxide (DMSO) and in 50% DMSO sequentially, and then frozen sections were prepared in 50% DMSO. Samples were thoroughly washed in 30% DMSO, and immersed sequentially in 50, 70, 90 and 100% acetone. They were transferred to isoamyl acetate solution and dried in liquid CO2 by the critical point drying technique, and were exposed to platinum vanadium. They were observed with a scanning electron microscope (Hitachi Ltd.).

2) Bacterial Observation

Cultured bacteria were fixed in 2.5% glutaraldehyde solution and centrifuged. The bacteria were collected and then fixed twice in 0.1 M phosphate buffered solution containing 1% osmic acid, washed in 0.1 M phosphate buffered solution, dehydrated sequentially in 50, 70, 80, 90 and 100% ethanol, and then resuspended in butyric alcohol. After they were lyophilized, platinum vanadium was evaporated onto the samples, which were then observed under the scanning electron microscope.

2. In vitro Preparation of Bacterial Biofilm

*P. aeruginosa* isolated from a patient with bronchiectasis was used. The procedure for bacterial biofilm formation is shown in Fig. 1. Sample bacteria cultured in tryptic soy broth (Eiken) at 37°C overnight were washed twice in physiological saline solution by centrifugation, and were resuspended in fresh physiological saline solution. Bacterial concentration was measured with a spectrophotometer (wavelength: 560 nm). A bacterial suspension was prepared to give a bacterial concentration of 10⁷ CFU/ml (OD=0.029). This bacterial suspension was diluted 10-fold in physiological saline solution with a piece of teflon (10 × 10 mm), and then incubated at 37°C for 4 to 6 days. Electron microscopic findings of bacteria which adhered to the teflon piece after incubation for 24 hours and 6 days are shown (Figs. 2 and 3).

The effects of human plasma on formation of a bacterial biofilm on a teflon piece were tested in the following way. After the teflon was immersed in a bacterial suspension in physiological saline solution, 0.5% human plasma was added on day 5 of incubation, and the incubation was continued at 37°C for another 24 hours. The bacterial biofilm formed in the presence of human plasma was compared with a biofilm formed in the absence of human plasma by observation under the scanning electron microscope.

3. In vivo Studies

To elucidate the clinical role of biofilm bacteria, some experiments were performed by using a mouse model. When the biofilm bacteria or floating bacteria were inoculated into the mouse lung through the trachea, the number of cells in broncho alveolar fluid (BALF), survival of mice, and the number of viable bacteria in the lung were counted. The details are given in “Results”.

4. Changes in Drug Sensitivity of Biofilm Bacteria

Ciprofloxacin (CPFX), tosufloxacin, cefclidin, meropenem, and clarithromycin (CAM) were used. The minimal inhibitory concentrations (MIC) was determined in accordance with the standard method established by the Japanese Chemotherapy Association. The minimal bactericidal concentrations (MBC) was the drug concentrations which had killed the inoculated bacteria 24 hours after the addition of drugs.

Biofilm bacteria were produced by the following method. After the teflon piece was incubated in a bacterial suspension at 37°C for 6 days, it was washed gently in physiological saline solution. It was then stirred at high speed with a vortex mixer for precisely 2 minutes in 2 ml of physiological saline solution. The bacterial suspension obtained was observed with an electron microscope. The bacteria produced a viscous substance, a glycocalyx, and several bacteria formed an aggregated mass of almost the same size (Fig. 4).

While this aggregated bacterial suspension was treated as the biofilm bacteria, the non-aggregated bacteria suspended in saline solution were treated as the floating bacteria. The antibacterial agents were added to the biofilm bacteria and floating bacteria at the final concentrations of 2 MIC or 2 MBC, and the suspensions were incubated at 37°C. A portion of bacterial suspension was collected 1, 2 and 4 hours after
the addition of the antibacterial agent. Ten fold serial dilutions were then prepared, and 0.1 ml of each suspensions was streaked on ordinary medium. After incubation at 37°C for 24 hours, colonies on agar plate were counted. The number of colony-forming units (CFU) were determined at each time point and compared with the number before adding the antibacterial agents. The survival rate was defined as the ratio of CFU to that before adding the antibacterial agent.

5. Statistical analysis

Differences among groups were compared by analysis of variance (Wilcoxon method), to assess statistical significance at p<0.05.

Results

1. Bacterial Biofilm in a Clinical Case

1) Microscopic findings of bacterial biofilm on airway surface

The patient had bronchiectasis with chronic *P. aeruginosa* infection. His chest X-ray is shown (Fig. 5). He had purulent sputum containing *P. aeruginosa* for more than 10 years, and he had been treated with various kinds of effective anti-*Pseudomonas* agents by all conceivable methods including pertracheal administration. However his clinical condition became gradually worse. At one time during the course of his disease, the tissue sample from his bronchus was scratched under bronchofiberscopy, and the bacterial condition on his airway surface was observed. Many aggregated *P. aeruginosa* were seen in many of the polysaccharides (glycocalyx), that is biofilm formation was observed. These bacteria adhered to the airway surface by means of the glycocalyx, and many neutrophils were observed outside the bacterial biofilm, but there was no neutrophil infiltration into the biofilm layer (Fig. 6). This phenomenon suggests that neutrophils are not able to interact with the biofilm and the bacterial biofilm is protected from phagocytosis by leukocytes. An electron micrograph of a single strain of *P. aeruginosa* on the bronchial surface in this case is shown (Fig. 7). The bacterial body was covered by a glycocalyx, and each strain aggregated with adjusted bacteria through glycocalyx. They also adhered to the airway surface through
Fig. 6 Photomicrograph of bronchial tissue of the patient shown in Fig. 5. (PAS stain, ×400)
Many pieces of biofilm containing *P. aeruginosa* (B) were seen along the bronchial epithelium (E). Outside the biofilm layer, many polymorphonuclear leukocytes are seen (P), but most of the PMNs did not enter into the biofilm layer.

Fig. 7 Scanning electron micrograph of a strain of *P. aeruginosa* on the surface of airway tissue. (×60,000)
The surface of the bacteria is rough with glyocalyx production and each strain adhered to adjusted strains through glyocalyx.

### Table 1 Biofilm Formation in Respiratory Diseases

<table>
<thead>
<tr>
<th></th>
<th>Chronic Bronchitis</th>
<th>Bronchiectasis</th>
<th>Diffuse Panbronchiolitis</th>
<th>Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>4</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>17</td>
<td>2</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

The glyocalyx. Thus, the bacterial biofilm was observed on the airway surface.

A bacterial biofilm was observed in 22 of 30 patients with chronic airway infection, whose pathogenic bacteria had persisted for over 3 months, even if they had received continuous treatment with antibacterial agents to which the bacteria were sensitive (Table 1). In detail, 8 patients had diffuse panbronchiolitis, 10 had bronchiectasis, 3 had chronic bronchitis and 1 had aspiration pneumonia with airway disturbance. In these cases, *P. aeruginosa* and *Klebsiella pneumoniae* were frequently isolated and *Staphylococcus aureus* was in one case.

2) **Clinical Findings of Airway Biofilm Disease**

The records of clinical findings and symptoms of these 22 patients with bacterial biofilm for last 2 years were reviewed. From 3 to 27 times of infectious exacerbations expressed by an increase in purulent sputum, productive cough and an increased wheezing with shortness of breath were observed in all cases.

The C-reactive protein (CRP) value and WBC count when the disease was the most exacerbated were plotted (Fig. 8). Neither CRP nor WBC in most cases were very markedly increased when the exacerbation was caused by the same strain that had persisted in the sputum, except in 2 patients with superinfection with *Haemophilus influenzae*. This findings suggests that the inflammatory response in the biofilm...
patients with exacerbation by the same strain as the persisting one is weaker. Some experimental studies on this problem were performed.

2. In vivo Experimental Study of P. aeruginosa Biofilm

1) Change in Cell Count in BALF

To observe the interaction between biofilm bacteria and leukocytes, the changes in cell counts in BALF when the biofilm bacteria or the floating bacteria were inoculated into mouse lungs through the trachea were examined. Forty-five 6-week-old ICR mice were used. Each group consisted of 5 mice. Either $5 \times 10^6$ CFU of floating bacteria or the same number of biofilm bacteria were inoculated into the lung and the changes in BALF cells with time were determined. The total cell count in BALF was $1.54 \times 10^5$/ml in normal mice, and most of the cells were alveolar macrophages. The total cell count in the group given floating bacteria increased to $56.1 \times 10^5$/ml at maximum 48 hours after the inoculation. Most of the cells were neutrophils. However, the count in the group given biofilm bacteria was only $4.9 \times 10^5$/ml at the same time (Table 2). There was a statistically significant difference between the two groups (p<0.01). Moreover, no leukocyte infiltration to the bacterial biofilm was found, only erythrocyte attachment to the surrounding biofilm piece was observed (Fig. 9). These findings suggest that the interaction between
Table 2  Changes in total cell count in BALF

<table>
<thead>
<tr>
<th>Time after Infection</th>
<th>0</th>
<th>6h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating Bacteria</td>
<td>1.54</td>
<td>2.1</td>
<td>37.3</td>
<td>56.1</td>
<td>12.5×10⁹/ml</td>
</tr>
<tr>
<td>Biofilm Bacteria</td>
<td>2.4</td>
<td>9.7*</td>
<td>4.9*</td>
<td>5.2*</td>
<td>5.2×10⁹/ml</td>
</tr>
</tbody>
</table>

*p<0.01

Fig. 11  Change in viable bacteria in mouse lung.

bacterial biofilm and leukocyte is weaker, and chemotaxis of leukocytes to the biofilm bacteria is also hard to find.

2) Survival Rate after Inoculation of Bacterial Biofilm

To determine the effect of biofilm bacteria on the host, the survival after inoculation of the biofilm bacteria was observed. Three types of *P. aeruginosa*, one the biofilm type in our saline-teflon system, one the floating type and the other the strain just cultured in broth were used. Three groups of twenty-six-week-old ICR mice were inoculated intratracheally with 10⁶ CFU of *P. aeruginosa*. The survival rate dropped to 0% or 5% in the groups given either floating bacteria or broth cultured bacteria 6 days after the inoculation. In contrast, it was 75% in the group given biofilm bacteria (p<0.05) (Fig. 10).

The results suggest that the virulence of biofilm bacteria is less than that of floating bacteria.

3) Changes in Viable Bacterial Count in Lung

Sixty-two six-week-old ICR mice were inoculated intratracheally with 10⁴ CFU of biofilm bacteria and the number of viable bacteria was compared with that of the floating bacteria. The bacteria in the lung tissue were almost eliminated on the 5th day after inoculation they could not be detected on days 6, 7, and 28. In the biofilm group, a small number of bacteria were still detected from lung tissue 28 days after the inoculation (Fig. 11). The results indicate that the biofilm bacteria are able to live in the lung tissue for a longer period. That may be due to weaker interaction with leukocytes, and such bacteria living in tissue may cause an infection to be repeated.

3. Interaction between Biofilm Bacteria and Antibacterial Agents

The killing effect of antibacterial agents on the biofilm bacteria and the floating bacteria were compared. When the floating bacteria were placed in contact with CPFX, the rate of survival of the bacteria decreased rapidly as a function of time in contact with CPFX, dropping to 0.04% in 4 hours. On the other
Fig. 12 Effect of CPFX on biofilm bacteria. The MBC of CPFX for P. aeruginosa was 0.4 μg.

Fig. 13 Interaction of CPFX with the biofilm bacteria to which human blood plasma was added to approximate in vivo conditions. The MBC of CPFX for P. aeruginosa was 0.4 μg.

In another experiment, human plasma was added to simulate in vivo conditions. This biofilm was placed in contact with CPFX at the concentration of 2 MBC for 4 hours. The survival rate for biofilm bacteria prepared in the presence of human plasma was 88.5% and it decreased only a little, whereas that for biofilm prepared in physiological saline solution alone was 32% (Fig. 13). The difference was statistically significant (p<0.05).

Other antibacterial agents were also tested for their effect on the bacteria. The experiment described above was used for a new cephem compound, cefclidin, which has potent in vitro anti-Pseudomonas activity. When biofilms prepared in the presence of human plasma were placed in contact with cefclidin at the concentration of 2 MIC for 4 hours, the survival rate was 98%, whereas that at 10 MIC was 87%. On the

手, the rate of survival of the biofilm bacteria decreased slowly, and was 42% after 4 hours of incubation (Fig. 12). The difference was statistically significant (p<0.05).

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Fig. 15 Effect of meropenem, a new kind of carbapenem, on the biofilm bacteria.
The MIC of meropenem for P. aeruginosa was 1.56 μg.

other hand, when the floating bacteria were placed in contact with cefclidin at the concentration of 2 MIC for 4 hours, the survival rate decreased rapidly to 1.8% (Fig. 14). The sensitivity of bacteria to the carbapenem compound meropenem was also examined. The rate of survival of the floating bacteria decreased to 0.7% after 4 hours of contact with 2 MIC of meropenem, but that of biofilms prepared with human plasma was 71% at 2 MIC, 58% at 5 MIC, and 45% at 10 MIC (Fig. 15). Thus, the rate of survival of P. aeruginosa inside the biofilm was higher than that of floating bacteria.

4. Effect of Clarithromycin on Biofilm Bacteria

The test P. aeruginosa did not exhibit sensitivity to CAM (MIC>200 μg/ml). The survival of neither the floating bacteria nor the biofilm bacteria in contact with 10 μg of CAM per ml was affected. In this experimental condition, the rate of survival of the biofilm bacteria in contact with 0.8 μg (2 MBC) of CPFX decreased to 42%. When 1.0 μg of CAM per ml was combined with CPFX, the survival rate for the biofilm bacteria decreased to 2.6%, and it was 0.1% when CPFX was combined with 10 μg of CAM per ml. The rate of survival of P. aeruginosa inside the biofilm decreased as a function of the CAM concentration used concomitantly with CPFX (Fig. 16).

Discussion

<Clinical Features>

In recent years, the prognosis of infectious disease has been considerably improved by development of new antibacterial agents. However, sometimes we have experienced intractable cases. Most of these patients obtained temporary clinical relief, but their causative pathogens persisted, even if effective agents were used, and there was often repeated exacerbation of the infection.

Typically such diseases are diffuse panbronchiolitis, and bronchiectasis due to P. aeruginosa or K. pneumoniae. We have observed a bacterial biofilm in most of these intractable cases. This suggests that bacterial biofilm plays an important role in determining the unfavorable prognosis in such patients. This
study was conducted to test this hypothesis.

Several authors reported the existence of bacterial biofilm in chronic osteomyelitis due to *Staphylococcus aureus*⁸, in endocarditis due to *Staphylococcus*, and in infections induced by medical devices.

However, the biofilm association had not been reported in airway disease until our report in 1990. We observed a bacterial biofilm on the airway surface in patients with chronic airway infection, especially in diffuse panbronchiolitis and bronchiectasia. *P. aeruginosa* was the most common biofilm pathogen. In fact, *P. aeruginosa* was eliminated in only 20 to 30% of chronic airway infections, even if new quinolones that were potent against *P. aeruginosa in vitro* were used⁹.

The clinical features of airway biofilm disease are characterized by difficulty in eradicating the pathogens in spite of concomitant treatment with antibacterial agents and repeated exacerbation of the infection. The main reasons for this problem may be the resistance of biofilm material to antibacterial agents that was shown in this study. Another reason may be the weak interaction of biofilm bacteria with the host phagocyte cells in the affected part as shown in our BALF examination.

Takeda, one of our group, recently showed less neutrophil chemotaxis to biofilm bacteria, than that to the floating bacteria by using the Boyden chamber method (unpublished). Stiver et al.⁹ reported that a mucoid exopolysaccharide produced by *P. aeruginosa* is thought to be antiphagocytic and to inhibit normal neutrophil chemotaxis in vitro. Our results in this examination indicate that the biofilm bacteria inhibit neutrophil chemotaxis in vivo.

The reasons for the weaker chemotaxis seen in in vivo experiment may be the indirect binding, a sandwich binding in other words, of biofilm bacteria to the host tissue. The biofilm bacteria, *P. aeruginosa* inside the biofilm, commonly attach to tissue through glycocalyx surrounding of the bacterial bodies. This is different from the direct binding that is usually seen in attachment of floating bacteria. In indirect binding, the stimulus for the host tissue may be less strong. Consequently, it induces less chemotaxis of neutrophils to the area of attachment.

Such weak chemotaxis of neutrophils may make a suitable environment for bacterial-living, and the biofilm bacteria may be able to live in tissue for a long time. In fact, biofilm bacteria living in lung tissue for a long time were observed in the mouse experiment in this study. They were isolated from mouse lungs for a longer period without any large infectious focus.

The survival rate after inoculation of biofilm bacteria into the mouse trachea was significantly higher than that after inoculation of the floating bacteria. The same result was obtained by intravenous inoculation into mice in our study. Less virulence of the biofilm bacteria is suggested. However, sometimes the strain released from the biofilm may be able to cause repeated infection in another part of the airway.

Morphologic switching from non-mucoid to mucoid is relevant to understanding the natural history of acquisition in patients with diffuse panbronchiolitis. Colonization of mucoid *P. aeruginosa* is commonly persists for a long time in such patients. In our clinical observation, patients with biofilm bacteria have never suddenly experienced progression in clinical features in spite of persistence of the pathogens. The features gradually progressed with repeated infectious exacerbations. Addition of such clinical experience to the results of survival studies observed in experimental mouse infection supports the concept of lower virulence of biofilm bacteria.

The role of virulence factors, such as exotoxin A and phospholipase C, in mucoid *P. aeruginosa* is still obscure. Baltimore et al.¹⁰ reported that the absence of bacteria in the lung parenchyma in cystic fibrosis and in animals is consistent with the concept that tissue damage may be due to elaborated toxins diffuse some distance airway. Hollsing et al.¹¹ reported that 100% of cystic fibrosis patients chronically colonized with *P. aeruginosa* had elevated antibody titers to phospholipase C. Haiby et al.¹² pointed out the role of immune complexes in tissue injury in cystic fibrosis patients. The relationship of exopolysaccharide
production by mucoid *P. aeruginosa* and the pathogenesis of lung infection remains of high priority.

Regarding the repeated infections which are often observed in patients with airway biofilm disease, we have not obtained any experimental data to explain this phenomenon. However, such an infectious exacerbation may be caused by floating type bacteria released from the biofilm. This hypothesis may be supported by the microscopic findings in the sputum of a patient with biofilm (Fig. 17). Many floating bacteria were found surrounding the piece of biofilm at an infectious exacerbation. According to our recent work (in press), the floating type bacteria are usually released from the biofilm. New infectious lesions in other parts of the airway surface may be caused by such floating bacteria, because floating type bacteria are more virulent, and attach to tissue directly.

**<Interaction with Antibacterial Agents>**

The interaction between biofilm bacteria and antibacterial agents has not been definitely reported, except for Fujimaki’s study\(^\text{13}\) that was performed with us. Therefore, first of all, we had to make biofilm bacteria with *P. aeruginosa in vivo* experimentally.

When *P. aeruginosa* that was cultured in tryptic soy broth was suspended in physiological saline solution in a test tube and a piece of teflon as foreign substance was put into this test tube, after incubation for 6 days, the surface of the bacteria adhering to the teflon surface was becoming rough by producing a glycocalyx. But the surface of bacteria floating in saline solution was smooth without any glycocalyx production. The reason why teflon was used in this study is simple. Teflon has often been used in medical devices or as a teflon coating, because it is the substance most resistant to bacterial attachment, so we dared to use it. From the results of our preliminary examinations, of course, we can use other material, such as vinyl, bone, tissue or dental prosthesis instead. In our saline-teflon system, the bacteria began to produce a glycocalyx after 3 days incubation and to aggregate with each other through the glycocalyx, and the biofilm on the teflon was completely formed after 6 days of incubation. The important point in this process is that biofilm formation is observed only with bacteria adhering to a foreign substance, and bacteria floating in saline solution are the smooth type without any glycocalyx production. Kobayashi\(^\text{14}\) showed that the bacteria begin to form biofilm after bacterial attachment to foreign material and it is a natural phenomenon for them to protect themselves from antibacterial substances such as antibiotics, neutrophils and natural stimuli.

The authors studied in detail the relationship between bacterial biofilm and antibacterial agents with this experimental system. There have been only a few observations in relation with biofilm and antibiotics. Marrie et al.\(^\text{15}\) reported a relationship between bacterial biofilm and antibacterial agents in clinical cases. They studied a patient with a pacemaker who had sepsis caused by *S. aureus*. The biofilm of *S. aureus* on the tip of the pacemaker could not be completely eliminated in spite of continuous therapy for 6 weeks.
Olson placed a catheter in urethra of a rabbit and infected it with *Escherichia coli*. Bacteria floating in the urine and those adhering to the bladder wall were both eliminated by amdinocillin at doses of 50 to 200 mg/kg, but a higher dose (400 mg/kg) of amdinocillin was needed to eliminate the biofilm bacteria formed on the surface of the catheter. Additionally, in *in vivo* experiments performed by Evans and Holmes, the MBC for pure cultured bacteria was 6.25 µg/ml for vancomycin, but the MBC for biofilm bacteria was above 400 µg/ml. However there is no definite demonstration of interaction between antibacterial agents and biofilm bacteria. Therefore an *in vitro* experiment on this problem was performed in this study. Our *in vitro* study demonstrated that *P. aeruginosa* organisms inside the biofilm was resistant to 2 MIC or 2 MBC of CPFX or tosufloxacin as well as to meropenem and cefclidin which have high *in vitro* antibacterial activity against *P. aeruginosa*.

The *in vivo* bacterial biofilm is able to be more resistant, because there are usually attached to it serum proteins, fibrin, thrombocytes and erythrocytes that contain human blood. Therefore a bacterial biofilm was prepared in human plasma. It showed more resistance to antibacterial agents than the bacterial biofilm prepared in physiological saline solution alone. Toumanen et al. reported that the bacterial growth rate decreased when E. coli was exposed to drugs to which they were weakly sensitive. Nichols et al. reported that diffusion of tobramycin into the bacterial cell was inhibited when tobramycin was bound to *Pseudomonas* exopolysaccharide. The most intractable factor seen in biofilm bacteria is the reduced ability of antibacterial agents to permeate the bacteria inside the biofilm.

Dull et al. found, in their *in vitro* study, that combined use of antibacterial agents and a digestive enzyme for polysaccharides, "dextranase", was more effective than antibacterial agents alone. Bayer et al. reported that *P. aeruginosa* was easily eliminated by antibacterial agents and the phagocytic activity of neutrophils, when the organisms were pretreated with alginate which cleaves alginate. Verginia et al. also reported that glycocalyx production by bacteria was inhibited when *P. aeruginosa* was treated with clindamycin and consequently such bacteria were easily eliminated by the phagocytic activity of neutrophils. Takeda et al. demonstrated that glycocalyx production by bacteria was inhibited when *P. aeruginosa* was exposed to a low dose of CAM and the adhesiveness of *P. aeruginosa* to tracheal cells was also inhibited in mice. Our *in vitro* study on the effect of CAM on a *Pseudomonas* biofilm demonstrated that the combined use of CPFX and CAM was more effective against biofilm bacteria than CPFX alone. One possible interpretation of this phenomenon is that CAM enables the bacterial biofilm to allow CPFX to penetrate through biofilm and eliminate bacteria inside it. Thus, the combined use of CAM and other antibacterial agents can be a powerful clinical tool against the bacterial biofilm. Kobayashi also found the combination of azithromycin and CPFX to be very effective against *P. aeruginosa* inside the biofilm in an *in vitro* study. The combination of CPFX and erythromycin, but not CPFX and josamycin or other 16-member macrolides had a similar effect. However, the reason for this phenomenon is currently obscure.

Further investigation of new treatment for infectious diseases associated with a bacterial biofilm is needed, including the use of macrolide compounds such as CAM or azithromycin for the treatment of intractable chronic respiratory infections.

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References


慢性気管感染症における細菌バイオフィルムの臨床的意義

吉卜大学第1内科
大垣 憲 隆

（平成5年10月12日受付）
（平成5年11月9日受理）

要 旨

細菌のバイオフィルム形成が感染症難治化要因の1つと考え、気道感染症における細菌バイオフィルムの臨床的意義を明らかにするため以下の実験を行った。

まず、難治性気道感染症患者の気道表面を顕微鏡的に観察し、気道表面に着いている細菌バイオフィルムを認めた。これら気道biofilm diseaseの多くは細菌芽胞あるいは肺管菌感染性びまん性汎気管支炎と気管支拡張症であった。次にマウス経気道的細菌接種実験では、バイオフィルム菌の場合、気管支肺胞洗浄液中の細胞数は少なく、好中球遊走はfloating菌の場合に比し弱かった。

また、in vitro実験で細菌バイオフィルムと抗菌剤の相互作用を観察した。細菌バイオフィルムを2MBC濃度のciprofloxacin（CPFX）あるいは2MIC-10MIC濃度のcefclidin、meropenemと接觸させた場合、バイオフィルム菌の生存率はfloating菌のそれに比し有意に高く、抗菌剤抵抗性であった。これら細菌バイオフィルムの対策について、clarithromycin（CAM）の細菌バイオフィルムに対する効果を観察した。CPFXにCAMを併用した場合、バイオフィルム菌の生存率はCPFX単独の場合に比し、急激に減少した。すなわち、CAMはバイオフィルム菌の対策の1つの方法と考えられた。

平成6年1月20日