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Effect of Adherence on Antimicrobial Susceptibility of *Pseudomonas aeruginosa, Serratia marcescens,* and *Proteus mirabilis*

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ABSTRACT

A simple method was used for testing the antibiotic susceptibility of adherent bacteria to plastic surfaces. *Pseudomonas aeruginosa, Serratia marcescens,* and *Proteus mirabilis* cells adhering to the bottom of a plastic tissue culture plate were incubated in serially diluted antibiotic solutions. After 24-h incubation the solutions were removed and a fresh medium without antibiotics was addded to each well. The viability of the cells was judged by their growth after a further 24-h incubation. In our assay system, we employed a short incubation time (1-h) involving adherence of bacteria to a surface for the purpose of minimizing the effect of the glycocalyx on antibiotic activity. Even if the bacteria did not form a biofilm, the minimal bactericidal concentrations for adherent bacteria (MBC^{AD}s) markedly elevated. The MBC^{AD}s of ofloxacin well correlated with the bacteriological eradication by ofloxacin treatment for urinary tract infections (UTIs) associated with indwelling urinary catheters, whereas the minimal inhibitory concentrations did not show a correlation. Kinetic studies showed that adherent *Pseudomonas aeruginosa* had a 2h-lag time before logarithmically growing when these bacteria were incubated in Mueller-Hinton broth without antibiotics. The tolerance demonstrated by adherent cells is likely to play a role in the difficulties encountered in the antimicrobial chemotherapy of biofilm infections. Moreover, our assay system was cosidered to be useful in the therapeutic selection of antibiotics for these infections.

Key words: *Bacterial adherence, Biofilm, Antibiotics, Bactericidal activity*

Indwelling prosthetic devices have become a common and indispensable part of modern medical care. Infection is the most frequent complication associated with these devices. Such infections are notoriously resistant to treatment, even with high doses of antibiotic agents, and tend to persist until the prosthetic devices are removed.

The devices are frequently colonized by bacteria which form biofilm[11,14]. Biofilm bacteria are known to be highly resistant to killing action by antibiotic agents[5]. The mechanisms involved in the protection of these bacteria from the killing effects of antibiotic agents have not been well characterized. Possible reasons are a glycocalyx barrier effect and a low growth rate[8].

The selection of therapeutic antimicrobial agents is usually based on the standard suspension culture minimal inhibitory concentration (MIC) test. However, the results of these standard tests do not always correlate with the bacteriological eradication to antimicrobial chemotherapy for the infections associated with indwelling prosthetic devices. These *in vitro* tests do not take into account the factors that are likely to affect the physiological functions of adherent bacteria *in vivo*. We used a simple method for evaluation of the effect of adherence on antibiotic susceptibility for adherent bacteria in the infections associated with indwelling prosthetic devices.

MATERIALS AND METHODS

1) Bacterial strains

A total 50 *Pseudomonas aeruginosa*, 50 *Serratia marcescens*, and 50 *Proteus mirabilis* clinical isolates were studied. These bacteria were isolated from patients with a catheter-associated UTI at the Hiroshima University Hospital from 1987 to 1992. A single strain was obtained from each patient. Medical records were reviewed to determine whether compromising host conditions (including urinary tract abnormalities, urinary tract instrumentation, and serious medical illnesses) were present at the time of the bacteremic episode.

2) Antimicrobial agents

The following antibiotic agents were used in this study : piperacillin (PIPC ; Toyama Chemical Co.,

Ltd., Tokyo, Japan), cefsulodin (CFS ; Takeda Pharmaceutical, Co., Ltd., Tokyo, Japan), gentamicin (GM ; Essex Japan, Co., Ltd., Osaka, Japan), amikacin (AMK ; Meiji Seika, Co., Ltd., Tokyo, Japan), ofloxacin (OFLX ; Daiichi Seiyaku, Co., Ltd., Tokyo, Japan), and tosufloxacin (TFLX ; Toyama Chemical Co., Ltd., Tokyo, Japan).

3) Suscptibility testing for adherent bacteria

We used the method of Miyake et al[13] with minor modifications. The overnight culture in Mueller-Hinton broth (MHB) was diluted with artificial urine[12] to 10^6cells/ml. A multiwell tissue culture plate (96-well, FALCON 3072, Becton Dickinson Labware, Lincoln Park, N.J.), each well of which received 100 µl (10^5cells/well) of the bacterial suspension, was centrifuged at 450×*g* for 10 min at 20°C. After incubation at 37°C for 1h, artificial urine was removed by aspiration, and 100 µl of serially diluted antibiotic solution was transferred to the wells from another plate in which the solution was prepared. Bacterial growth was assessed after 24h-incubation at 37°C. The minimal inhibitory concentration for adherent bacteria (MIC^{AD}) was determinated by visual turbidity and defined as the lowest concentration of antibiotic agents which inhibited growth completely. The antibiotic solution was then removed, and 200 µl of fresh broth without antibiotics was added to each well, followed by a further 24h-incubation at 37°C. The minimal bactericidal concentration for adherent bacreria (MBC^{AD}) was defined as the lowest concentration of antibiotics at which there was no bacterial growth.

4) Susceptibility testing for planktonic bacteria

All strains were tested by determination of MIC by a microdilution method. After preparation with artificial urine for 1h, bacterial suspension was diluted with MHB (10^6CFU/ml ; 100 µl/well). The MIC was defined as the lowest concentration of antibiotics which inhibited growth completely. The minimal bactericidal concentration (MBC) was defined at 99.9% killing of inoculum and determinated by spreading 10 µl of culture from each well onto a Mueller-Hinton agar plate for viable counts.

5) Killing and growth kinetic studies for adherent bacreria

The killing kinetics of adherent bacteria were studied by growth and time-kill curves. The overnight culture of *P.aeruginosa* IFO 12689 in MHB was diluted with artificial urine to 10^6cells/ml. A multiwell tissue culture plate, each well of which received 100 µl (10^5cells/well) of the bacterial suspension, was centrifused at 450×*g* for 10 min at 20°C. After incubation at 37°C for 1h, the artificial urine was removed, and 100 µl of fresh broth with GM was added to each well. *P.aeruginosa* cells were exposed to GM ranging from 0.125 to 2 µg/ml. Each well was treated with bovine trypsin for 20 min, and sonicated for 1 min at a low output to recover the adherent cells. Samples were obtained for colony counts at 0, 2, 4, 6, 10, 12, and 24h of incubation.

6) Killing and growth kinetic studies for planktonic bacteria

After preparation with artificial urine for 1h, a bacterial suspension of *P.aeruginosa* IFO 12689 was diluted with MHB to 10^5cells/well (10^6cells/ml) and exposed to the same concentrations of GM as the above experiment. Quantitative counts of viable planktonic bacteria were determinated by serial dilution.

7) Bacteriological eradication by OFLX treatment and MBC^{AD}

From 1987 to 1992, 30 men with urethral catheter associated UTI were treated with OFLX at the Department of Urology, Hiroshima University Hospital. Patient age ranged from 21 to 68, with a mean age of 49 years. All of these patients were checked to have pyuria of at least 5WBC/hyper per field, bacteriuria of at least 10^4cfu/ml and identifiable underlying urinary tract infection. Before the start of therapy, all organisms were identified using standard procedures and tests for antibiotic susceptibility (MIC and MBC^{AD}). Urine specimens were collected aseptically from the catheter by needle aspiration. The urethral catheters were not changed or removed routinely during therapy. The daily dose of OFLX was 300 mg t.i.d., and OFLX was administered for 7 days. The bacteriological eradication by OFLX treatment was compared with the MBC^{AD} of OFLX for isolated bacteria. The extended cumulative chi-squre test was used to examine the data for significant differences.

RESULTS

1) Susceptibility of *P.aeruginosa, S.marcescens*, and *P.mirabilis*

The antibiotic susceptibility of *P.aeruginosa, S.marcescens*, and *P.mirabilis* are shown in Table 1. The MBCs for these strains were equal to 1 or 2 times their MICs for all antibiotic agents. The aminoglycosides and the newer quinolones produced low MIC and MBC values. The MIC^{AD}s were also exactly the same as the MICs for all antibiotic agents. In most cases, however, the MBC^{AD}s were more than 128 times higher than their MIC^{AD}s. The concentration of antibiotics required to kill adherent cells was far higher than that required to kill planktonic cells.

2) Killing and growth kinetic studies of planktonic *P.aeruginosa*

The MIC of GM for planktonic cells was found to be 0.5 µg/ml, indicating that this strain is susceptible to this antibiotic. Fig. 1 shows the killing curves of the planktonic cells by various concentrations of GM. The 99.9% killing level was achieved 4h after exposure at 1xMIC (0.5 µg /ml). The killing effect increased with increasing concentration of the drug.

Table 1. Susceptibility of *P.aeruginosa, S.marcescens*, and *P.mirabilis*

Organism (No. of strains)	Antimicrobial agent	MIC (μg/ml) Range	50%	90%	MBC (μg/ml) Range	50%	90%	MIC^{AD} (μg/ml) Range	50%	90%	MBC^{AD} (μg/ml) Range	50%	90%
P.aeruginosa	PIPC	8-512	64	512	8-512	128	512	8-1024	64	512	1024-8192<	8192	8192<
(50)	CFS	1-32	16	32	2-64	16	32	1-64	16	32	1024-8192<	4096	8192<
	GM	0.5-4	0.5	2	0.5-4	0.5	2	0.5-8	0.5	2	8-256	32	128
	AMK	0.5-2	0.5	1	0.5-4	0.5	2	0.5-4	0.5	1	16-128	64	128
	OFLX	2-64	16	64	4-128	16	64	4-64	16	64	2048-2048<	2048	2048<
	TFLX	1-64	4	32	1-64	8	32	1-64	4	32	128-128<	128	128<
S.marcescens	PIPC	4-512	64	256	8-512	64	256	4-512	64	256	2048-8192<	8182<	8192<
(50)	GM	0.25-128	2	64	0.25-256	2	64	0.25-256	2	64	32-512	128	512
	AMK	0.25-64	1	8	0.25-128	1	16	0.25-128	1	16	16-256	64	256
	OFLX	0.25-64	2	32	0.25-128	4	32	0.25-64	4	64	1024-2048<	2048	2048
	TFLX	0.125-64	1	16	0.125-64	2	16	0.125-64	1	16	32-128<	128<	128<
P.mirabilis	PIPC	0.5-64	1	64	0.5-64	2	64	0.5-64	1	64	64-8192	256	4096
(50)	GM	0.25-2	0.5	2	0.25-2	0.5	2	0.25-2	0.5	2	32-256	32	256
	AMK	0.125-2	0.25	1	0.125-2	0.25	2	0.125-2	0.25	1	16-128	16	128
	OFLX	0.125-1	0.25	1	0.125-1	0.25	1	0.125-1	0.25	1	4-128	8	128
	TFLX	0.125-0.5	0.25	1	0.25-1	0.25	1	0.125-1	0.25	1	2-64	4	64

Susceptibility of *P.aeruginosa, S.marcescens,* and *P.mirabilis.*

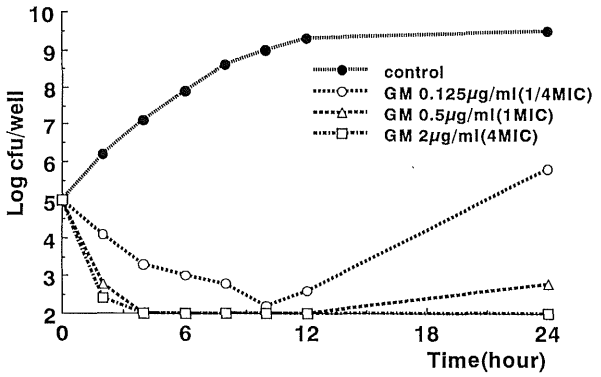

Fig. 1. Killing and growth kinetics of planktonic *P.aeruginosa* IFO 12689. GM was used at concentrations 0, 0.125, 0.5, and 2 μg/ml.

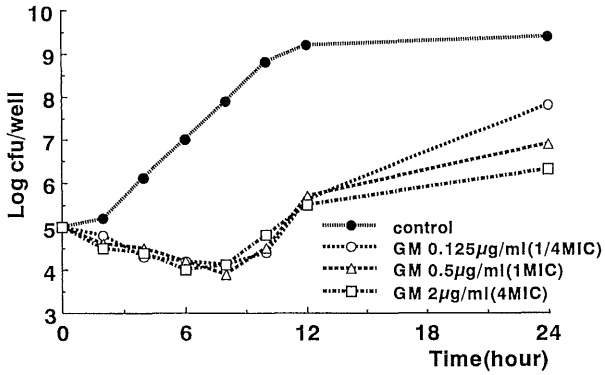

Fig. 2. Killing and growth kinetics of adherent *P.aeruginosa* IFO 12689. GM was used at concentrations 0, 0.125, 0.5, and 2 μg/ml.

3) Killing and growth kinetic studies of adherent *P.aeruginosa*

The MBC^{AD} of GM for the adherent cells was found to be 64 μg/ml. In the absence of GM, the adherent cells grew with a 2h-lag time before logarithmic growth which was not observed in the planktonic cells. Low bactericidal activity during 8h was also observed and there was no concentration-related effect for 0.125, 0.5 and 2 μg/ml during 12h. At various concentration, a regrowth was observed within 10h (Fig. 2).

4) Bacteriological eradication by OFLX treatment and MBC^{AD}

Ten *P.aeruginosa*, 8 *S.marcescens*, and 12 *P.mirabilis* were isolated from 30 patients. A single strain was obtained from each patient. The MBC^{AD} ranges of OFLX against these strains were as follows : *P.aeruginosa*, 2048-2048<μg/ml ; *S.marcescens*, 64-2048 μg/ml ; *P.mirabilis*, 4-128 μg/ml. The eradication was achieved in 7/7 strains indicating MBC^{AD}s of 4-64 μg/ml and in 0/10 strains indicating MBC^{AD}s of 2048-2048<μg/ml (Table 2). The MBC^{AD}s of OFLX well correlated with the bacteriological eradication by OFLX treatment for UTIs associated with indwelling urinary catheters ($p<0.05$), whereas the MICs did not show a correlation ($p=0.67$, Table 3).

Table 2. MBC^{AD} of OFLX and bacteriological eradication by OFLX treatment

MBC^{AD} (No. of strains)		eradication rate(%)
4-16	(6)	100
32-64	(1)	100
128-256	(8)	50
512-1024	(5)	20
2048-2048<	(10)	0

Table 3. MIC of OFLX and bacteriological eradication by OFLX treatment

MIC (No. of strains)		eradication rate(%)
0.125-0.25	(6)	50
0.5-1	(5)	60
2-4	(9)	44.4
8-16	(6)	33.3
32-64	(4)	0

DISCUSSION

It has been reported in a number of studies that bacteria growing in biofilms are more resistant to antibiotics than those growing in a suspension culture. Nickel et al[15] used a modified Robbins device as an *in vitro* infected urinary catheter model. A thick adherent biofilm with *P.aeruginosa* developed on the latex surface within 8h. After this colonization, sterile artificial urine containing 1000 μg/ml of tobramycin was flowed past the biofilm, and a significant proportion of the bacterial cells were found to be still viable after 12h of exposure to this very high concentration of an aminoglycoside antibiotic. The planktonic cells taken from the test system just before exposure of the biofilm to the antibiotic were completely killed by 50 μg/ml of tobramycin. Anwar et al[1] used an *in vitro* chemostat system to determine the interaction of *P.aeruginosa* with the antibiotic. The biofilm bacteria exhibited increased tobramycin resistance compared with that of the planktonic bacteria, and cells in old biofolms were more resistant than cells in young biofilms.

The mechanism of this resistance to antibiotics are suggested by the following : (1) the glycocalyx barrier effect, (2) a low growth rate, (3) nutrient deprivation[2]. Since bacterial adherence to a surface changes the bacterial physiology, the adherent cells are possibly more resistant to antibiotics than the planktonic cell. We modified the original method for measuring the antibiotic concentration required to kill adherent bacteria[13]. In this study, we used artificial urine and uropathogenic gram negative bacilli (*P.aeruginosa, S.marcescens,* and *P.mirabilis*) as a UTI model. Moreover, we employed a short incubation time (1h) involving adherence of the bacteria to a surface for the purpore of minimizing the effect of the glycocalyx on antibacterial activity. Examination by scanning electron microscopy after the short incubation time showed that the multiwell plate surfaces were almost confluently colonized by adherent bacteria, and these bacterial cells did not produce a glycocalyx (data not shown). We also found that the MBC^{AD}s for the uropathogenic adherent cells markedly elevated, even if they did not form a biofilm. In most cases, the MBC^{AD}s were more than 128 times higher than the MICs for planktonic bacteria. This low susceptibility of adherent bacteria to antimicrobial agents is henceforth likely to play an important role in the antibiotic resistance of biofilm bacteria.

Although the mechanism by which adherent cells can survive in the presence of high concentrations of antibiotics is not clear, several mechanisms have been suggested. Slow growth is the most standard condition that leads to a decreased susceptibility of bacteria to antibiotics in fluids[6] and on surfaces[7]. It is thought that the bacteria responsible for infections with prosthetic devices have low matabolic activity. In our experiments, adherent *P.aeruginosa* have a 2h-lag time before logarithmically growing when bacteria were incubated in MHB without antibiotics. Similar results have been obtained for biofilm bacteria colonized on a subcutaneous tissue in rat[4]. The adherent cells are probably different physiologically from the planktonic cells, and therefore their response to antibiotics may be altered, depending on the specific chemistry of the cells and the antibiotic's mechanism of action.

Urinary catheters are obviously the most common prosthetic device in use in the field of urology. The most significant improvement in the prevention of catheter-associated bacteriuria has been the conversion from open to closed sterile drainage systems[10]. However, the risk of infection increases with each catheterization, and bacteriuria develops in almost 100 per cent of prolonged catheterized patients[9,16]. Antibiotic prophylaxis[3] and urinary bladder irrigation with antibiotics[17] are generally used. However, these methods are not effective and long-term antimicrobial chemotherapy causes the emergence of resistant bacteria. In our study, the MBC^{AD}s of OFLX, but not the MICs, correlated with bacteriological eradication by OFLX treatment for UTI associated indwelling urinary catheters. Our assay system, therefore, was considered to be useful in the therapeutic selection for antibiotics of these infections. Moreover, we can conclude that the lack of effective antibiotic chemotherapy in these infections is due to lower dosing of antibiotics than that required for bacteriological eradication of adherent bacteria.

We found that uropathogenic adherent bacteria

were less susceptible to antibiotics, even if they did not form a biofilm. The mechanism of the effective protection of adherent bacteria from the killing effects of antibiotics is still unknown. The elucidation of this mechanism is needed to cure the infections associated with biofilm bacteria.

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