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Physical and Chemical Factors Affecting the Adherence of *Pseudomonas aeruginosa* to a Rabbit Cornea Cell Line (SIRC) Cells

Takashi IIDA[1)], Masafumi KATOH[2)] and Yoshiyasu MATSUO[3)]

1) Department of Ophthalmology, Hiroshima University School of Medicine, Hiroshima 734, Japan
2) Clinical Laboratory Center of Hiroshima City Medical Association, Hiroshima 730, Japan
3) Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734, Japan

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ABSTRACT

Adherence of *Pseudomonas aeruginosa* to SIRC cells was examined by the use of ^{14}C-lysine labeled organisms. Pretreatment of *P. aeruginosa* with heating, 3% formaldehyde, or ultraviolet caused a significant decrease in adherence to SIRC cells, whereas that with lipase, hyaluronidase, trypsin or protease did not. Treatment of SIRC cells with trypsin, protease, lipase or neuraminidase did not influence the adherence of *P. aeruginosa* to the cell. Treatment of *P. aeruginosa* with mannose or galactose inhibited the adherence, while that with fructose, lactose or glucose did not. Treatment of SIRC cells with galactosidase or mannosidase reduced the adherence of the organism. No correlation was demonstrated between the adhering ability and hydrophobicity of *P. aeruginosa*. The results suggest that both the viability in bacterial site and mannose and/or galactose molecules in cellular site are closely connected with the adherence of *P. aeruginosa* to SIRC cells.

P. aeruginosa is one of those bacteria which cause acute and severe corneal ulcer and the infection is difficult to treat[16)]. A number of workers[3,7,9,13,14)] reported on the corneal infection that correlation existed between various extracellular substances produced by *P. aeruginosa* and the virulence. Proteases and exotoxin A have been thought to be more necessary for the bacterial invasion into tissues than for the bacterial adherence to epithelial cell surface[21)]. On the other hand, some reports[25,29)] show that the bacterial adherence is closely related to the infectivity. Previously we[10)] demonstrated that protease producing strains adhered more avidly to SIRC cells than non-producing ones. However, another experiment[11)] using protease-deficient mutants suggested that there might be other factors besides protease in the adherence between *P. aeruginosa* and SIRC cells.

It is generally recognized[2,4,6,22)] that distinct substances which act as ligands for specific receptors on mammalian cells exist on bacterial surface and that non-specific interactions such as hydrophobicity[31)] and net surface charge are also necessary for bacterial adherence. The present paper describes participation of other factors besides protease in the adherence between *P. aeruginosa* and SIRC cells.

MATERIALS AND METHODS

Organism: Strains used were IFO 3455, NC 5 and PA 103 of *P. aeruginosa* which were maintained on slopes of tripticase soy agar.

Epithelial cell culture: Culture of SIRC cells was previously described in detail[10)]. Briefly, when the cells developed into a confluent monolayer, they were detached by 0.2% trypsin and 0.02% EDTA in phosphate buffered saline (PBS), washed once with and suspended in PBS at a density of 2.0×10^5 cells per ml. When

the cells should be treated with various enzymes, 0.05% EDTA in PBS was used for 30 min to detach the cells.

Labeling of bacteria: The bacteria were grown in tripticase soy broth on a shaking water bath at 37°C overnight. They were washed once with M-9 broth and suspended in M-9 broth supplemented with 0.5 μCi of ^{14}C-lysine per ml (Amersham Corp., U.S.A.) at an optical density of 0.59 at 570 nm (approximately 2.0×10^9/ml) on Coleman Junior spectrophotometer. After incubating at 37°C for 4 hr with shaking at 100 rpm, the bacteria were washed three times with and resuspended in PBS at a concentration of 2.0×10^8 organisms per ml.

Bacterial adherence: A mixture of 2.0 ml of the bacterial suspension and the same volume of the epithelial cell suspension was incubated at 37°C for 60 min on a shaking water bath at 100 rpm. One-ml volume of the mixture was filtered through a 10 μm pore size of polycarbonate membrane filter (Nucleopore Corp., U.S.A.) under vacuum. The filters were washed with 50 ml of PBS and dried to be placed in scintillation vials containing 10 ml of toluene with 0.04 g of 2,5-diphenyloxaole (PPO; Katayama Chemical Industries Ltd., Japan). The vials were counted in a liquid scintillation spectrophotometer (Packard Model 3320). The number of bacteria was enumerated by measuring scintillation count in a known number of bacteria. The number of adhering bacteria was calculated by subtracting the number of bacteria attached non-specifically on the filter in the absence of epithelial cells from the number of bacteria in the presence of them on the filter in the assay. Adherence was determined by dividing the number of adhering bacteria by the number of epithelial cells.

Pretreatment of bacteria: The bacterial suspension was killed by heating at 65°C for 30 min, by 3% formaldehyde at 37°C for 30 min or by ultraviolet irradiation. Ultraviolet irradiation was performed by exposing the bacterial suspension in a quartz tube to an ultraviolet lamp (GL 15) for 30 min at a distance of 20 cm. The radiation at the surface of the suspension was 750 μw/cm^2.

The bacteria were treated with various enzymes in PBS for 30 min at 37°C. The enzymes used were lipase (from wheat germ, type I; Sigma Chemical Co., U.S.A.), hyaluronidase (from bovine testes, type IV; Sigma), trypsin (from bovine pancreas, type III; Sigma) and protease (from *B. subtilis*, type VIII; Sigma).

The following saccharides were used for the adherence inhibition study; D-mannose (Katayama Chemical Industries Ltd., Japan), D-fructose (Katayama Chemical), lactose (Ishizu Pharmaceutical Co., Ltd., Japan), D-glucose (Tokyo Kasei Kogyo Co., Ltd., Japan) and galactose (Katayama Chemical). Each saccharide was added to the bacterial suspension in PBS to achieve a final concentration of 2.5% (wt/vol) and incubated for 30 min at 37°C. The bacteria were washed twice with PBS and subjected to the adherence assay.

Pretreatment of SIRC cell: SIRC cells (2.0×10^5/ml) were incubated together with various enzymes for 10 min at 37°C. The enzymes used were trypsin, protease, lipase, neuraminidase (from *Cl. perfringens*, type V; Sigma), mannosidase (from jack beam, type III; Sigma) and galactosidase (from *E. coli*, grade VIII; Sigma). After the treatment, the cells were washed twice with PBS.

Hydrophobicity: Hydrophobicity of *P. aeruginosa* was determined according to the method of Rosenberg et al[23]. Briefly, the bacteria were incubated for 24 hr in tripticase soy broth, washed twice with and suspended in PBS at the optical density of 0.4 to 0.6 at 500 nm. A 3 ml-volume of bacterial suspension was added to various volumes of n-hexadecane (Wako Pure Chemical Industries, Ltd., Japan) in a small test-tube. 10 min after incubation at 37°C, the tube was shaken vigorously for 120 sec. After separating the mixture into two phases, the aqueous phase was carefully collected and the light absorbance was measured by using a spectrophotometer at 500 nm (100—50 type, Hitachi Co., Ltd., Japan).

RESULTS

Effect of time and temperature of incubation on the bacterial adherence: The optimum time of incubation for the adherence was determined by incubating SIRC cell suspension (1.0×10^5 cells/ml) with *P. aeruginosa* suspension (1.0×10^8 organisms/ml) for various periods of time at 4°C or 37°C (Fig. 1). The mean number of attached bacteria increased progressively along with incubation time up to 60 min at 37°C and

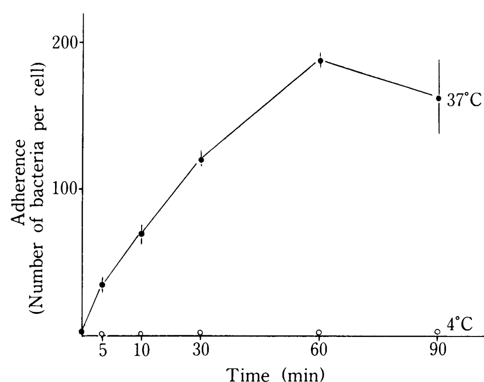

Fig. 1. Effect of incubation time on adherence of *P. aeruginosa* IFO 3455 to SIRC cells. Adherence assay was carried out at 4°C and 37°C. Bars indicate mean ± standard deviation.

Table 1. Effect of bacterial killing on adherence of *P. aeruginosa* IFO 3455 to SIRC cells

Method of killing	Treatment	% Adherence
Heat	37°C 30 min	100
	65°C 30 min	44
Formaldehyde	None	100
	3% 30 min	0
Ultraviolet	None	100
	GL 15 30 min[1]	54

1) distance: 20cm

was followed by a slight decrease. A slight adherence was observed at 4°C. Subsequently, a temperature of 37°C and a time of 60 min were used for the adherence assay.

Effect of physical and chemical treatments of bacteria on the bacterial adherence: Table 1 shows the effect of bacterial killing on the adherence of *P. aeruginosa* strain IFO 3455 to SIRC cells. The bacteria treated with 3% formaldehyde demonstrated a 100% decrease in adherence when compared with untreated control. Heat-treated and ultraviolet-irradiated bacteria showed moderate decrease in adherence.

The effects of enzymatic treatment of *P. aeruginosa* on the adherence to SIRC cells are shown in Table 2. Pretreatment of *P. aeruginosa* with hyaluronidase slightly increased the adherence, whereas lipase inhibited the adherence to a small extent. Neither trypsin nor protease affected the bacterial adherence.

Table 2. Adherence of enzyme-treated *P. aeruginosa* IFO 3455 to SIRC cells

Treatment	% Adherence
Control (pH 7.0)	100
Lipase (1.6mg/ml)	79
Hyaluronidase type IV (0.55mg/ml)	137
Trypsin (1.0mg/ml)	115
Protease (1.0mg/ml)	104

After treatment with each enzyme for 30 min at 37°C, the bacteria were washed twice with and resuspended in PBS.

Table 3. Adherence of *P. aeruginosa* IFO 3455 pretreated with various carbohydrates to SIRC cells

Carbohydrate	% Adherence
Control	100
Mannose	56
Fructose	92
Lactose	86
Glucose	82
Galactose	49

After treatment with each carbohydrate at a final concentration of 2.5% for 30 min at 37°C, the bacteria were washed twice with and resuspended in PBS.

As shown in Table 3, pretreatment with mannose and galactose caused 44% and 51% decrease in adherence respectively, while only a slight inhibition of the adherence was observed with other sugars.

Effect of enzymatic pretreatment of SIRC cell on the bacterial adherence: Table 4 shows the effects of various enzymatic treatments of SIRC cells on the adherence of *P. aeruginosa*. No significant effect of pretreatment of the cell with trypsin, protease, lipase or neuraminidase was demonstrated on the adherence of *P. aeruginosa* to SIRC cells. The cells treated with mannosidase and galactosidase moderately reduced the bacterial adherence (Table 5).

Hydrophobicity: The adherence of *P. aeruginosa* to n-hexadecane was investigated in order to

Table 4. Effect of various enzyme treatments of SIRC cells on adherence of *P. aeruginosa* IFO 3455

	Treatment	% Adherence
Exp. 1	Control (pH 7.0)	100
	Trypsin (2.5μg/ml)	100
	Protease (2.5μg/ml)	110
	Lipase (10.0μg/ml)	102
Exp. 2	Control (pH 5.5)	100
	Neuraminidase (120μg/ml)[1)]	111

After treatment with each enzyme for 10 min at 37°C, SIRC cells were washed twice with and resuspended in PBS.
1) Phosphate-buffered saline at pH 5.5 was used in the treatment with neuraminidase.

Table 5. Effect of various enzyme treatments of SIRC cells on adherence of *P. aeruginosa* IFO 3455

	Treatment	% Adherence
Exp. 1	Control	100
	Mannosidase (2.5μg/ml)	77
Exp. 2	Control	100
	Mannosidase (10.0μg/ml)	83
	Galactosidase (1.0μg/ml)	65
Exp. 3	Control	100
	Galactosidase (1.0μg/ml)	52

After treatment with mannosidase or galactosidase for 10 min at 37°C, SIRC cells were washed twice with and resuspended in PBS.

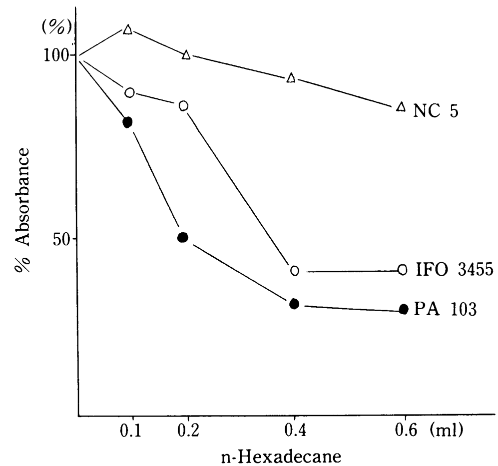

Fig. 2. Adherence of *P. aeruginosa* to n-hexadecane. Results were expressed as the percentage of the initial absorbance at 500 nm of the aqueous suspension.

assess the correlation between the hydrophobicity and adherence ability of bacteria (Fig. 2). Strains PA 103 and IFO 3455 exhibited high affinity to n-hexadecane, whereas strain NC 5 showed low affinity.

DISCUSSION

Infectious process generally takes place in the following sequence; attachment to, colonization on epithelium, and invasion into deeper tissues. Adherence of bacteria to epithelial cells is the prerequisite for establishment of the infection[8,30], and reports[25,29] suggest that adherence of bacteria to epithelial cells *in vitro* closely correlates to bacterial infectivity *in vivo*. Our previous work[10] demonstrated that protease-producing strains of clinical isolates of *P. aeruginosa* adhered to SIRC cells much more as compared with non-producing ones. This finding agrees with the above suggestion, because the isolation of protease-producing strains is higher than that of non-producing ones in *P. aeruginosa* infection[17,24], and *P. aeruginosa* protease is well known as a virulent factor, especially in the corneal infection[3,7,14,15]. However, another idea about the adherence of *P. aeruginosa* to SIRC cells arose from our recent study[11] in which experimentally produced protease-deficient mutants of *P. aeruginosa* adhered to SIRC cells slightly less than the parental strain. There will be other factors besides protease which contribute to the adherence of *P. aeruginosa* to the cells.

A low level of adherence was noted after the treatment of *P. aeruginosa* with heating, formaldehyde or ultraviolet. Fader et al[6] reported that heat-killed *Klebsiella pneumoniae* reduced the adhering ability because of depiliation. Eden and Hansson[5] remarked that the adhering ability of *Escherichia coli* decreased after formaldehyde treatment without destroying pili. In our previous study[10], no reduction in the adherence of *P. aeruginosa* was observed after a vigorous washing of the bacteria to remove pili so that the pili might not contribute to the bacterial adherence. Bacterial viability may be associated with the adhering ability of *P. aeruginosa* to

SIRC cells.

No significant interference with the adherence was observed on *P. aeruginosa* treated with lipase, hyaluronidase, trypsin or protease. Woods et al[34] described that protease or trypsin treatment of *P. aeruginosa* led to decrease in adherence to buccal cells. Fader et al[6] reported on a reduced adhering ability of trypsin-treated *K. pneumoniae* to bladder epithelial cells. They explained that the decrease in adherence would be due to depiliation. On the other hand, Bartelt and Duncan[1] demonstrated that hyaluronidase treatment of a *streptococci* strain 203 increased the ability to adhere to epithelial cells and suggested that hyaluronic acid capsule of the organism would interfere with the adherence to some extent.

Regarding the effect of pretreatment of host cells with neuraminidase on bacterial adherence, Yamazaki et al[35] showed that the treatment markedly enhanced the adherence of *Eikenella corrodens* to buccal epithelial cells. They proposed two possible mechanisms; firstly, epithelial cell surface receptors for the adherence might be masked with sialic acid, and secondly, the negative charge of sialic acid might have some influence on the adherence. On the contrary, Sobeslavsky et al[32] reported that the treatment of tracheal epithelial cells with neuraminidase reduced the adsorption of *Mycoplasma pneumoniae* and believed that neuraminic acid might serve as a cell receptor for *M. pneumoniae*. In the present study, the pretreatment of SIRC cells with neuraminidase did not affect the adherence of *P. aeruginosa* to the cells. Zawaneh et al[36] observed no alteration in the adherence of group B *streptococci* to epithelial cells treated with neuraminidase.

Many investigators[18-20,26,27] reported that certain receptors on epithelial cell surface for bacterial adherence could be inhibited by various sugars. Schaeffer et al[26] showed that the adherence of *E. coli* to uroepithelial cells was completely inhibited by the treatment of the organisms with D-mannose, suggesting that mannose-containing carbohydrates on the host epithelial cells are involved in adherence. Ofek et al[18,19] remarked that the ability of *E. coli* to adhere to human epithelial cells was directly related to its ability to bind mannose residues. Our present study revealed that *P. aeruginosa* treated with mannose or galactose resulted in a significant reduction in adherence to SIRC cells. In addition, the treatment of SIRC cells with mannosidase or galactosidase reduced the bacterial adherence. These findings suggest that termination of receptors on SIRC cells may be compounds of mannose- or galactose-like molecules. According to Sharabi and Gilboa-Garber[27], mannose- or galactose-philic lectin of *P. aeruginosa* adsorbed protozoa. Adhesins on bacterial surface are known to be M-protein in *Streptococcus pyogenes*[2], and pili in *K. pneumoniae*[6], *E.coli*[5], *Vibrio cholerae*[33] or *Proteus*[28]. On the other hand, receptors for adherence on epithelial cells are reported to be related to D-mannose[18-20], neuraminic acid[32] or ℓ-fucose-containing structure[12]. Although we could not identify any adhesion on *P. aeruginosa* in the present study, there is a possibility that lectin may serve as an adhesin of *P. aeruginosa*. Physical or chemical pretreatments of the bacteria affected the bacterial adherence to SIRC cells, but treatments with various enzymes did not, which fact suggests that the killing of *P. aeruginosa* with heating, formaldehyde or ultraviolet may destroy molecules on bacterial surface which specifically interact with receptors on SIRC cell surface. Besides adhesin-receptor interaction, non-specific attraction such as surface hydrophobicity[31] or net surface charge is involved in bacterial adherence. In the present study, however, no correlation was noted between the adherence of *P. aeruginosa* to SIRC cells and the microbial hydrophobicity.

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