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Role of *Pseudomonas aeruginosa* Culture Filtrates in the Association, Invasion, and Cytotoxicity Against Cloned Cells from Murine Corneal Epithelium and KB Cells

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Purpose: To clarify the effect of *Pseudomonas aeruginosa* culture filtrates on the association with, invasion into, and cytotoxicity against cloned cells from murine corneal epithelial cells and KB cells.

Methods: Simian virus 40-transformed murine corneal epithelial (MCE) cells were established. Murine corneal epithelial cells and KB cells were infected with a protease-positive strain, IID1117 (Pa IID1117), and a protease-negative strain, IID1130 (Pa IID1130) of *P. aeruginosa*, and then tested for association and invasion of Pa IID1117. The cytotoxicity test was performed by incubating the cells with culture filtrate.

Results: Association of Pa IID1117 with KB cells pretreated with Pa IID1130 was significantly promoted. After pretreatment with culture filtrate, invasion was more effective into MCE cells than into KB cells. When infecting bacteria (Pa IID1117) were pretreated with protease inhibitor, invasion of the bacteria into MCE cells and KB cells clearly decreased. The cellular damage induced by the culture filtrate of Pa IID1130 was greater than the damage by that of Pa IID1117.

Conclusion: These results suggest that association of *P. aeruginosa* with MCE cells and KB cells was influenced by the culture filtrates other than proteases, and that invasion of *P. aeruginosa* into MCE cells and KB cells was promoted by protease.
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Key Words: Association, invasion, KB cells, *Pseudomonas aeruginosa*, SV40-transformed murine corneal epithelial cell.

Introduction

Pseudomonas aeruginosa, a widely occurring opportunistic pathogen, is often found in nosocomial infections and is responsible for opportunistic infections of the respiratory tract, cornea, burned skin, and other sites.[1] *P. aeruginosa* is a leading cause of corneal infections, especially in users of soft contact lenses and in immunocompromised or aged individuals.[2–7] Bacterial keratitis associated with the use of contact lenses is rapidly progressive and difficult to treat, and can cause vision impairment.[8]

P. aeruginosa has been considered an extracellular bacterium for a long time. The organism adheres to host tissues, and it is believed that the infectious process then involves destruction of the underlying tissue by the release of extracellular toxic substances, and that the stimulation of the host immune response can exacerbate tissue damage, leading to erosive disease.[9] Fleiszig et al[10] reported that *P. aeruginosa* could invade corneal epithelial cells in a murine experimental model of corneal wound infection.

P. aeruginosa produces many extracellular products, such as proteases and toxins, which are candidates for important virulence factors in keratitis. These products include exotoxin A, exoenzyme S, phospholipase C, rhamnolipid, Las B elastase, Las A elastase, alkaline protease, and pyocyanin.[11] We used

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proteinase-positive and proteinase-negative strains of *P. aeruginosa* and, by a gentamicin survival assay, studied their association and invasion of murine corneal epithelial cells and a cloned KB cell line. A cytotoxicity assay using the trypan blue dye exclusion method was also performed, and the mechanism of interaction of corneal epithelial cells and KB cells with *P. aeruginosa* is discussed.

Materials and Methods

Bacterial Strains and Culture Medium

Two strains of *P. aeruginosa*, IID1117 (Pa IID1117) and IID1130 (Pa IID1130), were kindly provided by the Institute of Medical Science, Tokyo University. Pa IID1117 is elastase-positive and protease-positive and forms mucoid colonies. Pa IID1130 is elastase-positive and protease-negative and also forms mucoid colonies. These bacteria were inoculated and maintained on Tryptic Soy agar (Difco Lab, Detroit, MI, USA) slant.

In infection experiments, bacteria were cultured in Tryptic Soy broth at 37°C for 8 hours with shaking. The culture was centrifuged at 1,100 *g* for 15 minutes, and then the bacteria were resuspended in phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}, PBS(−). In a preliminary experiment, we determined a standard curve for bacterial concentration by measuring absorbance at 550 nm.

For preparation of culture filtrate, a loopful of Pa IID1117 or Pa IID1130 was inoculated into Tryptic Soy broth, and the bacteria were cultured at 37°C for 24 hours with shaking. The cells were removed by centrifugation at 1,600 *g* for 15 minutes, and the supernatant was further filtered through a membrane filter with pore size of 0.22 μm.

In some experiments, the culture filtrate was further fractionated using the Centricon 10 concentrator (Amicon, Beverly, MA, USA). This concentrator separated the fraction with molecular weight of more than 10 kilodaltons (>10 kDa fraction) from the fraction with molecular weight of less than 10 kDa (<10 kDa fraction). These fractionated culture filtrates were transferred to small test tubes and stored in a refrigerator until use.

Bacterial and Extracellular Protease Activities

The protease activity was detected using casein agar plates, which contained 10 g of casein (from milk; Wako Pure Chemical, Osaka) 4.4 g of sodium citrate, 2.5 g of Bacto yeast extract (Difco), 10 g of polypeptone (Wako), 1.0 g of glucose, 15 g of agar and 0.02 M $CaCl_2$ in 1,000 mL of distilled water.[12] A loopful of Pa IID1117 or Pa IID1130 was inoculated on the casein agar plate and incubated at 37°C. Alternatively, a round piece of filter paper (diameter: 8 mm) containing 1 mL of culture filtrate was put on the casein agar plate, and incubated at 37°C for 20 hours.

Epithelial Cell Lines

The KB cell line is a cloned line of epithelial cells originating from a carcinoma of the human nasopharyngeal cavity.[13] In order to create a cloned cell line of the corneal cells, murine corneal epithelial cells were prepared according to previously described methods, with some modifications.[14,15] Briefly, a conventional mouse of the BALB/c strain was sacrificed according to the Guidelines for Animal Experimentation of the Faculty of Medicine, Tottori University (Guide for the Care and Use of Laboratory Animals), and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and both eyes were aseptically removed. Corneal tissue was separated by perforating the corneal margin with a 26-gauge needle and cutting with a Springlehandle knife, and then transferred to a plastic dish (Falcon #3001; Nippon Becton Dickinson, Tokyo) containing Dulbecco's modified Eagle's medium (DMEM). Corneal epithelial tissue was stripped off from the stroma with forceps. A piece of corneal epithelial tissue and a piece of corneal stroma were separately suspended in DMEM supplemented with 10% fetal calf serum (DME-FC) and incubated at 37°C and 5% CO_2 in air. After incubation for a few weeks, the cells became confluent, and they were then infected with Simian virus 40 (SV40) (SV68C strain) according to the method described elsewhere.[16] An SV40-transformed cell clone with the capability of constant cell division was selected as a cloned epithelial cell line (designated as MCE cells). MCE cells had several characteristic cellular markers essential for epithelial cells along with the SV40 large-T antigen. They were positive to type VI collagen and common cytokeratin but negative to fibronectin, which is a marker for fibroblasts.

The MCE cells grew slowly in the plastic dish (Falcon #3002), and usually the medium was changed to fresh DMEM once a week. When the cells became confluent, the medium was discarded and 0.5 mL of PBS(−) containing 0.05% trypsin and 0.02% EDTA was added to the plate, which was then incubated at 37°C for 15 minutes. After the cells were detached from the plate by pipetting, 5 mL of fresh medium was added. The cells were collected by centrifugation at 400 *g* and resuspended in fresh medium, and a given number of cells were used for infection experiments.

For the morphological study, MCE cells on glass coverslips were rinsed and fixed with 2.5% glutaral-

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dehyde buffered with 0.1 M phosphate buffer (pH 7.3), and postfixed with osmium-tetroxide in the same buffer. After conductive-staining with 2% tannic acid and 1% osmium tetroxide, they were dehydrated in a graded series of ethanol, immersed in isoamyl acetate, and critical point dried in CO_2. The dried samples were coated with platinum using an ion-sputtering coater and observed under a scanning electron microscope (S-430; Hitachi, Tokyo). For fluorescence microscopy, MCE cells were treated with fluorescein isothiocyanate (FITC)-conjugated anti-T antigen rabbit serum (Cedarlane Lab, Ontario, Canada) and observed by a fluorescence microscope (Olympus, Tokyo).

In Vitro Association and Invasion Assays

Either KB cells or MCE cells (2.5×10^5 cells/1 mL DMEM) were dispersed in a plastic dish (Falcon #3047) and incubated at 37°C and 5% CO_2 in air for at least 24 hours. At that time, the cells were confluent and attached to the surface of the dish.

After the medium was removed, 1 mL of antibiotic-free Ham's F-12 containing a 1.0×10^7 colony forming unit (CFU) of Pa IID1117 was added to the dish. The dish was centrifuged at 150 *g* for 5 minutes to allow the bacteria to easily contact the cells. Following 1 hour of incubation at 37°C and 5% CO_2 in air, the inoculum was removed and the dishes were washed three times with PBS(−) to remove nonassociated bacteria. After the washing, the association assay or invasion assay was performed.

For the association assay, 1 mL of 0.25% Triton X-100 (Wako) was added to the dish, which was kept at 37°C for 10 minutes, and then mixed well by pipetting. The viable organisms were assessed by the colony counting method.

For the invasion assay, 0.5 mL of gentamicin (200 μL/mL) was added for 2 hours to kill extracellular bacteria, and then cells were washed once with PBS(−) to remove the antibiotic. The cells were lysed and viable organisms were assessed as described for the association assay.

To examine the effect of the culture filtrate on association and invasion, 2.5×10^5 epithelial cells in DME were dispersed in a plastic dish (Falcon #3047) and incubated at 37°C with 5% CO_2 in air for 24 hours. After the medium was removed, 0.5 mL of serially diluted culture filtrate of Pa IID1117 or Pa IID1130 was added to the cells and incubated for 1 hour. The cells were washed once with PBS(−) before the 1-hour infection period for the association and invasion assays using Pa IID1117.

For testing the effect of protease inhibitor (Protease Inhibition Cocktail; Sigma-A1drich, St. Louis, MO, USA), Pa IID1117 was pretreated with protease inhibitor for 10 minutes before infection with the cells. One milliliter of antibiotic-free Ham's F-12 medium containing 1.0×107 CFUs of the protease inhibitor-treated bacteria was added to a monolayer of 2.5×10^5 cells of each of the cell lines. After 1 hour of incubation, the association and invasion assays were performed.

Cytotoxicity Test

Either MCE cells or KB cells (2.0×10^5 cells/5 mL DMEM) were dispersed in a dish (Falcon #1007) and then the culture filtrate of either strain of *P. aeruginosa* was added at a concentration of 10% (V/V). The plate was incubated at 37°C and 5% CO_2 in air.

At various intervals, 5-μL aliquots of the culture were removed to small test tubes, to each of which 5 μL each of 0.3% trypan blue solution was added, and viable cells were assessed by the trypan blue dye exclusion test.

Statistical Analysis

The Student *t*-test was used for statistical analysis to test for differences between the groups.

Results

Corneal Epithelial Cells

Simian virus 40-transformed murine corneal epithelial (MCE cells) grew slowly and took about 2 days for one cell division. The morphology of MCE cells, with microvilli-like projections on the apical cell surface, is shown in Figures 1A and 1B. The SV40 T antigen was revealed by staining the cells with FITC-conjugated anti-T antigen rabbit serum and observing the cells by fluorescence microscopy (Figure lC).

Association and Invasion of P. aeruginosa

The association and invasion of Pa IID1117 or Pa IID1130 with KB cells and MCE cells are shown in Figure 2. The association of Pa IID1117 and Pa IID1130 did not differ in KB cells and MCE cells. However, the invasion of Pa IID1130 into KB cells was significantly less than that of Pa IID1117 into the same cells. When KB cells and MCE cells were in contact with Pa IID1130 for more than 1 hour in invasion assays, some of the infected cells were damaged and detached from the plate, and the total number of intracellular bacteria might thereby have decreased. We, therefore, used Pa IID1117 as the infecting bacteria for the following experiments.

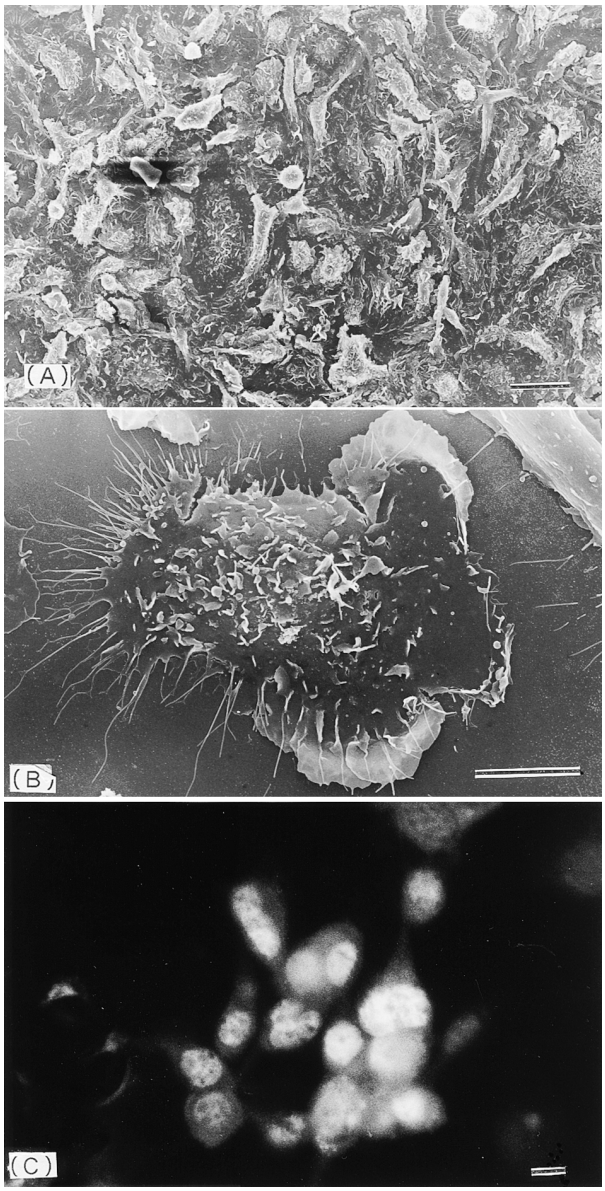

Figure 1. Simian virus 40-transformed murine corneal epithelial (MCE) cells. (**A**) Photograph of cultured MCE cells. Bar = 40 μm. (**B**) Scanning electron micrograph of typical MCE cell showing microvilli-like projections on the apical cell surface. Bar = 10 μm. (**C**) Fluorescence microscopy of MCE cells treated with fluorescein isothiocyanate-conjugated anti-T antigen rabbit serum. T antigen is observed in cell nuclei. Bar = 10 μm.

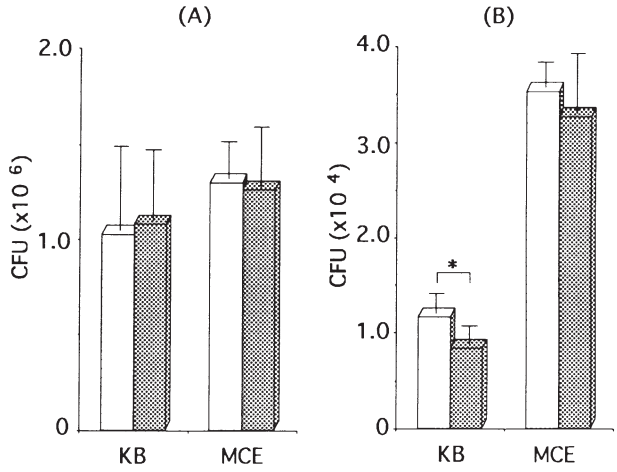

Figure 2. Infections of KB cells and murine corneal epithelial (MCE) cells by *Pseudomonas aeruginosa* IID1117 and *P. aeruginosa* IID1130. Association (**A**) and invasion (**B**) of Pa IID1117 (open columns) or Pa IID1130 (closed columns) were assessed as described in Materials and Methods. Each column shows the mean and standard deviation (shown by bar) of three separate experiments performed in duplicate. CFU: colony forming unit; KB: KB cells; MCE:MCE cells; $*P < .01$.

Protease Activity of the Bacteria and its Effect on Infection

Since bacterial association with and invasion into the cells is affected by the protease activity of the bacterial products, the bacteria were pretreated with a protease inhibitor and then used to infect the cells. The association of the protease inhibitor-treated Pa IID1117 with KB cells and MCE cells was slightly, but not significantly, less than the association of untreated Pa IID1117 (Figure 3A). However, the invasion of the protease inhibitor-treated Pa IID1117 into KB cells and MCE cells clearly decreased in comparison with the invasion of untreated Pa IID1117 into both types of cells (Figure 3B).

The bacterial strains used in this study, Pa IID1117 and Pa IID1130, have almost the same characteristics, except for protease activity. As shown in Figure 4A, protease activity was detectable on casein agar plates. Pa IID1117 made a turbid zone, the inside of which became clear on the medium, but no turbid zone was observed around the Pa IID1130 colony. When the casein was partially digested, it formed a turbid precipitate in the agar medium, while the final products of casein became transparent. A similar turbid zone was observed around a round filter paper containing the culture filtrate of Pa IID1117, but the culture filtrate of Pa IID1130 did not make a precipitate zone (Figure 4B).

Effect of Culture Filtrate on Association and Invasion

The association and invasion of Pa IID1117 with KB cells were influenced by the culture filtrates of Pa IID1117 and Pa IID1130 (Figure 5). The maximum association of Pa IID1117 with KB cells was observed

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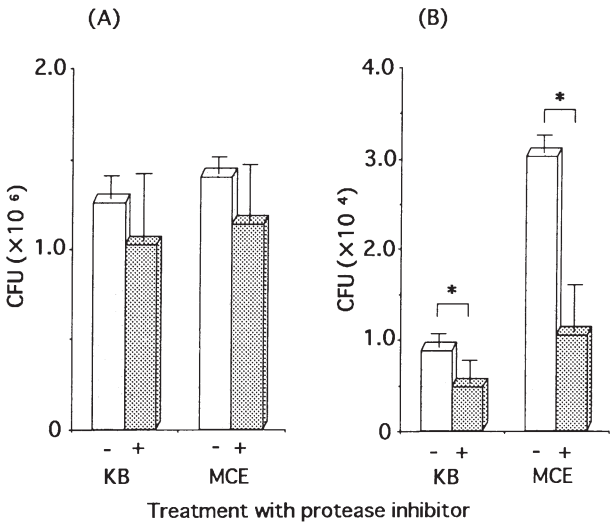

Figure 3. Effect on association and invasion of treatment of *Pseudomonas aeruginosa* with protease inhibitor.Pa IID1117 was incubated with protease inhibitor, and then, used to infect KB cells and MCE cells. Association (**A**) and invasion (**B**) assays were done, and each column shows the mean and standard deviation of three separate experiments performed in duplicate. +: treatment with protease inhibitor; –: treatment with medium alone. CFU: colony forming unit; KB: KB cells. $*P < .01$.

when KB cells were pretreated with the culture filtrates of Pa IID1117 and Pa IID1130 at a dilution of 10^9-fold (Figure 5A). The association of Pa IID1117 with KB cells preincubated with the culture filtrate of Pa IID1130 was higher than that with KB cells preincubated with the culture filtrate of Pa IID1117 (Figure 5A). The maximum invasion of Pa IID1117 into KB cells preincubated with the culture filtrates of Pa IID1117 and Pa IID1130 was observed at a dilution of 10^5-fold (Figure 5B). At that dilution, the CFU of Pa IID1117 in KB cells pretreated with the culture filtrate of Pa IID1130 was greater than that in KB cells pretreated with the culture filtrate of Pa IID1117.

The association of Pa IID1117 with MCE cells occurred maximally when the cells were preincubated with the culture filtrate of either Pa IID1117 or Pa IID1130 at a dilution of 10^9-fold (Figure 6A). However, the invasion of Pa IID1117 into the cells was maximal when the cells were preincubated with the culture filtrate of Pa IID1130 diluted 10^5-fold and when they were preincubated with the culture filtrate of Pa IID1117 diluted from 10^5-fold to 10^9-fold (Figure 6B).

The association of Pa IID1117 with KB cells pretreated with the culture filtrate of Pa IID1130 was greater than that with KB cells pretreated with the culture filtrate of Pa IID1117 and with MCE cells pretreated with the culture filtrate of Pa IID1117 or Pa IID1130 (Figures 5A and 6A). The Invasion of Pa IID1117 into KB cells was significantly less effective than that into MCE cells. However, there was no difference at a dilution of 10^5-fold between the maximum invasion of Pa IID1117 into the cells pretreated with the culture filtrate of Pa IID1117 and that with the culture filtrate of Pa IID1130 (Figures 5B and 6B).

Cytotoxicity of Culture Filtrate

The trypan blue dye exclusion method revealed marked cytotoxicity of the culture filtrates of Pa

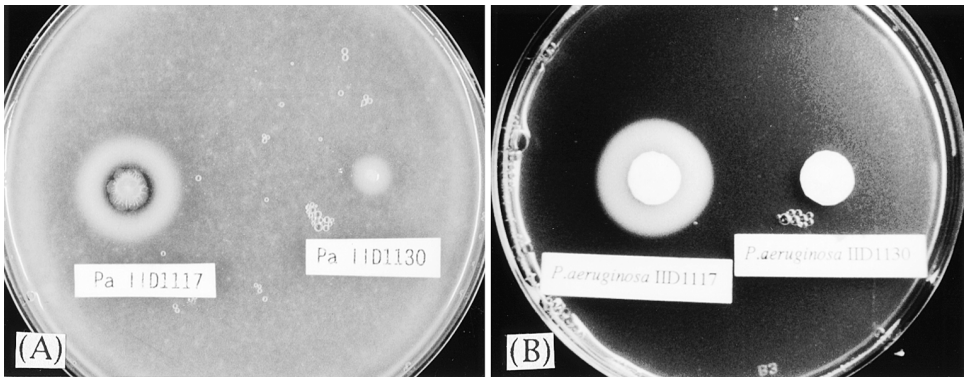

Figure 4. Protease activities of *Pseudomonas aeruginosa* IID1117 and Pa IID1130 and of culture filtrates of both strains of bacteria. (**A**) Loopful of Pa IID1117 or Pa IID1130 was separately spotted on casein agar plates, and incubated at 37°C for 2 days. Turbid casein precipitate was observed around colony of Pa IID1117. Clear (transparent) zone, which was due to complete digestion of casein, appeared around colony of Pa IID1117. There was neither a turbid nor a clear zone around colony of Pa IID1130. (**B**) Round pieces of filter paper containing culture filtrates of each strain were placed on casein agar plate and plate was kept at 37°C for 20 hours. Turbid precipitate was observed around filter paper containing culture filtrate of Pa IID1117, but no precipitate was observed around the filter paper containing culture filtrate of Pa IID1130.

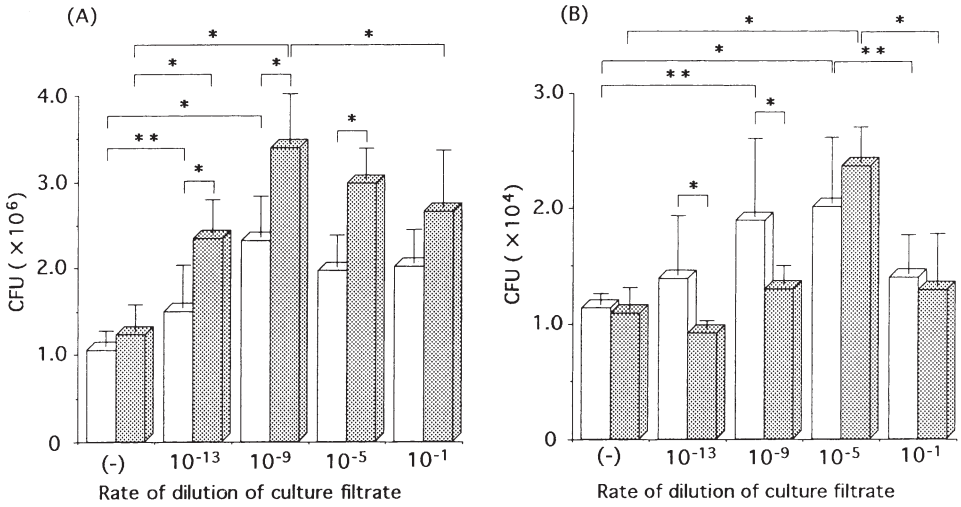

Figure 5. Infection of KB cells by *Pseudomonas aeruginosa* IID1117. KB cells were treated with serial dilutions of culture filtrates of Pa IID1117 (open columns) or IID1130 (closed columns) followed by infection with Pa IID1117. Association between cells and bacteria (**A**) and invasion of cells by bacteria (**B**) were tested. Each column shows the mean and standard deviation (shown by bar) of three separate experiments performed in duplicate. (−): treatment of cells with fresh medium. $*P < .01$, $**P < .05$.

IID1117 and Pa IID1130 against KB cells and MCE cells (Figure 7). The survival rates of KB cells and MCE cells after 4 hours of incubation with the culture filtrate of Pa IID1117 were both about 70%, and the survival rate for cells incubated with the culture filtrate of Pa IID1130 was less than 60%. The cytotoxicity of the culture filtrate of Pa IID1130 was greater than that of the culture filtrate of Pa IID1117.

When the culture filtrate was fractionated by Centricon and used for cytotoxicity assays, both KB cells and MCE cells were more severely damaged by the >10 kDa than by the <10 kDa fraction of Pa IID1130 (Figure 8). The cytotoxic effect of the culture filtrate was destroyed by heating at 95°C for 30 minutes (Figure 9).

Discussion

There have been many reports describing the infection of cultured cells by *P. aeruginosa.* Primary murine corneal epithelial cells,[8] primary rabbit epi-

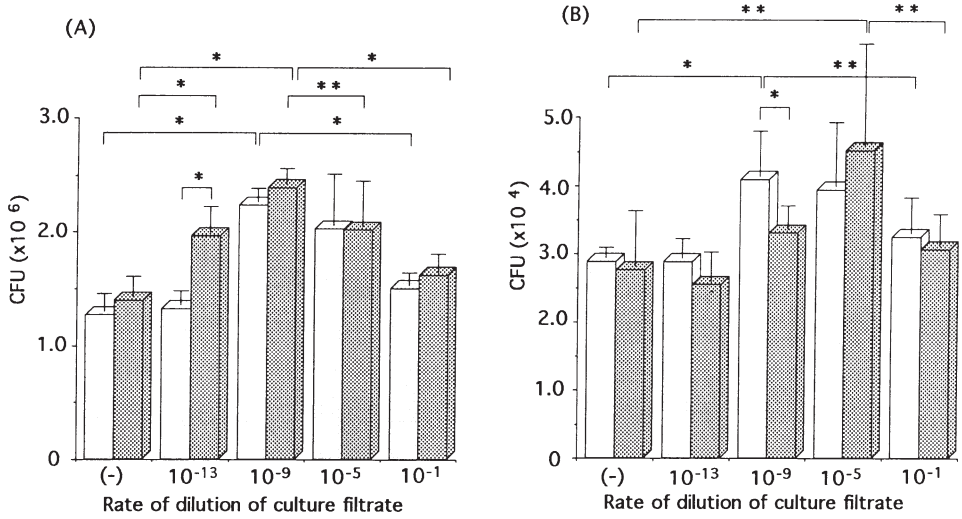

Figure 6. Infection of MCE cells by *Pseudomonas aeruginosa* IID1117. Murine corneal epithelial cells were treated with culture filtrates of Pa IID1117 or IID1130, and then association (**A**) and invasion (**B**) were assessed. (−): treatment of cells with fresh medium. $*P < .01$, $**P < .05$.

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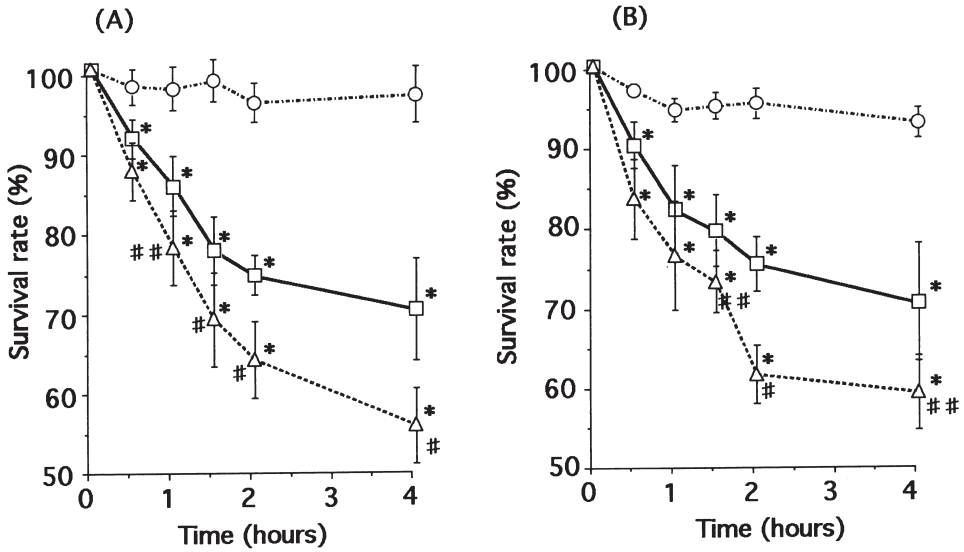

Figure 7. Cytotoxicity test. KB cells (**A**) and murine corneal epithelial simian virus 40-transformed cells (**B**) were incubated with culture filtrates of Pa IID1117 (□) and Pa IID1130 (△), respectively, at a final concentration of 10%. Control experiment (○) was done by adding 10% fresh medium. At various intervals, viable cell numbers were assessed by trypan blue dye exclusion test. Each point represents the mean and standard deviation of three separate experiments performed in duplicate. $^{*}P < .01$, $^{**}P < .05$; both versus survival rate of control cells. $^{\#}P < .01$, $^{\#\#}P < .05$; both versus survival rate of KB cells or MCE cells treated with culture filtrate of Pa IID1117.

thelial cells,[10] SV40-transformed rabbit corneal epithelial cells[17,18] and a human bronchial cell line (BEAS-2B, ATCC CRL9609)[19] have been used for *P. aeruginosa* infection. We established SV40-transformed murine corneal epithelial cells (MCE cells) and used them, along with a cloned epithelial cell line from a human nasopharyngeal carcinoma (KB cells), for testing the infection by *P. aeruginosa.*

There was no difference between the association of Pa IID1117 and Pa IID1130 with MCE cells or KB

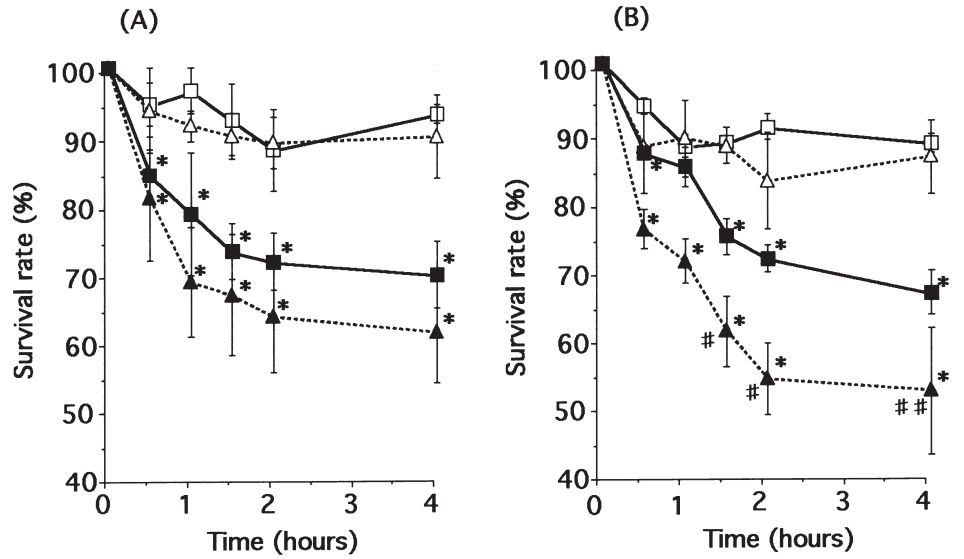

Figure 8. Cytotoxicity test of fractionated culture filtrate. KB cells (**A**) and simian virus 40-transformed murine corneal epithelial cells (**B**) were incubated with fraction with molecular weight of less than 10 kilodaltons (<10 kDa fraction) (□) and >10 kDa fraction (■) of culture filtrates of Pa IID1117, or with <10 kDa fraction (△) and > 10 kDa fraction (▲) of culture filtrate of Pa IID1130. Viable cell numbers were assessed. Symbols are same as shown in Figure 7. $^{*}P < .01$, $^{**}P < .05$, $^{\#}P < .01$, $^{\#\#}P < .05$.

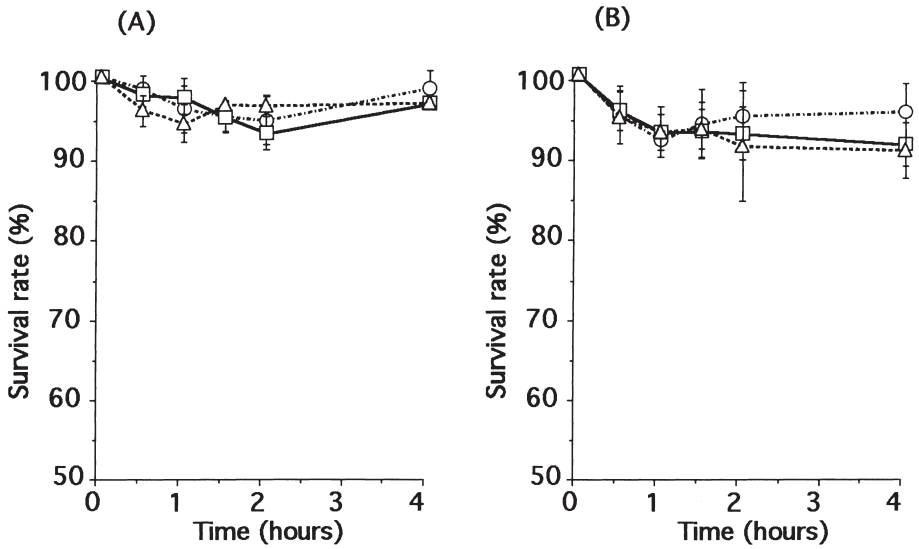

Figure 9. Cytotoxicity test of heat-inactivated culture filtrate. Pa IID1117 (□) and IID1130 (△) culture filtrates, and fresh medium (○) were heated at 95°C for 30 minutes. Target cells were KB cells (**A**) and murine corneal epithelial (MCE) cells (**B**), and number of viable cells was assessed after various times of incubation with heat-treated culture filtrates.

cells, and between the invasion of Pa IID1117 and Pa IID1130 into MCE cells. However, invasion of Pa IID1117 into KB cells was more efficient than that of Pa IID1130 into the same type of cells (Figure 2B). Protease may play a role in invasion into KB cells. Moreover, the invasion of both bacteria was more efficient into MCE cells than into KB cells. These differences might be due to species differences of the original tissues, MCE cells being from mouse corneal epithelium and KB cells from human epithelial cells of nasopharyngeal carcinoma. It is well-known that the receptor molecules against *P. aeruginosa* products are found on the surface of murine cells, but that no receptor is found on the human cells. The *P. aeruginosa* products, when bound to the receptors on murine cells, might promote the association and invasion of bacteria.

It has been reported that *P. aeruginosa* exoproducts could contribute directly to the keratitis caused by *P. aeruginosa* through the toxic effects on corneal cells, and indirectly through the activation of corneal proteases.[20–22] We examined whether the association of bacteria with cells was affected by the bacterial exoproducts released into the culture medium. In view of the results of the association experiments, it might be considered that the culture filtrate of Pa IID1130 contained association-promoting products other than proteases that acted efficiently on KB cells. These findings were also supported by the fact that pretreatment of the bacteria with protease inhibitor did not have any effect on the association between the bacteria and the cells (Figure 3A).

The mechanism of the association of *P. aeruginosa* with the corneal epithelium is not yet clear. Panjwani's study showed that the phosphatidylserine and phosphatidylinositol present in mucous or on the cell surface may function as receptors.[23] It is possible that the receptor molecule is exposed by bacterial enzymes or toxins, and that the bacteria might thereby be able to attach easily to the cells.

The association and invasion of Pa IID1117 into MCE cells and KB cells was maximal at a dilution of the culture filtrate of 10^9- or 10^5-fold (Figures 5B and 6B). The cells were damaged at higher concentrations of the culture filtrates, and detached easily from the surface of the plate. Therefore, it may be considered that the cellular damage by the culture filtrate was induced in a dose-dependent manner. The invasion of Pa IID1117 into MCE cells pretreated with the culture filtrates of Pa IID1117 and Pa IID1130 was significantly greater than that into the culture filtrate-treated KB cells. The invasions of bacteria into both cell lines pretreated with the culture filtrate of Pa IID1117 at a dilution of 10^9-fold were significantly greater than those into the same cells pretreated with the culture filtrate of Pa IID1130. However, there was no difference in the number of CFU between the culture filtrate of Pa IID1117 and Pa IID1130 at a dilution of 10^5-fold. In these experiments, invasion of Pa IID1117 into KB cells and MCE cells increased when the cells were pretreated with the culture filtrate. Thus, the exoproducts in the culture filtrate might play a role in damaging the cells, and along with proteases, the bacteria could easily invade the cells.

The invasion of protease inhibitor-treated Pa IID1117 into MCE cells was significantly less efficient than that of nontreated Pa IID1117 into the same cells, indicating that invasion of Pa IID1117 is dependent on the bacterial protease activity. The protease inhibitor

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used in these experiments was a cocktail of inhibitors of serine protease, cysteine protease, aspartic protease, metallo-protease and aminopeptidase according to the manufacturer's description. An inhibitor of matrix metallo-proteinase, which promotes the repair of tissue damage, also inhibited *P. aeruginosa* protease activity.[24]

Using the trypan blue dye exclusion method, it was found that the cytotoxicity caused by the culture filtrate of Pa IID1130 was stronger than that caused by the filtrate of Pa IID1117 (Figure 7). These cytotoxic substances in the culture filtrate of Pa IID1130 might be proteins with a molecular weight of >10 kDa. According to the IID list of the culture collection, Pa IID1117 is a protease-positive and elastase-positive strain, while Pa IID1130 is a protease-negative and elastase-positive strain. However, no details of the other exoenzymes and exotoxins are mentioned. It is possible that Pa IID1130 might produce exoenzymes and/or exotoxins that are more toxic for the cultured cells than those produced by Pa IID1117. These substances might include exotoxin A, exoenzyme S, or the hemolysins (phospholipase C and rhanmolipid).[11]

By using primary murine corneal epithelial cells and *P. aeruginosa* isolates, Fleiszig et a1[10] found that a significant inverse correlation was observed between the ability to induce cytotoxicity and epithelial cell invasion, and suggested that there were two different types of *P. aeruginosa*-induced disease; one caused by strains that were cytotoxic and the other involving bacteria that could enter epithelial cells. Comparing these results with our results, it might be considered that Pa IID1117 is an invasive strain and Pa IID1130 is a cytotoxic strain.

In this experiment, our results suggest that protease plays an important role in invasion into the cells and that some of the exoproducts other than proteases can be cytotoxic and act on the association and invasion. Further studies are needed on the isolation of active substances from the culture filtrate, which might promote association and invasion in the early stage of pseudomonal infection.

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