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ORIGINAL ARTICLE

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Pretreatment of *Pseudomonas aeruginosa* with a sub-MIC of imipenem enhances bactericidal activity of neutrophils

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Abstract The influence of the pretreatment of *Pseudomonas aeruginosa* strain O1 (PAO-1) with a sub-minimum inhibitory concentration (MIC) of imipenem on bactericidal activity, phagocytosis, the production of oxygen radical intermediates, and the induction of apoptosis in murine peritoneal neutrophils, as well as the catalase activity in the bacteria in comparison with that of ceftazidime-treated bacteria were studied. Bacteria treated with imipenem at $\frac{1}{4}$ MIC were killed at significantly higher rates by neutrophils than ceftazidime-treated and nontreated bacteria. However, antibiotic-treated bacteria showed similar numbers of bacteria-phagocytized neutrophils to those in untreated bacteria. Imipenem pretreatment of bacteria led to an increase in the production of oxygen radical intermediates by neutrophils and the inhibition of neutrophilic apoptosis following incubation, whereas these features did not occur in neutrophils incubated with nontreated and ceftazidime-treated bacteria. The catalase activity of bacteria was not suppressed by pretreatment with either antibiotic at $\frac{1}{4}$ MIC. These findings suggest that the exposure of *P. aeruginosa* to a sub-MIC of imipenem enhances the susceptibility of the bacteria to neutrophilic killing and effectively modifies the physiological activities of neutrophils, but does not decrease bacterial catalase activity. These actions may account for the postantibiotic leukocyte enhancement (PALE) effect of a sub-MIC of imipenem in the host.

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Introduction

The minimum inhibitory concentration (MIC) and a variety of other parameters are used to express the concentrations of antibiotics that inhibit growth or kill clinically significant numbers of bacteria in vitro. However, clinical efficacy is often observed even when an antibiotic is present at a sub-MIC within a site of bacterial infection.[1–3] Such in vivo activity is considered to be attributable, not to the direct bactericidal effect of the antibiotic, but rather, to the postantibiotic effect (PAE) that is observed in vitro.

The PAE is defined as the suppression of bacterial growth that persists after a short period of exposure of microorganisms to antibiotics, and the effect is often observed with protein synthesis inhibitors such as aminoglycosides, macrolides, quinolones, and carbapenems.[2,4–7] In vivo, the PAE is considered to be associated with the influence of antimicrobial agents on bacterial structure and metabolism, which causes microorganisms to become more susceptible to killing by humoral and cellular defense factors such as complement, antibodies, and phagocytes. The PAE involving phagocytes is referred to as postantibiotic leucocyte enhancement (PALE).[6,7]

Imipenem is a carbapenem antibiotic that is effective against *Pseudomonas aeruginosa*, a major pathogen causing opportunistic infections in both immunocompetent and immunocompromised hosts. Imipenem is known to induce an in vitro PAE for *P. aeruginosa* at concentrations of four to ten times its MIC.[5,8] However, very little has been reported about the PALE effect of imipenem at sub-MICs against *P. aeruginosa*,[9] and the mechanism of its PALE has not been elucidated completely. In the present study, we compared the PALE of imipenem at a sub-MIC against *P. aeruginosa* via neutrophils with that of ceftazidime by using an in vitro bactericidal assay with murine peritoneal neutrophils. This was done in order to investigate the cellular

mechanisms of PALE which result from the interaction between neutrophils and bacteria pretreated with a sub-MIC of antibiotic.

Materials and methods

Antibiotics

Imipenem (Banyu Pharmaceutical, Tokyo, Japan) and ceftazidime (Glaxo SmithKline, Tokyo, Japan) were used. Imipenem was dissolved in 0.05 M 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.2), and ceftazidime was dissolved in 0.05 M phosphate buffer (pH 7.5) at appropriate concentrations.

Bacterial strains

P. aeruginosa strain O1 (PAO-1) and *Escherichia coli* strain NIHJ were used. The bacteria were cultured in Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, MI, USA), with continuous shaking at 37°C for 18 h, centrifuged, and suspended in Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA). The bacterial concentration was measured by determining the absorbance of a bacterial suspension at 530 nm. The number of viable bacteria, determined by plating 0.2 ml of an appropriate dilution of the suspension on Mueller-Hinton agar (MHA; Difco) plates, was expressed as cfu/ml.

Killed *E. coli*, which were used as an inducer of neutrophils, were prepared by incubating the bacterial suspension in 0.6% formalin for 2 h at room temperature, followed by centrifuging, two washes with PBS, and suspension in PBS, at a final concentration of 10^8/ml.

Animals

Six-week-old male ddy mice were purchased from Japan SLC (Shizuoka, Japan) and maintained in a specific pathogen-free environment at the Laboratory Animal Centre for Medical Science. Experiments were permitted by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine and were performed according to the ethical guidelines of the Committee. The mice were killed by carbon dioxide asphyxiation.

Preparation of neutrophils

Neutrophils were collected by lavage – using cold Hank's balanced salt solution (HBSS; Nissui Pharmaceutical, Tokyo, Japan) containing 10 units heparin (Pharmacia Upjohn, Tokyo, Japan) – of the peritoneal cavity of mice 6 h after a peritoneal injection of 1 ml of killed bacteria. After three washes with cold HBSS, the cells were resuspended in HBSS. Viability of the cells was evaluated by trypan blue (Sigma). More than 95% of the cells consisted of characteristic neutrophils, as determined by morphologic observation of Giemsa (Merck Japan, Tokyo, Japan) staining.

Pretreatment of *P. aeruginosa* with sub-MIC of antibiotics

To avoid the influence of antibiotics that are absorbed by neutrophils, bacteria were pretreated with a sub-MIC of each antibiotic. The MIC was determined according to the National Committee for Clinical Laboratory Standards recommended method for agar dilution.[10] The MICs of imipenem and ceftazidime against PAO-1 were 6.25 μg/ml and 0.78 μg/ml, respectively. Cultured bacteria (0.1 ml) were cultured in 10 ml of either MHB as a control, or MHB containing a final concentration of $\frac{1}{4}$ MIC of imepenem or ceftazidime, which caused morphological changes, with gentle shaking for 4 h. The bacteria were centrifuged, washed twice with PBS, and suspended in PBS at a final concentration of 2×10^8 cfu/ml. After treatment with a sub-MIC of each antibiotic, smears stained with methylene blue (Sigma) were examined by light microscopy (BH-2; Olympus, Tokyo, Japan). Shapes of imipenem- and ceftazidime-pretreated bacteria were noted as spherical or filamentous forms. The filamentous form was approximately two times larger than the size of nontreated bacteria.

Assay for the bactericidal activity of neutrophils

The PALE of antibiotics at a sub-MIC against PAO-1 was evaluated by an in vitro killing assay. Briefly, 5×10^5 neutrophils suspended in 1 ml of warmed HBSS containing 10% fresh serum obtained from syngeneic mice and 0.1% gelatin were mixed with antibiotic-treated or nontreated bacteria in culture tubes coated with silicon (Sigma). The ratio of bacteria to neutrophils (multiplicity of infection; MOI) was adjusted to 1:1 (MOI, 1). The tubes were incubated in a shaking water bath at 37°C for 4 h. Aliquots were taken in triplicate from the incubation mixtures at 0, 60, 120, and 240 min. The number of viable bacteria in each sample was determined by plating 0.1 ml of an appropriate dilution of the sample on MHA plates and converting into log scale. The values for results are expressed as the mean log cfu/ml ± SD in triplicate cultures.

Assay for phagocytosis by neutrophils

The phagocytosis of antibiotic-treated and nontreated bacteria by neutrophils was assayed by fluorescence microscopy. Briefly, neutrophil suspensions (5×10^5/ml) in HBSS containing 10% mouse serum were mixed with bacteria stained with Syto 9 dye (green fluorescent, for staining live cells) and propidium iodide (PI; red fluorescent, for staining dead cells) (LIVE/DEAD Bac Light Bacterial viability kits; Molecular Probes, Eugene, OR, USA) at an MOI of 5 at 37°C for 10 min and centrifuged at 900 *g* at 4°C for 10 min to remove unbound bacteria. The cell pellet was resuspended in 1 ml of PBS and cell numbers were counted by fluorescence microscopy (BX-60; Olympus), using a hemocytometer at 100 × magnification. We defined a neutrophil incorporating more than two bacteria as a bacteria-phagocytized neutrophil. The viable bacteria-phagocytized neutrophils could be readily distinguished from free neutro-

phils. Values for results were expressed as the mean number of bacteria-phagocytized neutrophils ± SD in triplicate counting.

Assay for production of oxygen radical intermediates (ORI)

The production of ORI by neutrophils was measured according to the chemiluminescence method,[11] with luminol (Sigma), using phorbol 12-myristate 13-acetate (PMA, Sigma) as a trigger. In brief, 0.2 ml of the reaction mixture, containing 10^5 neutrophils in HBSS supplemented with 0.2 mM luminol, was preincubated with antibiotic-treated or nontreated bacteria at an MOI of 5 in a black microplate (Thermo Labsystems, Franklin, MA, USA). The chemiluminescence of luminol was measured by using Fluoroskan (Ascent FL; Thermo Labsystems) every 2 min at 37°C for 60 min following the addition of 0.1 μg/ml of PMA to the reaction mixture. Values for results were expressed as the percent chemiluminescence of luminol calculated using the following formula: chemiluminescence of luminol at 30 and 60 min after incubation ÷ that at 0 min × 100.

Assessment of neutrophil apoptosis

To quantify neutrophils apoptosis in response to bacterial infection, neutrophils were specifically stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI (Apoptosis detection kit; Wako Pure Chemical Industries, Osaka, Japan) to detect apoptotic or dead cells following incubation. Neutrophils were incubated with/without antibiotic-treated or nontreated bacteria at an MOI of 1, with gentle shaking at 37°C for 60 min. After 30 and 60 min, the percentage of apoptotic cells was determined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Two-parameter histograms are shown in quadrants 1–4 of Fig. 4. In Fig. 4, quadrant 2, with high Annexin V and high PI staining, was the late stage of apoptotic cells, while quadrant 4, with high Annexin V and low PI staining, was the early stage of apoptotic cells.

Assay for catalase activity of *P. aeruginosa*

The catalase activity of antibiotic-treated and nontreated bacteria was assayed by a modification of the method of Beers and Sizer.[12] Briefly, bacteria were centrifuged, suspended in 67 mM phosphate buffer (pH 7.2), and destroyed by sonication for the preparation of crude enzymic materials. The reaction mixture (3 ml), containing 0.1 M phosphate buffer and 10 mM H_2O_2, was preincubated in a quartz cuvette at 37°C for 5 min. A material sample (30 μl) was added to the reaction mixture. The reduction rate of H_2O_2 (ΔA/min) was measured continuously by recording the adsorption decrease at 240 nm at 37°C for 10 min. Protein concentrations (Pc) were determined by the method of Biuret[13] and expressed as mg/ml. Values for results were expressed as the reduction rate of H_2O_2 per mg protein of sample (μmole/min per mg), calculated by using the following formula: ΔA/min × 3 ÷ 0.0394 (millimolar absorption coefficient of H_2O_2) ÷ Pc.

Statistical analysis

The statistical significance of differences between the two experimental groups was determined by Wilcoxon's *t*-test. A P value of less than 0.05 was considered significant.

Results

Bactericidal activity of neutrophils against antibiotic-treated *P. aeruginosa*

To examine whether pretreatment of bacteria with a sub-MIC of antibiotic enhanced bactericidal activity by neutrophils, the time course of in vitro bacterial growth in cultures of antibiotic-treated and nontreated *P. aeruginosa* was assessed in the presence or absence of neutrophils. The bacterial numbers in cultures for each experiment were increased for the 3-h incubation period. However, as shown in Fig. 1a, the number of bacteria treated with $\frac{1}{4}$ MIC of imipenem was significantly decreased in the presence of neutrophils when compared with the number in the absence of neutrophils; there was a mean reduction of log 0.242, log 0.277, and log 0.235 cfu/ml after 1 h, 2 h, and 3 h of incubation, respectively. Similar significant results were also observed when bacteria were pretreated with $\frac{1}{8}$ MIC of imipenen, but not when the bacteria were not pretreated with antibiotic (Fig. 1b) or when they were treated with less than $\frac{1}{8}$ MIC (data not shown). However, no significant differences were observed between the growth rates of $\frac{1}{4}$ MIC ceftazidime-treated and nontreated bacteria in the presence or absence of neutrophils (Fig. 2a,b). The mean reduction in the number of ceftazidime-treated bacteria was log 0.1, log 0.07, and log 0.1 cfu/ml after 1 h, 2 h, and 3 h of incubation, respectively. These results revealed that imipenem pretreatment at a sub-MIC rendered *P. aeruginosa* more amenable to the bactericidal activity of neutrophils.

The cytotoxicity of antibiotic-treated or nontreated bacteria to neutrophils was also evaluated, by staining viable cells with trypan blue at intervals of 60 min after incubation. At 1 h after incubation, the percentages of viable cells were 81.2%, 84.2%, and 78.1% in imipenem-treated, ceftazidime-treated, and nontreated bacteria, respectively. There was no significant difference between antibiotic-treated and nontreated bacteria. Subsequently, the viable percentage of neutrophils in these respective bacteria was almost the same.

Phagocytosis of antibiotic-treated *P. aeruginosa* by neutrophils

To examine whether the morphologic changes in bacteria caused by pretreatment with a sub-MIC of antibiotics had

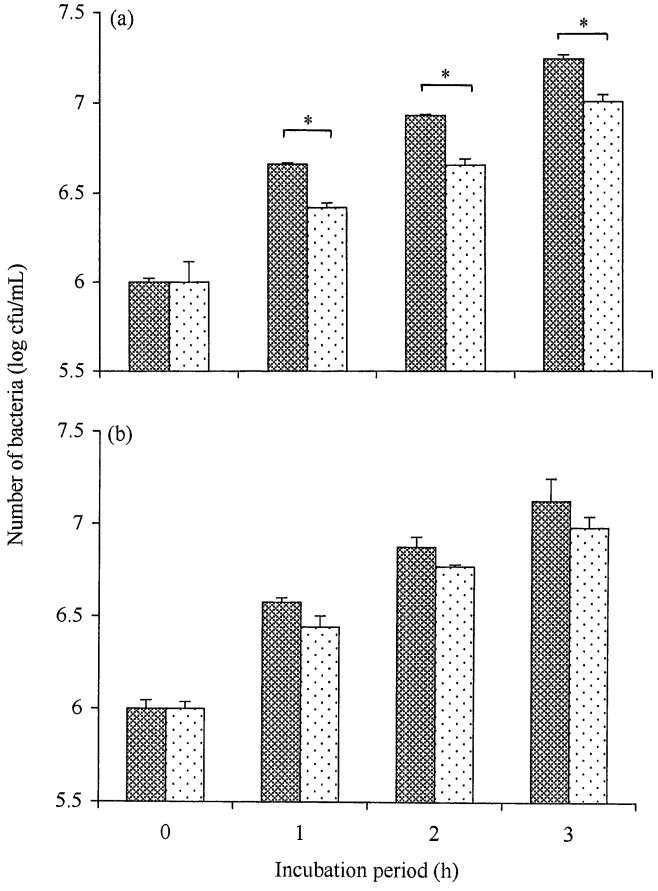

Fig. 1a,b. Bactericidal activity of neutrophils against imipenem-treated *Pseudomonas aeruginosa* at a sub-minimum inhibitory concentration (MIC). **a** $\frac{1}{4}$ MIC-treated, and **b** nontreated bacteria were incubated in the presence (*dotted bars*) or absence (*net patterned bars*) of neutrophils at a multiplicity of infection (MOI) of 1. Values for results are expressed as the mean log cfu/ml ± SD of bacteria in triplicate cultures. * Significant compared with the absence of neutrophils ($P < 0.05$)

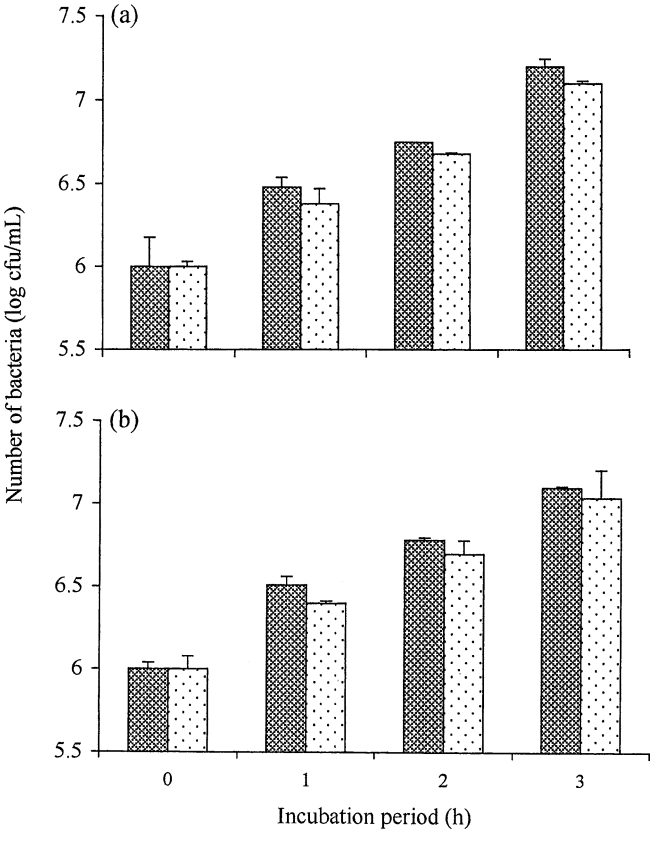

Fig. 2a,b. Bactericidal activity of neutrophils against ceftazidime-treated *Pseudomonas aeruginosa* at a sub-MIC. **a** $\frac{1}{4}$ MIC-treated and **b** nontreated bacteria were incubated in the presence (*dotted bars*) or absence (*net patterned bars*) of neutrophils at an MOI of 1. Values for results are expressed as the mean log cfu/ml ± SD of bacteria in triplicate cultures

an influence on the phagocytosis of bacteria by neutrophils, fluorescence-labeled bacteria were incubated with neutrophils. As shown in Table 1, imipenem- and ceftazidime-treated bacteria were phagocytized by neutrophils to the same extent as that shown for the respective nontreated bacteria. The percent phagocytosis of bacteria treated with each antibiotic, and the nontreated bacteria, was always in the range of 10% to 23%. We then compared the number of phagocytized antibiotic-treated bacteria per neutrophil with that of nontreated bacteria. There was no significant difference between antibiotic-treated and nontreated bacteria (the average number of bacteria per bacteria-phagocytized neutrophil was four; data not shown).

Effect of antibiotic-treated *P. aeruginosa* on production of ORI by neutrophils

After ingesting bacteria and other foreign materials, neutrophils rapidly produce ORI with bactericidal activity. We measured the production of ORI by neutrophils after the phagocytosis of antibiotic-treated and nontreated bacteria. As shown in Fig. 3a, the production of ORI by neutrophils 30 min after the phagocytosis of imipenem-treated bacteria was significantly increased over that for nontreated bacteria. Imipenem generated luminol chemiluminescence in a dose-dependent manner (data not shown). However, the production of ORI by neutrophils after the phagocytosis of ceftazidime-treated bacteria gradually decreased in proportion to incubation time, and was lower than that after the phagocytosis of nontreated bacteria (Fig. 3b).

Effect of antibiotic-treated *P. aeruginosa* on induction of neutrophil apoptosis

In general, many neutrophils progressively undergo apoptosis in systems of in vitro culture. However, suppression of neutrophil apoptosis is reported to occur after the phagocytosis of certain extracellular bacteria.[14] Whether the suppression of neutrophilic apoptosis was related to PALE against imipenem-treated bacteria was investigated using flow cytometric analysis with Annexin V-PI. The percentages of apoptotic neutrophils at 30 min after the phagocytosis of imipenem-treated bacteria was lower than that observed after the phagocytosis of nontreated bacteria

Table 1. Phagocytosis of antibiotic-treated and nontreated *Pseudomonas aeruginosa*

Antibiotic	Concentration	Number of bacteria-phagocytized neutrophils ± SD ($\times 10^4$/ml)
NT	0 MIC	30.0 ± 6.68
Imipenem	$\frac{1}{4}$ MIC	30.0 ± 6.68
NT	0 MIC	19.5 ± 2.38
Ceftazidime	$\frac{1}{4}$ MIC	20.0 ± 1.83

NT, no treatment with antibiotics; MIC, minimum inhibitory concentration

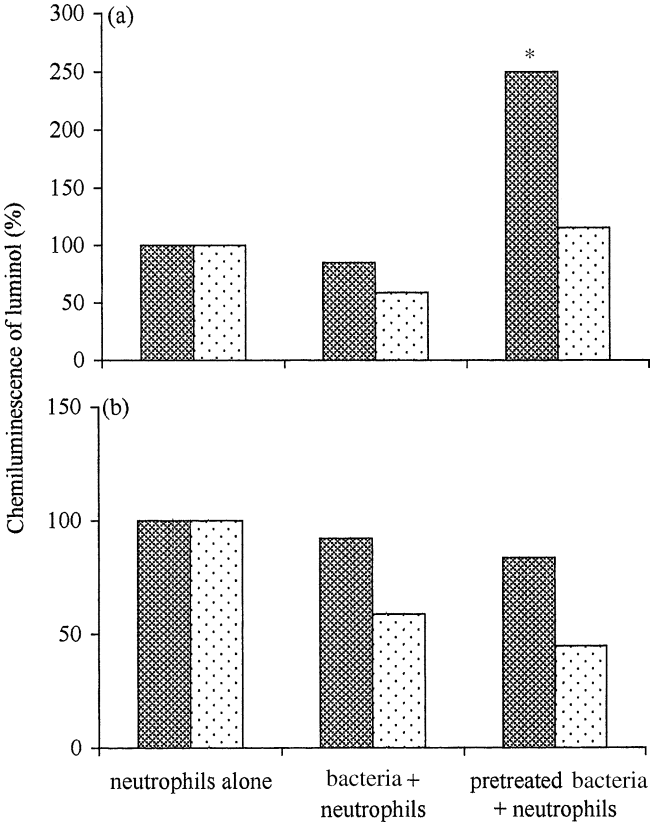

Fig. 3a,b. Effect of antibiotic-treated *Pseudomonas aeruginosa* on production of oxygen radical intermediates. Neutrophils were incubated with or without **a** bacteria pretreated with $\frac{1}{4}$ MIC imipenem or **b** bacteria pretreated with ceftazidime at $\frac{1}{4}$ MIC and nontreated bacteria. Values for results are expressed as the percent chemiluminescence of luminol at 30 min (*net patterned bars*) and 60 min (*dotted bars*) after incubation. *, Significant compared with neutrophils alone ($P < 0.05$)

(Fig. 4a). In contrast, an increase in the percentage of apoptotic cells was observed after the phagocytosis of ceftazidime-treated bacteria, with the percentage being higher than that of nontreated bacteria (Fig. 4b).

Effect of pretreatment with a sub-MIC of antibiotic on catalase activity in *P. aeruginosa*

To determine whether pretreatment with antibiotic at a sub-MIC changed bacterial catalase activity, the activity was determined by determining the reduction rate of H_2O_2. Bacteria pretreated with imipenem showed significantly

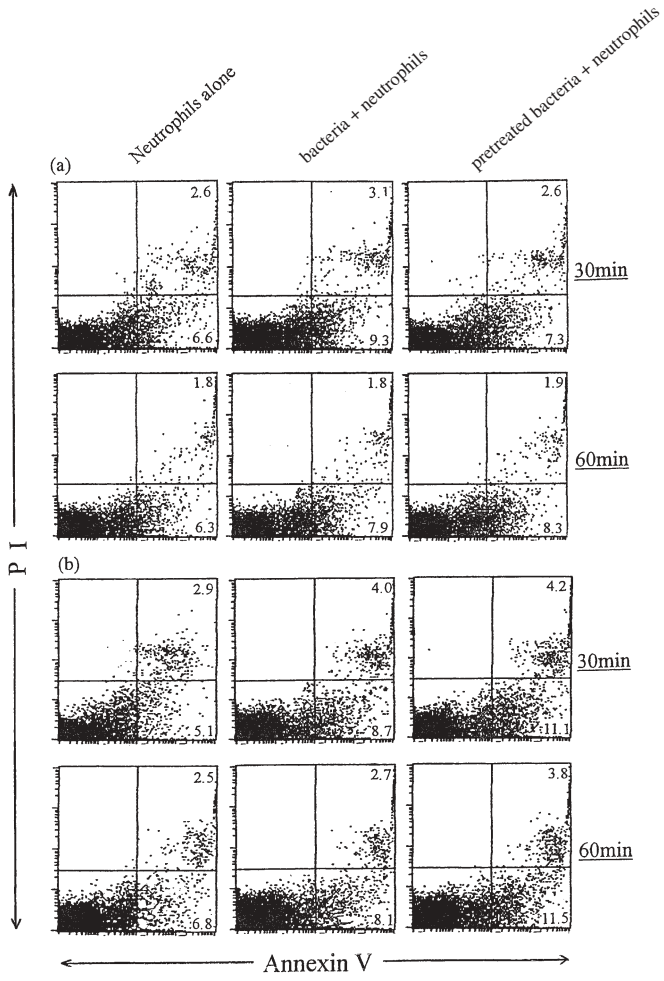

Fig. 4a,b. Comparison of induction of neutrophilic apoptosis after phagocytosis of antibiotic-treated and nontreated *Pseudomonas aeruginosa*. Neutrophils were incubated with or without $\frac{1}{4}$ MIC of **a** imipenem- or **b** ceftazidime-treated and nontreated bacteria and stained with Annexin V and propidium iodide (*PI*). The figures show the percentages of apoptotic cells at early (*quadrant 4*) and late stages (*quadrant 2*)

higher increases in catalase activity than nontreated bacteria, in a dose-dependent manner ($\frac{1}{8}$ MIC, 5.29 ± 0.51; $\frac{1}{4}$ MIC, 6.49 ± 0.71; control, 4.34 ± 0.22 μmole/min/mg). However, the catalase activity of ceftazidime-treated bacteria did not change in comparison with that in nontreated bacteria ($\frac{1}{4}$ MIC, 4.72 ± 0.31; $\frac{1}{2}$ MIC, 4.65 ± 0.23; control, 4.32 ± 0.31 μmole/min/mg).

Discussion

Post antibiotic effect and PALE are often offered as explanations for the phenomenon that antibiotics exert antibacterial activity at sub-MICs in vivo. PALE is mainly known to occur with aminoglycosides, macrolides, and carbapenems. In general, it is thought that PALE results from intracellular penetration, causing changes to the surface structures of bacteria (such as hydrophobicity and adhesiveness) or influencing the production or secretion of

exotoxins and enzymes.[2,4,5,15] However, the bacterial characteristics and host defense factors which mediate the presence or absence of PALE with these antibiotics has not been elucidated. In the present study, we compared the PALE of imipenem at sub-MICs against *P. aeruginosa* with that of ceftazidime, and attempted to investigate the cellular mechanism of PALE. Pretreatment with imipenem at a sub-MIC induced a significantly greater increase in PALE against *P. aeruginosa* than that induced by ceftazidime.

The differences in the antimicrobial activities of imipenem and ceftazidime against gram-negative bacteria are based on differences of binding affinity to the penicillin-binding protein (PBP) between the two antibiotics.[5] Imipenem binds more strongly to PBP-2, which is involved in the alteration of bacterial morphology, so that it causes rods to take on a spherical form. By contrast, ceftazidime binds more strongly to PBP-3 and PBP-1a and preferentially inhibits septation, resulting in alteration of morphology to elongated filamentous forms. Upon the removal of ceftazidime, rapid septation probably occurs (up to 12 separate cells per filament), as shown with piperacillin-induced *E. coli* filaments.[16] It seems that the rapid increase in bacterial numbers results in impairment of PAE.[5] However, the present study showed that the increased number of ceftazidime-treated *P. aeruginosa* (1.1- to 1.6-fold increase) was similar to that of nontreated bacteria (1.1- to 1.7-fold) after incubation with/without neutrophils. This finding indicates that a rapid increase in bacterial number may not be responsible for the impairment of PALE by neutrophils against ceftazidime-pretreated *P. aeruginosa*, and the spherical form of *P. aeruginosa* may be more susceptible to killing by neutrophils.

There is some evidence that the activity of catalase in bacteria like *P. aeruginosa*, known as scavengers of ORI, is related to virulence[17,18] and is suppressed subsequent to pretreatment with cell-wall active antibiotics at sub-MICs.[15] Based on these findings, we speculated that a decrease in catalase activity after pretreatment with antibiotics was responsible for the enhancement of bacterial killing by neutrophils. However, we showed a tendency toward increasing catalase activity in imipenem-treated bacteria in proportion to the sub-MIC of the antibiotic. Furthermore, there is a report that the enhancement effect of cell-wall active antibiotics at sub-MICs on bacterial killing by granulocytes was dependent on the bacterial strains and independent of catalase activity.[19] These findings indicate that the activity of catalase in PAO-1 does not directly influence the enhancement of PALE by neutrophils.

The four neutrophil-related factors that induce PALE against imipenem-treated bacteria are: (1) enhanced bacterial phagocytosis by neutrophils, (2) enhanced generation of ORI by neutrophils, (3) suppression of bacterial cytotoxicity against neutrophils, and (4) increased complement-dependent cellular killing by neutrophils. (1) Antibiotic-treated bacteria showed similar numbers of bacteria-phagocytized neutrophils to those in nontreated bacteria. Therefore, the sub-MIC of antibiotics had no influence on bacterial phagocytosis that was mediated by scavenger receptors. This finding is in agreement with an earlier report that enhanced phagocytosis is not involved in the occurrence of PALE.[2] (2) A significant increase in the generation of ORI was observed only in neutrophils which phagocytized imipenem-treated bacteria. Taken together with previous observations that the activation and enhanced generation of ORI play an important role in antimicrobial activity against *P. aeruginosa*,[20,21] we can speculate that increased ORI production may only be involved in the enhancement of PALE by pretreatment with imipenem at sub-MIC against *P. aeruginosa*. (3) The cytotoxicity of PAO-1 against neutrophils was not significantly suppressed by pretreatment with/without imipenem or ceftazidime. It is not possible that enhancement of PALE against imipenem-treated bacteria occurred in association with the suppression of bacterial cytotoxicity against neutrophils, and this has been noted previously.[22] (4) It has been reported that imipenem at sub-MICs increased complement-dependent cellular killing of bacteria such as *E. coli*, *P. aeruginosa*, and *Serratia marcescens*.[23] However, we found that inactivation (treatment at 56°C for 30 min) of fresh mouse serum before use did not cause any change in the enhancement of PALE by imipenem (data not shown). Therefore, it appears unlikely that the sequential activation of complement by imipenem-treated bacteria has an influence on PALE.

In this study, the percentage of apoptotic neutrophils after the phagocytosis of imipenem-treated bacteria was lower than that observed after the phagocytosis of ceftazidime-treated bacteria and nontreated bacteria. *P. aeruginosa* possesses a type-III secretion system and multidrug-resistant efflux systems (MexAB-OprM), dedicated to overcoming the host defense.[22,24–26] These systems allow the translocation of four cytotoxins (ExoS, ExoT, ExoU, and ExoY) and invasive factor(s) into the eucaryotic cytosol and cause invasion, apoptosis, or intoxication of target cells.[22,24–28] Indeed, we have observed that the invasion of imipenem-treated PAO-1 at sub-MICs was markedly decreased compared with that of ceftazidime-treated or nontreated bacteria, using a gentamicin survival assay with MDCK cells[29] (data not shown). Furthermore, it has been reported that neutrophils which phagocytized *P. aeruginosa* or *Staphylococcus aureus* did not become apoptotic.[14] Our findings in the present study, taken together with these lines of evidence, suggest that pretreatment of *P. aeruginosa* with imipenem at a sub-MIC may markedly delay the induction of neutrophilic apoptosis through the type-III secretion system[30] as compared with findings for ceftazidime-treated and nontreated bacteria, and such a suppressive effect of imipenem pretreatment on neutrophilic apoptosis may contribute to the enhancement of PALE.

There are reports of excessive influxes of neutrophils, at sites of infection with *P. aeruginosa*, which are unable to eliminate the bacteria.[27,28] The phagocytized bacteria may temporarily downregulate the production of cytokines, which influences the survival of phagocytes and produces cytotoxins through the apparatuses mentioned above, causing host-cell intoxication. Therefore, the effect of imipenem at sub-MICs on cytokine production by neutrophils and on cytotoxin production by bacteria needs to be investigated further.

Novelli and coworkers[7] showed that pretreatment of *P. aeruginosa* with 4 MIC of meropenem produced a greater PALE than that with imipenem. The final reasons for the greater effect of meropenem than that of imipenem are not clear. However, taking into consideration the finding that meropenem exhibits higher affinity for all PBPs except PBP-1A in *P. aeruginosa* than imipenem,[31] its enhanced PALE may be related to phenotypic modifications with changes on the bacterial cell surface, causing bacteria to be more susceptible to phagocytic killing.

In conclusion, this study has demonstrated that imipenem, at a sub-MIC, shows enhancement of PALE on *P. aeruginosa*. However, the clinical significance of the PALE of imipenem at sub-MICs has not been established. Based on evidence that carbapenems have rapid initial bactericidal activity against *P. aeruginosa*[32] and the ability to produce a significant PAE against *P. aeruginosa*,[4,5,33] an accurate understanding of these features may assist in the design of more rational dosage schedules for carbapenems.

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