Efficacy of Calcium-EDTA as an Inhibitor for Metallo-β-Lactamase in a Mouse Model of Pseudomonas aeruginosa Pneumonia

Nobumasa Aoki,1,2 Yoshikazu Ishii,1* Kazuhiro Tateda,1* Tomoo Saga,1 Soichiro Kimura,1 Yoshiaki Kikuchi,1 Tetsuo Kobayashi,2 Yoshinari Tanabe,2 Hiroki Tsukada,2 Fumitake Gejyo,2 and Keizo Yamaguchi1

Department of Microbiology and Infectious Diseases, Faculty of Medicine, Toho University School of Medicine, Tokyo 143-8540, Japan,1 and Division of Infection Control and Prevention, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan2

Received 15 April 2010/Returned for modification 20 June 2010/Accepted 12 August 2010

In this study, we have evaluated the efficacy of calcium-EDTA (Ca-EDTA) as an inhibitor of bacterial metalloenzymes, such as metallo-β-lactamase (MBL) and other proteases, in a mouse model of Pseudomonas aeruginosa pneumonia. The simultaneous presence of Ca-EDTA (32 μg/ml) reduced the MICs of imipenem (IPM) in all MBL-producing P. aeruginosa isolates (IMP-1, -2, -7, and -10 and VIM-2) but not non-MBL-producing strains. In the pneumonia model, mice were intranasally infected with MBL-producing P. aeruginosa and kept under conditions of hyperoxia to mimic ventilator-associated pneumonia. With both intranasal and subcutaneous administrations, Ca-EDTA significantly potentiated survival benefits of IPM compared to those of IPM alone. Ca-EDTA combination therapy induced a significant reduction of the bacterial burden in the lungs (P < 0.05). Furthermore, the inhibition activity of Ca-EDTA against MBL activity was confirmed by using the purified IMP-1 enzyme, which was characterized by a 50% inhibitory concentration (IC50) of 55 ± 8.2 μM. Finally, the protective effects of Ca-EDTA were demonstrated by culture supernatant-induced epithelial cell damage and acute lung injury in mice. These data suggest the therapeutic potential of Ca-EDTA not only by the blocking of MBLs but also by neutralizing tissue-damaging metalloproteases in P. aeruginosa infections.

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections. In particular, this organism is a major cause of respiratory damage and death in patients with several types of pulmonary diseases. Recent epidemiological data demonstrated that P. aeruginosa is a leading cause of ventilator-associated pneumonia (VAP) and is associated with high mortality rates (1, 8, 33, 36). The estimated prevalence of VAP in intensive care unit (ICU) setting ranges from 5 to 67%, and the mortality rates are 24 to 76%, according to population studies (1, 8, 11, 18, 34, 35, 37). It is also well known that P. aeruginosa is intrinsically resistant to a variety of antibiotics and tends to acquire resistance during and after antimicrobial treatment (24, 45). Of several resistant mechanisms, metallo-β-lactamase (MBL)-producing P. aeruginosa is becoming a serious global concern (15, 46). MBLs confer resistance to essentially all β-lactams, including carbapenems, and their catalytic activities are generally not neutralized by commercially available β-lactamase inhibitors such as clavulanic acid and tazobactam (41). The presence of zinc in the active site of the enzymes is a characteristic feature of MBLs, which enable this group of enzymes to hydrolyze a broad range of β-lactams, even newly developed carbapenem antibiotics (26).

EDTA is a metallo-chelator and is known to have intrinsic antimicrobial activity. Such activities include direct antimicrobial effects, potentiating the activity of other classes of antibiotics, detoxication, and neutralization of bacterial toxins/enzymes. One of the recognized modes of action of EDTA is to potentiate other antibiotics by a disruption of the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria (19, 44). Through this disruption, the membrane becomes more permeable to other agents. Moreover, EDTA has striking potentiating activities with some β-lactam antibiotics against MBL-producing microorganisms, because the zinc-containing active site of MBL is inactivated by EDTA (25, 46). Interestingly, EDTA is also expected to reduce the pathogenicity and virulence of P. aeruginosa, since this organism produces a variety of metalloenzyme virulence factors such as alkaline protease and elastase (38). These metalloproteases exhibit strong proteolytic activities for cells and tissues and are likely dangerous key substances for tissue destruction and the development of infections (3, 40).

Despite these beneficial effects, the toxicity of EDTA prevented this compound from clinical use. An important exception is calcium disodium EDTA (Ca-EDTA), a complex of EDTA and the calcium ion. This compound was created for an injectable form of chelator with reduced toxicity, which has been approved for the treatment of lead intoxication (7, 23). Ca-EDTA is used intravenously, and the dose is 1,000 to 1,500 mg/m2 per day (or 25 to 75 mg/kg of body weight per day) for 5 days. It can be administered continuously or in two to four
divided doses. The major potential toxicity with Ca-EDTA is renal nephrotoxicity, neurotoxicity, and hypocalcemia. In this study, we evaluated the efficacy of Ca-EDTA as an inhibitor of bacterial metalloenzymes, such as MBL and other forms of proteases, in a mouse model of P. aeruginosa pneumonia. To our knowledge, this is the first report examining the effect of Ca-EDTA on a mouse model of multiple-drug-resistant P. aeruginosa pneumonia.

MATERIALS AND METHODS

Bacterial strains. We used P. aeruginosa PAO1 (a kind gift from Barbara H. Iglewski) and 24 clinical isolates of P. aeruginosa. Ca-EDTA was purchased from Nisshin Pharmaceutical Co. (Yamagata, Japan). Powders were dissolved in acetic acid to a concentration of 32 µg/ml for lead intoxication patients (27). MICs were determined by the broth dilution method according to the instructions of the Clinical Laboratory Standards Institute (10). P. aeruginosa ATCC 27853 was used as a reference strain.

Table 1. Effects of Ca-EDTA (32 µg/ml) on MICs of CAZ, IPM, and AMK

<table>
<thead>
<tr>
<th>Strain</th>
<th>MBL</th>
<th>CAZ</th>
<th>CAZ + Ca-EDTA</th>
<th>IPM</th>
<th>IPM + Ca-EDTA</th>
<th>AMK</th>
<th>AMK + Ca-EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 IMP-1</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>4</td>
<td>1</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>10 IMP-1</td>
<td>&gt;256</td>
<td>8</td>
<td></td>
<td>256</td>
<td>8</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>17 IMP-1</td>
<td>&gt;256</td>
<td>16</td>
<td></td>
<td>128</td>
<td>32</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>43 IMP-1</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>256</td>
<td>32</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>44 IMP-1</td>
<td>&gt;256</td>
<td>64</td>
<td></td>
<td>128</td>
<td>8</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>1630 IMP-1</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>128</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2769 IMP-2</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td></td>
<td>256</td>
<td>16</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>16 IMP-7</td>
<td>&gt;256</td>
<td>64</td>
<td></td>
<td>16</td>
<td>1</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>63 IMP-7</td>
<td>&gt;256</td>
<td>64</td>
<td></td>
<td>16</td>
<td>8</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>68 IMP-7</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>128</td>
<td>8</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>11 IMP-10</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>128</td>
<td>8</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>34 IMP-10</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
<td>256</td>
<td>16</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>40 IMP-10</td>
<td>&gt;256</td>
<td>16</td>
<td>&gt;256</td>
<td>256</td>
<td>32</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>46 IMP-10</td>
<td>&gt;256</td>
<td>16</td>
<td></td>
<td>256</td>
<td>16</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>58 VIM-2</td>
<td>64</td>
<td>2</td>
<td></td>
<td>32</td>
<td>2</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>1631 VIM-2</td>
<td>64</td>
<td>32</td>
<td></td>
<td>256</td>
<td>16</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>14 Negative</td>
<td>8</td>
<td>8</td>
<td></td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>26 Negative</td>
<td>8</td>
<td>8</td>
<td></td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>28 Negative</td>
<td>32</td>
<td>32</td>
<td></td>
<td>32</td>
<td>32</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>30 Negative</td>
<td>4</td>
<td>4</td>
<td></td>
<td>16</td>
<td>16</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>31 Negative</td>
<td>16</td>
<td>16</td>
<td></td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>32 Negative</td>
<td>32</td>
<td>32</td>
<td></td>
<td>32</td>
<td>32</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>42 Negative</td>
<td>16</td>
<td>16</td>
<td></td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>61 Negative</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td></td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>64 Negative</td>
<td>32</td>
<td>32</td>
<td></td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

P. aeruginosa pneumonia model in the setting of hyperoxia. BALB/c mice (female, 6 to 8 weeks old) were purchased from Charles River Japan. Mice were quarantined for 1 week after receipt. Mice were housed in separate cages under a constant temperature (23°C) with a 12-h light/dark cycle and were given standard laboratory food and water ad libitum. All animal experiments were performed according to the instructions of the Toho University Animal Center (permission NO-169). Mice were anesthetized intramuscularly with 50 mg/kg of ketamine and 10 mg/kg of xylazine, and 30 µl of the bacterial suspension (strain 10) (1 × 106 to 2 × 108 CFU/mouse) was then administered intranasally (43). For hyperoxic exposure, mice were kept under hyperoxic conditions (90% of O2) for the indicated times in airtight chamber (42). The oxygen concentration in the chamber was maintained by a constant flow of oxygen, which was monitored with an in-line oxygen analyzer (model D2; Beckman, Fullerton, CA). Therapies were initiated 2 h after the induction of pneumonia. In mouse survival experiments, mice were used, divided into four groups, and administered saline as a control, Ca-EDTA, IPM, and IPM plus Ca-EDTA. Total daily doses were divided into 2 doses and administered every 12 h. IPM (50 mg/kg/day) was injected subcutaneously, and Ca-EDTA was administered subcutaneously (200 mg/kg/day) or intranasally (300 mg/kg/day). Treatments or placebo was continued until 48 h after inoculation, and the survival was monitored twice a day for 5 days. For comparison of the lung bacterial burden, 36 mice were split into groups of six, administered saline as a control or IPM, intranasally administered Ca-EDTA plus IPM, and subcutaneously administered Ca-EDTA plus IPM. Ca-EDTA was used at two doses, 50 and 100 mg/kg. Four hours after the induction of pneumonia, mice were sacrificed by CO2 asphyxiation, and their lungs were removed for analysis. After removal of the lungs, they were homogenized in 1 ml saline by using a tissue homogenizer (Omni International). Homogenates (10 µl) were inoculated onto Mueller-Hinton agar after serial 1:10 dilutions.

Effects of Ca-EDTA on culture supernatant-induced lung injury. To assess the protease-blocking activity of Ca-EDTA in an in vivo model, bacterial culture supernatant-induced acute lung injury was employed. Filter-sterilized culture supernatants of P. aeruginosa PAO1 were inoculated into the lungs to induce acute lung injury, and the mice were then kept under hyperoxic conditions (95% of O2) for 72 h. To prepare a culture supernatant, P. aeruginosa PAO1 at approximately 1 × 106 CFU/ml was inoculated into Muller-Hinton broth and incubated at 37°C for 24 h. The culture medium was centrifuged at 5,000 × g for 15 min and filter sterilized through a lipopolysaccharide (LPS)-free filter (ED-Disk 25, 0.2 µm; Kanto Chemical Co., Inc., Tokyo, Japan). Sixty mice were used.
for this experiment and were divided into three groups. Thirty microliters of the supernatants was intratracheally inoculated into each mouse. One group was administered control medium in place of the supernatants. The other two groups received 100 mg/kg of Ca-EDTA or saline twice a day subcutaneously, and survival was monitored for 5 days.

Effects of Ca-EDTA on protease activity in culture supernatant of P. aeruginosa. We used a synthetic substrate, N-Suc-(Ala)₃-p-nitroanilide (pNA) (Sigma), in order to assess elastase-type endoprotease in the culture supernatants of P. aeruginosa PAO1 (14). The culture supernatants were prepared as described above. N-Suc-(Ala)₃-pNA (1 mM) was dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 1 mM CaCl₂, 1 mM MgCl₂, 0.01% Brij 35, and 0.01% NaN₃. Culture supernatants were added to this substrate solution (50% volume). The solution was incubated at 37°C for 5 h with or without Ca-EDTA, and the degradation of the substrate was read at 405 nm.

Effects of Ca-EDTA on cell viability in culture supernatant-induced cell damage. The human lung alveolar epithelial cell line A549 was obtained from the ATCC, and the cells were seeded into wells (2 x 10⁴ to 3 x 10⁴ cells/well) of a 96-well plate. The cells were incubated in a chamber containing 5% CO₂ at 37°C for 48 h, with or without 10% of the supernatant of P. aeruginosa, as described above. The change of morphology at 6 h and the viability of epithelial cells at 48 h were studied in the presence of different concentrations of Ca-EDTA. Cell viability was examined by using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay using TetraColor One (Seikagaku Kogyo, Tokyo, Japan).

Statistical analysis. All data are presented as means ± standard deviations (SD). Statistical significance was determined by using the unpaired, two-tailed t test. Survival curves were constructed by the Kaplan-Meier method and were analyzed by a log-rank test. The analysis was implemented by using SPSS software (SPSS Inc., Chicago, IL). For all tests, differences were considered statistically significant when P values were <0.05.

RESULTS

Effects of Ca-EDTA on MICs of CAZ, IPM, and AMK (Table 1). The effects of Ca-EDTA on the MICs of several antibiotics were examined with 25 clinical isolates of P. aeruginosa, 9 of which were MBL negative and the others of which were MBL positive. Ca-EDTA did not inhibit the growth of these organisms, even at 10,000 μg/ml of this compound. As shown in Table 1, the addition of Ca-EDTA drastically reduced the MICs of CAZ and IPM in all MBL-positive strains but not MBL-negative strains. As 16 clinical isolates with several types of MBLs (IMP-1, -2, -7, and -10 and VIM-2) were used in this study, Ca-EDTA effects are likely to be substantially similar for all MBL-positive strains. In contrast, no potentiating effects of Ca-EDTA were observed for the MICs of AMK.

IC₅₀ of Ca-EDTA. Purified IMP-1 was used to analyze the inhibitory activity of Ca-EDTA in vitro. The IC₅₀ of Ca-EDTA against IMP-1 was calculated to be 55 ± 8.2 μM.

Effects of Ca-EDTA on survival of mice with P. aeruginosa pneumonia. To assess the effects of Ca-EDTA on the survival of mice, we have applied the P. aeruginosa pneumonia model of hyperoxia, which mimics VAP in the clinical setting. As shown in Fig. 1, the control mice started to die at 24 h, and all mice died by 72 to 84 h after the intranasal inoculation of P. aeruginosa. Ca-EDTA alone had no effect on survival with either intranasal or subcutaneous administration. IPM treatment delayed the death of mice and increased the survival rate from 0% to approximately 30% at the end of observation, although this was not statistically significant. Importantly, the effects of combining Ca-EDTA with IPM were striking: 100% survival was achieved for this group, regardless of the route of administration of Ca-EDTA. Consistent with the survival data, the bacterial burden was significantly reduced for the combination of IPM and Ca-EDTA (Fig. 2). IPM treatment alone did not induce a significant reduction of bacterial numbers in the lungs. In contrast, the simultaneous administration of Ca-EDTA at both 50 and 100 mg/kg significantly enhanced the clearance of organisms from the lungs. The reduction in the bacterial burden was more than 1 log when the mice were treated with 100 mg/kg of Ca-EDTA subcutaneously in addition to IPM. These results demonstrated the therapeutic efficacy of Ca-EDTA in combination with IPM, which was well correlated with the reduction in the pulmonary bacterial burden.

Protease-blocking activity of Ca-EDTA in P. aeruginosa culture supernatant. Next, we examined whether Ca-EDTA neutralized the protease activity in the culture supernatants of P. aeruginosa, since it is well known that this organism produces a variety of metalloproteases, which may be associated with the virulence of P. aeruginosa. The filter-sterilized culture supernatant of P. aeruginosa PAO1 was used as a bacterial protease-rich medium. As shown in Fig. 3, Ca-EDTA suppressed protease activity in a concentration-dependent manner. In the presence of 1, 16, and 64 μg/ml of Ca-EDTA, the protease

FIG. 1. P. aeruginosa mouse pneumonia model in the setting of hyperoxia. Mice were inoculated with P. aeruginosa strain 10 and kept under hyperoxic conditions (90%). Therapies were initiated 2 h after the induction of pneumonia by intranasal administration (a) and subcutaneous administration (b) and continued until 48 h after inoculation. Mice were divided into four groups and administered saline as a control (open circles), Ca-EDTA (closed circles), IPM (open squares), and IPM plus Ca-EDTA (closed squares). *, P < 0.01 compared with IPM monotherapy.
activities were suppressed to 80%, 60%, and 50% of the control, respectively.

**Effects of Ca-EDTA on cell viability in culture supernatant-induced cell damage.** We also examined the antiprotease effects of Ca-EDTA with a cell viability assay (Fig. 4 and 5). Human alveolar epithelial cells (A549) were incubated with or without 10% of the culture supernatants for the indicated times, and the changes of morphology and viability of cells were then examined. As shown in Fig. 4, the addition of the culture supernatant induced changes of cell morphology, characterized by rounding, shrinking, and detachment. The coin-cubation of culture supernatants with Ca-EDTA protected cells from those cytotoxic effects by culture supernatants in a Ca-EDTA concentration-dependent manner (Fig. 4). Cell viability decreased to 20.0% of the control in the presence of culture supernatant, while the cell viability was restored to 46.5% by the addition of 8 μg/ml of Ca-EDTA (*P* < 0.001).

**Effects of Ca-EDTA on lethality of mice in culture supernatant-induced acute lung injury.** To examine the effects of the metalloprotease-blocking activity of Ca-EDTA, we used the culture supernatant-induced acute lung injury model in the setting of hyperoxia. The inoculation of the culture supernatant into the lungs induced the death of mice, and most of the mice died by 120 h (Fig. 5). In contrast, Ca-EDTA-treated mice demonstrated significantly higher survival rates at the end of observation (*P* < 0.05).

**DISCUSSION**

The present study demonstrates the effect of combining IPM with Ca-EDTA in the mouse pneumonia model of multiple-drug-resistant *P. aeruginosa*. The presence of Ca-EDTA at 32 μg/ml strikingly reduced the MICs of IPM and CAZ in MBL-producing isolates but not non-MBL-producing isolates. Importantly, we have observed almost the same survival benefits of Ca-EDTA with both intranasal and subcutaneous administrations. Finally, our results indicate that Ca-EDTA also suppressed the protease activity of *P. aeruginosa* culture medium, and these effects may be associated with the protection of alveolar epithelial cells *in vitro* and improvement of survival in the culture supernatant-induced injury model. Together, the present data suggest an application of Ca-EDTA for certain types of infectious diseases caused by MBL and/or tissue-destructive metalloenzyme producers.

EDTA has been reported to express several antibacterial activities, principally by chelating metal ions from microorganisms. Banin et al. previously suggested that EDTA caused rapid dispersion and eradication of bacteria from biofilms, which was mediated by the chelation of several divalent cations that are required to stabilize the biofilm matrix (5). Exploiting the antibiofilm property of EDTA, several investigators reported effective uses of this compound for catheter lock solutions (31, 39). EDTA can also deprive the LPS of cations (for example, magnesium and calcium), resulting in the permeabilization and disorganization of the outer membrane (44). EDTA has been widely used to detect MBL producers in clinical isolates because chelating zinc inactivates these enzymes (21, 28, 41). Bedenic et al. reported previously that the combination of EDTA and carbapenems prolonged the post-antibiotic and post-β-lactamase inhibitor effect on MBL-producing *P. aeruginosa* isolates, although no clinical application of EDTA was discussed because of its toxicity (6). Compounds with less-toxic but residual chelating activity may be beneficial for the treatment of refractory infectious diseases characterized by biofilm formation and MBL production. Since no apparent toxicity or death of mice was observed for the group treated with higher doses of Ca-EDTA (subcutaneous dose of 100 mg/kg and intranasal dose of 300 mg/kg), this compound may be a potential candidate for therapeutic development.

Importantly, the present data demonstrate that Ca-EDTA sensitizes MBL producers (Table 1). There is concern that plasmid-mediated MBL genes will spread from non-glucose-
fermenting organisms (*P. aeruginosa* and *Acinetobacter* spp.) to members of the Enterobacteriaceae (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens*, and *Escherichia coli*) (30, 46). A recent report from South Korea demonstrated that 11.4% of IPM-resistant *P. aeruginosa* isolates and 14.2% of IPM-resistant *Acinetobacter baumannii* isolates possessed MBLs (20). Another report from the SENTRY antimicrobial surveillance program in Japan indicated that 10.8% of the tested carbapenem-resistant strains possessed MBLs (15). Given that MBLs will hydrolyze virtually all classes of β-lactamase and given the lack of development of newer MBL-stable β-lactam antibiotics or MBL inhibitors, their continued spread may become a clinically significant problem.

MBL-producing *P. aeruginosa* and *A. baumannii* isolates are frequently associated with the “panresistant” phenotype, which is characterized by resistance to all β-lactams (including carbapenems), fluoroquinolones, and aminoglycosides. Presently, MBL-producers that exhibit a colistin-susceptible-only resistance pattern are increasingly detected worldwide and constitute a threatening situation for the patient. In *vitro* studies reveal that tigecycline and colistin are the only agents with consistent activity against certain panresistant MBL-producing strains (12, 28, 47). Although investigators have reported several options for combination treatment (for example, β-lactams plus aminoglycosides and β-lactams plus polymyxin) against panresistant organisms, whether these combinations yield outcomes that are improved over those with a single agent alone remains to be determined (32). Recently, we have reported the synergistic effects of colistin combined with IPM or rifampin in a mouse model of ventilator-associated pneumonia caused by panresistant *P. aeruginosa* (2). The antibiotics (for example, carbapenems, cephalosporins, and penicillins) that are the most effective partners for Ca-EDTA against panresistant organisms remain to be determined.

A variety of organisms, including *P. aeruginosa*, are known to produce metalloenzymes, such as alkaline protease and elastase, which play important roles in the pathogenesis of these infections (3, 4, 16, 38). Leidal et al. reported previously that alkaline protease and elastase from *P. aeruginosa* have proteolytic activity to efficiently degrade host chemokines, such as

---

**FIG. 4.** Changes of morphology of epithelial cells in the presence of *P. aeruginosa* PAO1 supernatants and Ca-EDTA. (A) Cellular morphologies were examined by light microscopy after 6 h of incubation with different concentrations of Ca-EDTA. (B) Cell viability was measured by an MTT assay. Ca-EDTA was added at the chosen concentrations, and cell viability was assigned a percentage of the control, with 100% representing the absorbance without supernatants. *, *P* < 0.01 versus without Ca-EDTA.

**FIG. 5.** Effects of Ca-EDTA on lethality of mice in culture supernatant-induced acute lung injury. Mice were intranasally inoculated with 30 μl of culture supernatants of *P. aeruginosa* PAO1 and kept under hyperoxic conditions (95%). No death of mice was observed when the control medium (no bacterial incubation) was instilled (open circles). Mice were inoculated with the filter-sterilized supernatants of *P. aeruginosa* and then administered saline (n = 20) (open squares) or Ca-EDTA (n = 20) (closed squares) twice a day for 2 days. *, *P* < 0.05 compared with the saline control.
RANTES and monocyte chemotactic protein 1 (22). These data suggest that these metalloproteases may contribute to the pathogenesis of *P. aeruginosa* infections not only through the destruction and/or degradation of cells and/or tissues but also through the modulation of host inflammatory and immunological processes. The present data demonstrate that Ca-EDTA expresses its protective activity from concentrations of 1 to 2 µg/ml in maintaining cell viability and morphology. Since Ca-EDTA effects were partial in the culture supernatant-mediated assays, even at higher concentrations, nonmetalloenzyme virulence factors may be involved in cytotoxicity and cellular damage by *P. aeruginosa*.

Since Ca-EDTA has been a mainstay against lead intoxication for more than 50 years, there are data accumulated from clinical experiences (9). Although adverse effects such as nephrotoxicity and allergic reaction have been reported, Ca-EDTA has been used safely at the recommended dose (13). Osterloh and Becker previously reported the pharmacokinetics of Ca-EDTA, in which maximum human serum concentrations of 51 µg/ml and a half-life (t½) of 0.7 to 10.8, depending on renal function, were demonstrated (27). In preliminary experiments, we examined the MICs of IPM with serial concentrations of Ca-EDTA. The potentiating effects of Ca-EDTA were observed from 2 µg/ml, and the maximum effects were obtained between the concentrations of 32 and 64 µg/ml. There were similar tendencies regardless of the type of metallo-beta-lactamase used (data not shown). In view of these results, the concentrations of Ca-EDTA used for the experiment were effective and within the accessible range for clinical practice. We also demonstrated the potentiating effects of Ca-EDTA in *vivo*. In a pneumonia model with pneumonia caused by an IMP-1-producing strain, IPM and Ca-EDTA cotherapy resulted in a drastic improvement of the survival rate and reduction of lung bacterial burdens. Additionally, both intranasal and subcutaneous administrations of Ca-EDTA produced similar effects, which suggested a good penetration of this compound into the lungs. These data consistently demonstrate the possibility of Ca-EDTA for clinical applications. Further investigation of Ca-EDTA as a chelating agent against life-threatening infectious diseases by MBL- and/or metalloenzym-producing organisms, including pharmacokinetic-pharmacodynamic and safety profiles, is warranted.

ACKNOWLEDGMENTS

We thank Barbara H. Iglewski for the kind gift of *P. aeruginosa* PAO1. We express our deep appreciation to Tse Hsien Koh and Robert A. Bonomo for their critical comments and careful reviewing of the manuscript.

REFERENCES


