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Antimicrobial Agents and Chemotherapy, 54(2), pp.757-762; 2010

Efficacy of clarithromycin against experimentally induced pneumonia caused by clarithromycin-resistant *Haemophilus influenzae* in mice

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ABSTRACT

Clarithromycin is a 14-member lactone ring macrolide with potent activity against *Haemophilus influenzae*, including ampicillin-resistant strains. We evaluated the *in vivo* efficacy of clarithromycin at 40 mg/day and 100 mg/day for 3 days in the treatment of a murine model of pneumonia using a macrolide-resistant *H. influenzae* strain, which was also ampicillin resistant. The MIC of clarithromicin was 64 μg/ml. The viable bacterial counts in infected tissues after treatment with 100 mg/kg clarithromycin were lower than the counts obtained in control and 40 mg/kg clarithromycin-treated mice. Concentrations of macrophage inflammatory protein (MIP)-2 and interleukin (IL)-1β in bronchoalveolar lavage fluid (BALF) samples of mice treated at both concentrations were lower than in the control group. Pathologically, following infection, clarithromycin-treated mice, particularly at a dose of 100 mg/kg, showed lower numbers of neutrophils in alveolar walls, and inflammatory changes had apparently improved, whereas large aggregates of inflammatory cells were observed within alveoli of control mice. In addition, we demonstrated that clarithromycin has bacteriological effects against intracellular bacteria at levels below the MIC. Our results indicate that clarithromycin may be useful *in vivo* for macrolide-resistant *H. influenzae*, and this phenomenon may related the good penetration of clarithromycin into bronchoepitherial cells. We also believe that conventional drug susceptibility tests may not reflect the *in vivo* effects of clarithromycin.

INTRODUCTION

Haemophilus influenzae is a gram-negative bacillus that is a commensal inhabitant of the human nasopharynx, and that may be isolated from most of the human population.

Non-typeable *H. influenzae* (NTHi) strains cause a variety of infections, including otitis media, and acute exacerbation of chronic bronchitis, pneumonia and meningitis, primarily in pediatrics (7, 10, 18). The incidence of beta-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains of *H. influenzae* has recently exhibited a marked increase in some countries, particularly Japan (13, 28, 34). Macrolides are increasingly used for the treatment of respiratory tract infections, and in the 1990s, the "new" macrolides clarithromycin (CAM) and azithromycin (AZM) were made available (30). The new macrolides have an expanded spectrum of activity, including fastidious gram-negative bacilli, such as *H. influenzae* and *Neisseria* spp (20, 23).

CAM is a 14-member lactone ring macrolide antibiotic that has been used for the treatment of various infectious diseases. The antimicrobial activity of macrolides is generally considered to be through the inhibition of microbial protein synthesis by acting on the 50S subunit of the 70S ribosome (36). In addition, macrolide antibiotics exert anti-inflammatory effects by inhibiting of the production of proinflammatory cytokines (22, 33, 35). Moreover, the pharmacokinetics (PK) of macrolides is characterized by a combination of low serum concentrations, and high tissue

concentrations (40), with advanced-generation macrolides being highly concentrated within polymorphonuclear leucocytes. Following phagocytosis of pathogens at the infection site, these cells are exposed to very high intracellular concentrations of antibacterial agent.

Macrolide resistance in several pathogens was recently evaluated because of increases in worldwide macrolide consumption (14, 27). However, pharmacodynamic (PD) models and susceptibility breakpoints derived from studies with other classes of drugs, such as the beta-lactams and aminoglycosides, do not adequately explain the clinical utility of antibacterial agents that achieve high intracellular concentrations, such as macrolides and fluoroquinolones (1). Some authors have suggested that *in vitro* resistance is less useful for guiding clinical decisions. In addition, the mechanisms of airway epithelium invasion by *H. influenzae* have been reported (11, 16, 29), and the characteristics are thought to contribute to escape from antibiotics and to long-term persistence in the airway. We believe that macrolides also have good clinical effects *in vivo* against these intracellular bacteria due to their excellent tissue penetration and intracellular penetration.

The focus of this study was to investigate the *in vivo* efficacies of CAM in an experimental pneumonia model using CAM-resistant *H. influenzae*.

MATERIALS AND METHODS

Bacteria

Clinically isolated NTHi strain 4437, which had been stored in tripticase soy broth with 10% glycerol stocks maintained at -80°C at Nagasaki University Hospital, was spread on chocolate agar plates (Nissui Pharmaceutical, Tokyo, Japan) and incubated overnight (18-24 h) at 37°C in 5% CO_2.

Laboratory animals

Six-week-old male ddY-specific pathogen-free mice (body weight, 16-20 g) were purchased from SLC Japan (Tokyo, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University (Nagasaki, Japan). Experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

Murine model of *H. influenzae* respiratory infection

We used the intubation model of NTHi pneumonia in mice, as reported previously (26). Briefly, disposable, sterile, plastic cut-down intravenous catheters (3-Fr, 1.0-mm diameter; Atom, Tokyo, Japan) were used for tracheal intubation. The intubation procedure was performed under pentobarbital anesthesia. The blunted end of the inner needle of an intravenous catheter (Angiocath; Becton Dickinson Vascular Access, Sandy, UT, USA) was inserted through the oral cavity, with the outer sheath and attached tube at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was then removed, and the outer sheath was gently pushed to place the plastic tube into the main bronchus. Organisms were instilled at 7 days after intubation. *H. influenzae* were cultured on chocolate α agar plates (Nissui Pharmaceutical) and incubated overnight (18-24 h) at 37°C in 5% CO_2, and the organisms were then suspended in normal saline. Final numbers of bacteria were approximately 2×10^9 colony-forming units (CFU)/ml, as determined by the optical density method. Infection was induced by intratracheal inoculation of 0.05 ml of a bacterial suspension under anesthesia with pentobarbital sodium.

Bacteriological and histopathological examination

Mice were divided into 3 groups: control mice (no therapy); mice treated with 40 mg/kg clarithromycin twice a day (12, 25); and mice treated with 100 mg/kg clarithromycin twice a day (39). Mice were treated for 3 days, and then sacrificed at 12 h after the final treatment. Tubes were removed and the lungs were excised under aseptic conditions. Lungs were homogenized in 1.0 ml of phosphate-buffered saline and cultured quantitatively by serial dilution on chocolate α agar plates (Nissui Pharmaceutical), followed by incubation overnight (18-24 h) at 37°C in 5% CO_2. For histopathological examination, lung specimens were fixed in 10% formalin-buffered solution.

Bronchoalveolar lavage (BAL) and cytokine enzyme-linked immunosorbent assay (ELISA)

BAL was performed as described previously (38). Briefly, mice were treated for 3 days, and sacrificed at 12 h after the final antibiotic administration. The chest was opened to expose the lungs and trachea, and a disposable sterile plastic cutdown intravenous catheter was inserted into the trachea. BAL was performed 3 times sequentially using 1.0 ml of saline each time. Recovered fluid fractions were pooled for each animal. Total cell counts were performed by

Turk staining. For differential cell counts, cells were centrifuged at 850 rpm for 2 min onto slides, which were then stained with Diff-Quick stain. Differential cell counts were performed by counting 100 cells. Concentrations of macrophage inflammatory protein (MIP)-2 and interleukin (IL)-1β in BAL fluid (BALF) were assayed using mouse cytokine ELISA test kits (R&D Systems, Minneapolis, MN, USA).

Antimicrobial agents

CAM was kindly provided by Taisyotoyama Pharmaceutical Co., Ltd. (Tokyo, Japan). CAM was dissolved in dimethyl sulfoxide, prepared according to the manufacturer's instructions and frozen at -80°C until use.

Antibiotic susceptibility testing

Susceptibility of *H. influenzae* to CAM was tested in duplicate for each isolate at 10^5 CFU/ml, and was determined using the broth dilution method with *Haemophilus* Test Medium, according to Clinical and Laboratory Standards Institute (CLSI) recommendations (6). Production of β-lactamase was confirmed by nitrocefin test (Showa Chemical, Tokyo, Japan). Strains were

classified according to CLSI AMP susceptibility criteria: susceptible strain (AMP minimum inhibitory concentration (MIC) ≤1 μg/ml), intermediate strain (AMP MIC=2 μg/ml), and resistant strain (AMP MIC≥4 μg/ml).

Antibiotic examination

At 24 h after challenge with *H. influenzae*, CAMwas administered orally twice a day. Individual doses were either 40 mg/kg or 100 mg/kg, and treatment was administered for 3 days. Each group of mice was killed by cervical dislocation at 12 h after the final drug administration. Bacteriological examination and BALF analysis was performed using the methods described above.

Three-hour invasion assay with NCI-H292 cells

The NCI-H292 epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C under 5% CO_2 in fully humidified air and were subcultured twice weekly. Cells were seeded in a 12-well plate at 5 × 10^5 cells/well. When confluent, cells were incubated in RPMI 1640 medium containing 0.5%

fetal bovine serum for 24 h. Cells were then rinsed with serum-free RPMI 1640 medium and exposed to bacteria. Bacterial suspensions at about 6×10^6CFU/ml were inoculated at 10 µl/well. Invasion assay was performed as reported previously (17, 32). Briefly, cell monolayers were infected and incubated at 37°C under 5% CO_2 for 3 h, washed 3 times with phosphate buffer solution (PBS), and treated with gentamicin (Sigma, Tokyo, Japan) at a concentration of 200 µg/ml for 2 h in order to kill extracellular bacteria. CAM at concentrations below the MIC (2 µg/ml to 64 µg/ml) was mixed with gentamicin. Cell monolayers were washed 3 additional times with PBS, and viable intracellular bacteria were released by incubation with 0.5 ml of 1% Triton X-100 (Sigma-Aldrich) in PBS for 15 min. Samples were harvested and vortex agitated for 1 min in order to lyse cells. Viable bacteria were serially diluted and plated onto chocolate agar (Nissui, Tokyo, Japan) for colony counting.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between the numbers of viable bacteria in the lungs were evaluated by analysis of variance. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

In vitro susceptibility

For *H. influenzae* clinical strain 4437, the MIC of CAM was 64 μg/ml. This strain was a BLNAR strain, and the AMP MIC was 16 μg/ml.

Changes in viable bacterial numbers over time (Figure1)

Respiratory infection occurred in all intubated mice with inoculation. The number of viable bacteria increased to 6.0±1.2 (log_{10} CFU/ml) 3 days after inoculation in the control group, and there were no significant differences between the control group and the 40 mg/kg treatment group (5.8±0.8 (log_{10} CFU/ml)). Conversely, the number of viable bacteria decreased significantly in the 100 mg/kg treatment group (4.6.±1.0 (log_{10} CFU/ml)). These results indicate that CAM has *in vivo* bacteriological effects against the macrolide-resistant strain, as determined by conventional *in vitro* drug susceptibility tests.

BALF analysis

Total cell counts (Figure 2A) and neutrophil counts (Figure 2B) were significantly lower in both the 40 mg/kg treatment group and 100 mg/kg treatment group, particularly in the 100 mg/kg treatment group. To estimate further effects of CAM, inflammatory cytokine levels in BALF

were analyzed. IL-1β (Figure 3A) and MIP-2 (Figure 3B) were significantly decreased in both treatment groups, particularly in the 100 mg/kg treatment group. These data indicate that CAM has dose-dependent anti-inflammatory effects against the acute inflammation induced by macrolide-resistant *H. influenzae*.

Histopathological examination

In non-treated mice, bronchioles and adjacent alveoli were filled with neutrophils, epithelial cells and inflammatory cells (Figure 4A). Conversely, in treated mice, although mild inflammatory changes were evident in the 40 mg/kg treatment group (Figure 4B), inflammation had improved after 3 treatment days, particularly in the 100 mg/kg treatment group (Figure 4C).

Intracellular bacteriological effects at various CAM concentrations

In conventional drug susceptibility tests, such as the microdilution method, PK and PD are not reflected in the antimicrobial effects against the pathogen that has invaded the bronchoepithelial cells. We performed 3 h invasion assay to investigate the intracellular bacteriological effects of CAM against macrolide-resistant *H. influenzae*, which was determined to be a highly resistant strain based on the CLSI judgment criteria. The number of intracellular *H. influenzae* in the

control group (non-treatment group) was 4.8±1.2 (log_{10} CFU/ml). We hypothesized that CAM shows bacteriorogical effects against macrolide-resistant *H. influenzae* at concentrations below MIC, as CAM attains higher concentrations in lung tissue than in serum. As shown in Figure 5, CAM reduced the number of viable bacteria at less than half the MIC. This data indicates that CAM can eradicate macrolide-resistant *H. influenzae* at levels below the MIC, and may be able to prevent persistent and recurrent infection by this pathogen. In addition, the difference between our results and conventional drug susceptibility test results may be due to the good penetration of CAM into bronchoepithelial cells.

DISCUSSION

Macrolides and beta-lactams are the most commonly prescribed antimicrobials to treat infection by *H. influenzae*. Antimicrobial resistance is a growing problem among *H. influenzae*. BLNAR was first observed in the 1980s (8, 21, 24) at very low frequency in United States (3, 9, 13), but has rapidly become more common to account for 25 to 30% of isolates in Japan and the other Asian countries (13, 28, 34). As safe and well-tolerated antibiotics, macrolides play a key role in the treatment of community-acquired respiratory tract infections (RTIs) against not only beta-lactam-susceptible strains, but also resistant strains such as BLNAR. Their broad spectrum of activity against gram-positive, gram-negative and atypical pathogens, has led to the

widespread use of macrolides for empiric treatment of RTIs.

CAM is a 14-member lactone ring macrolide antibiotic, and although its increased utility has been compromised by intrinsic and acquired resistance to CAM, treatment failures are uncommon. Generally, *in vitro* resistance is based on the results of using susceptibility breakpoints developed by the CLSI. This susceptibility data is considered useful for determining epidemiological trends of resistance, but *in vitro* resistance is less useful for guiding clinical decisions, and does not necessarily indicate a lack of clinical efficacy.

The discrepancy may be based on the characteristic features of macrolides. It is known that macrolides are able to transfer and accumulate intracellularly, and show intracellular bactericidal effects. Moreover, macrolides are readily taken up by phagocytes, lymphocytes and epithelial cells (4, 30). The concentration of macrolides in respiratory tract tissues and fluids has thus been shown to be higher than serum concentrations, resulting in the possibility of increased activity against organisms localized to these extra-plasma sites. The PD parameter of CAM has not yet been fully studied. CAM is considered concentration dependent by some investigators and concentration independent by others (5). Tessier et al (37) demonstrated AUC/MIC was the most reasonable predictor of CAM efficacy by using experimental *S. pneumoniae* pneumonia model. But they also showed that time above MIC and C_{max}/MIC was also important parameter correlated with the change of bacterial load. In the present study, although we did not

demonstrate which way is better 1 dose or twice a day in CAM administration, 100mg/kg treatment group showed improvement both in pathology and the inflammatory mediators, indicating that CAM may be able to show the dose, and concentration dependent efficacy against *H. influenzae* pneumonia.

Moreover, macrolides also have anti-inflammatory properties that improve clinical outcomes via extramicrobial mechanisms, leading to improvements in symptoms and overall quality of life among patients with a variety of respiratory conditions. None of these benefits are reflected by *in vitro* drug susceptibility testing.

Drug concentration of both in epithelial lining fluid (ELF) and alveolar macrophages (AM) is important to treat extracellular and intracellular bacteria. CAM is extensively concentrated in both ELF and respiratory phagocytes, reaching levels that are between one and three orders of magnitude higher than in plasma (2, 23). Antibacterial potency is driven by the concentration of active agents at the site of infection, and thus in the case of macrolides, efficacy may be increased substantially by the tissue penetration described previously. This aspect of macrolide pharmacology is not taken into account by traditional drug susceptibility tests, such as the microdilution method. Thus, the most likely rationale is that *in vitro* resistance MICs are misleading, leading to underestimation of the clinical efficacy of these therapeutic agents.

In the present study, to investigate the differences between the *in vivo* effects and the results of

conventional drug susceptibility of CAM against macrolide-resistant *H. influenzae*, as determined by *in vitro* susceptibility test, we demonstrated experimentally lower respiratory tract infection. The viable bacterial number was reduced significantly in the 100 mg/kg treatment group. In addition, CAM showed dose-dependent effects on improving the number of inflammatory cells and levels of inflammatory cytokines. Interestingly, however, CAM did not decrease viable bacterial number in the 40 mg/kg treatment group. CAM may be able to suppress the accumulation of neutrophils and other inflammatory cells through its immunomodulatory effects, thereby contributing to inflammatory improvement.

It has been shown that antimicrobial drug therapy based on PK and PD is necessary for the treatment of infectious diseases (7). β-lactams, the most common antibiotics to treat respiratory infections, cannot eradicate bacteria that have invaded airway epithelial cells. Considering the pathology of airway infection by highly invasive *H. influenzae* strains, antimicrobial agents that readily penetrate airway epithelium to which bacteria show susceptibility is necessary. We performed cell invasive assay to estimate the effects of CAM against intracellular *H. influenzae* using a macrolide-resistant strain. Our data indicate that CAM can reduce intracellular viable bacterial number at concentrations lower than MIC, which was determined by the microdilution method. However, in this study, we did not measure intracellular CAM concentrations, and this effect may indicate intracellular concentrations higher than MIC, thus contributing to the good

in vivo effects against *H. influenzae*, even in macrolide-resistant strains. Some authors were reported that CAM achieves high concentration not only in ELF but also intracellular space. Rodvold et al. reported that the steady-state concentrations of CAM in ELF and AM obtained in intrapulmonary samples during bronchoscopy and bronchoalveolar lavage from 40 healthy of nonsmoking adult volunteers were analyzed, and CAM was extensively concentrated in ELF (34.4µg/ml at 4 h to 4.6µg/ml at 24 h) and AM (480µ/ml at 4 h to 99µg/ml at 24 h) (31). However we did not measure the intracellular concentration of CAM in this time, our result may reflect the high penetration into the bronchial epithelial cell of CAM, from the viewpoint of PK. We also consider that it may be useful information clinically because *H. influenzae* is known to invade into the respiratory epithelial cells and tend to escape the antibiotics effect.

Unfortunately, *in vitro* resistance data has a strong impact on the drug selection process, as macrolide therapy is largely empirical. This issue is not easy to resolve, as existing PD data use serum or plasma as an index for microbiological efficacy, which may not be appropriate for macrolides when they are used against organisms with higher MICs that reside in bodily fluids with drug concentration profiles that differ from those of serum. As time passes and more organisms become resistant, and therefore have higher MICs, this situation will become more confused, and one can predict that the macrolide class of antibiotics will probably be replaced by the ‘‘respiratory’’ quinolones for empirical therapy of respiratory tract infections. This may

relegate the macrolides to the role of adjunctive agent in the treatment or prophylaxis of infections believed to be caused by intracellular pathogens. This would be unfortunate if it is based upon erroneous resistance data, and could deprive patients of an acceptable and somewhat unique (due to its high tissue penetration and immunological properties) class of agents; of course, the entire situation is further complicated by host immune function.

In conclusion, macrolide therapy remains a reasonable treatment for respiratory infection, even when macrolide-resistant *H. influenzae* is the causative pathogen. Advanced macrolides penetrate extensively within the respiratory tract and alveolar cells, and have anti-inflammatory properties that improve clinical outcomes via extramicrobial mechanisms. None of these benefits are reflected by *in vitro* testing. Thus, conventional drug susceptibility tests do not sufficiently reflect the *in vivo* efficacy of macrolides.

Although we demonstrated the efficacies of CAM against CAM resistant *H. influenzae*, the data is limited because we did not analyze PK/PD and these results based on only experimental *H. influenzae* pneumonia model. In fact, some papers which reported the clinical treatment failure of macrolide resistant *S. pneumonia*e were published recently (15, 19). A randomized, prospective trial is needed to establish a causal relationship between in *vitro* resistance and clinical treatment in macrolide. We should treat macrolide resistant *H. influenzae* carefully, and continuous monitoring of macrolide resistant pathogen will also important.

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FIGURE LEGENDS

Figure 1: Number of viable organisms in lower respiratory organs. Mice were inoculated with 1×10^8 CFU/ml of *H. influenzae* strain 4437. At days 3 after infection, control mice, and mice treated at 40 mg/kg and 100 mg/kg (oral administration, twice/day) were compared. Numbers of viable bacteria were significantly lower in the 100 mg/kg treatment group. Data are expressed as means ± SD for four experiments. *$P < 0.05$ vs. control.

Figure 2: Changes in the total cell counts in BALF, total cell counts (A) and neutrophils (B) between control mice, and mice treated with 40 mg/kg and 100 mg/kg treatment (oral administration, twice/day). Cells were stained with Turk. Data are expressed as means ± SD for four experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

Figure 3: Changes in inflammatory cytokine levels in BALF, IL-1β (A) and MIP-2 (B) between control mice, and mice treated with 40 mg/kg and 100 mg/kg treatment (oral administration, twice/day). Data are expressed as means ± SD for four experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

Figure 4: High-power magnification of the lung after 3 days of treatment (×200, hematoxylin and eosin). (A) Control group, untreated; (B) 40 mg/kg treatment group (oral administration, twice/day); (C) 100 mg/kg treatment group (oral administration, twice/day). Inflammatory changes improved in both treatment groups, particularly in the 100 mg/kg treatment group.

Figure 5: Intracellular bactericidal effects of various CAM concentrations against strain 4437. Intracellular viable bacterial number decreased significantly when the strain was treated CAM at 1×MIC, 0.5×MIC and 0.25×MIC.

Data are expressed as mean ± SD for three times experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

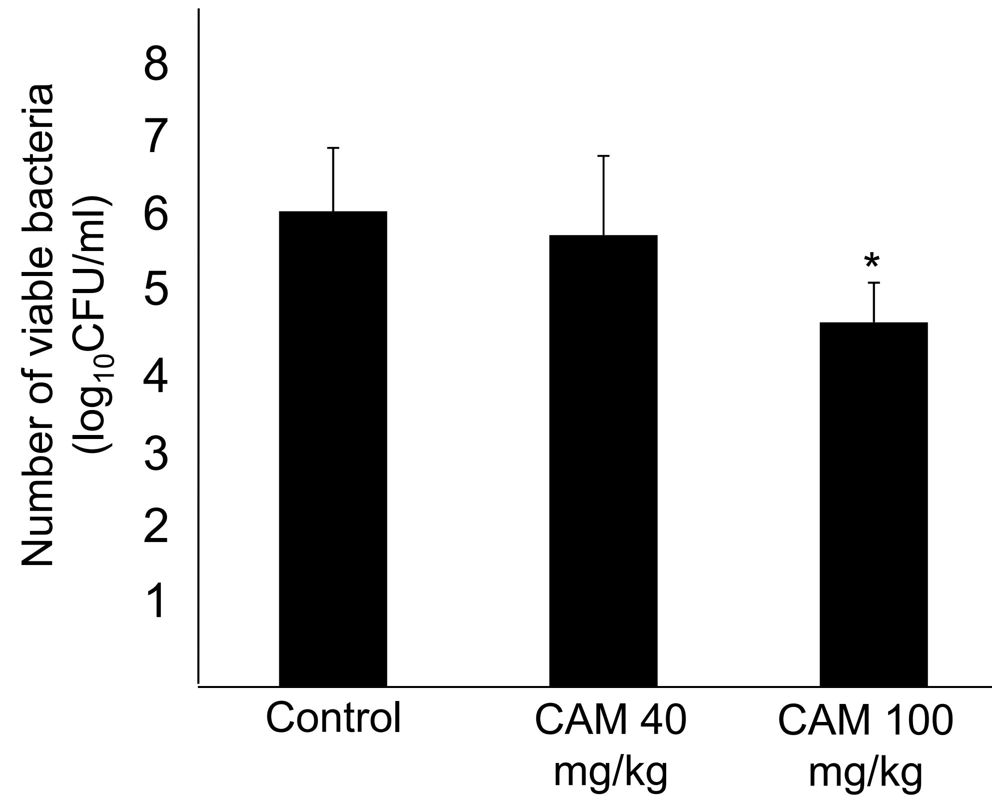
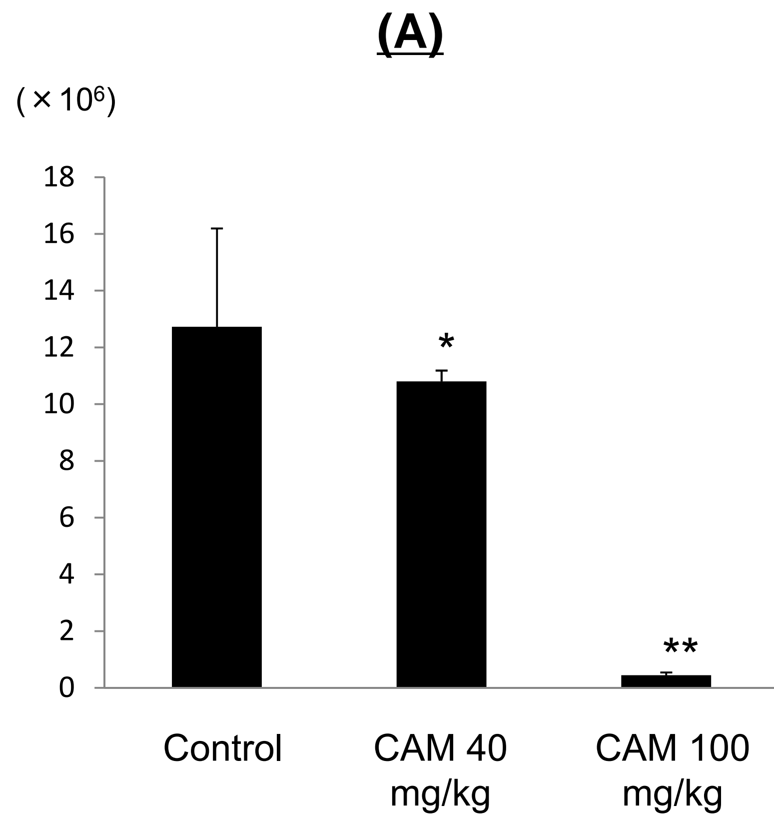


Fig. 1



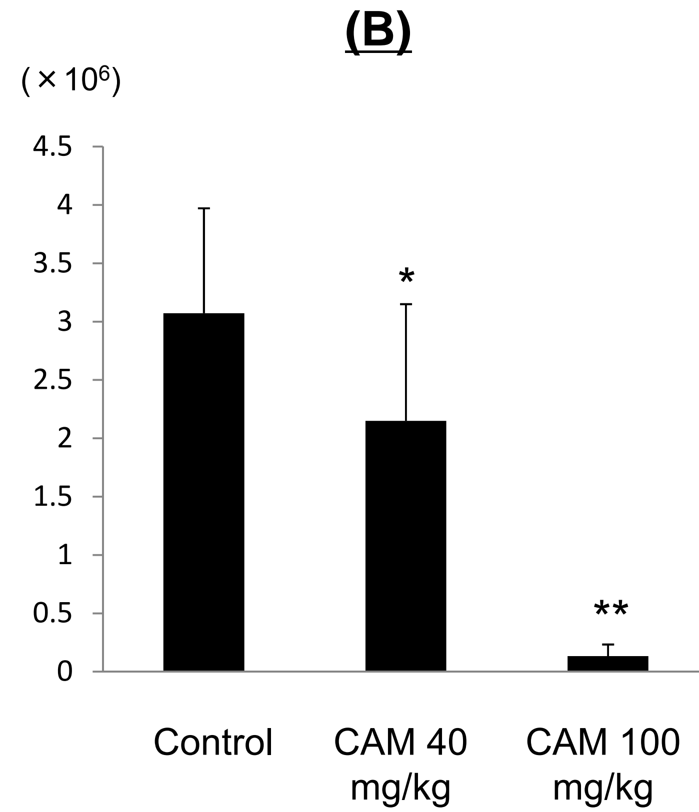


Fig. 2

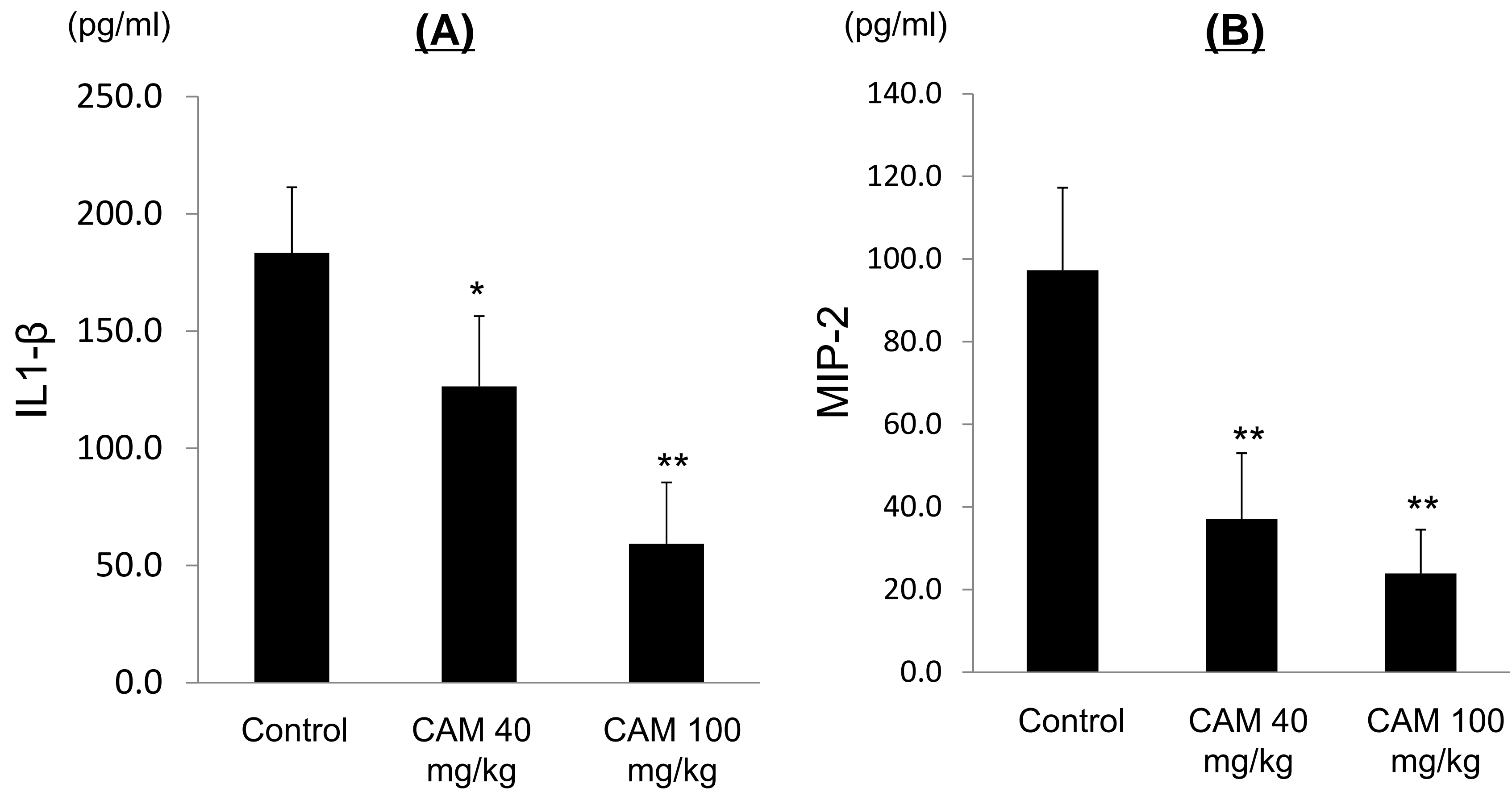
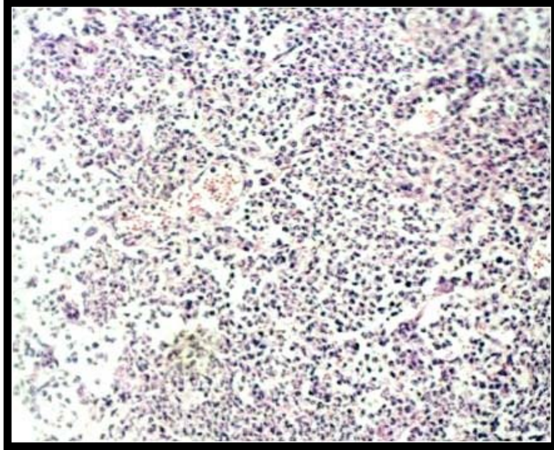


Fig. 3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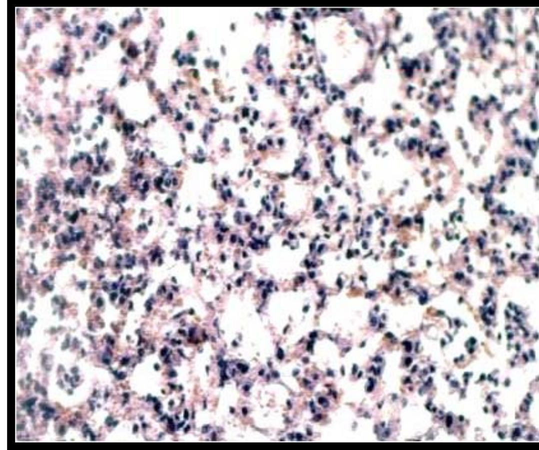
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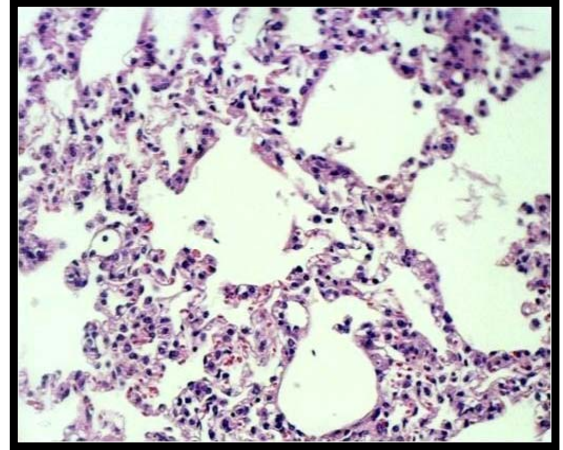
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Fig. 4

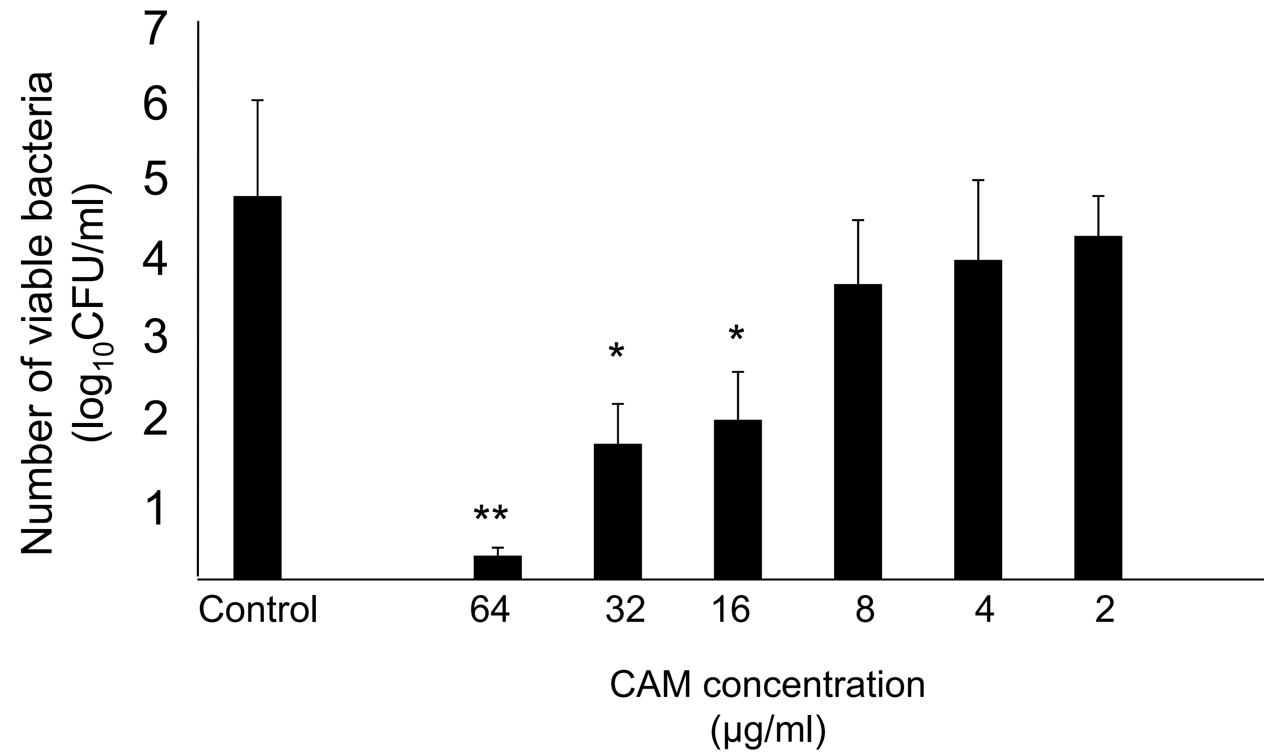

Fig. 5