In vivo efficacy of KRP-109, a novel elastase inhibitor, in a murine model of severe pneumococcal pneumonia

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Abstract

KRP-109 is a novel specific inhibitor of neutrophil elastase (NE).Various

studies suggest that NE inhibitors reduce lung injury associated with systemic

inflammatory response syndrome (SIRS). In this study, the efficacy of KRP-109

was examined using a murine model of severe pneumonia induced by

Streptococcus pneumoniae (*S. pneumoniae*).   Female mice (CBA/J, aged 5

weeks) were inoculated intranasally with   penicillin-susceptible *S. pneumoniae*

(ATCC49619 stain, 2.5×10^8 CFU/mouse). KRP-109 (30 or 50 mg/kg) or

physiological saline as a control was administered   intraperitoneally every 8 h

beginning at 8 h after inoculation, and survival rate was evaluated over 7 days.

Histopathological and bacteriological analyses of the lung, and bronchoalveolar

lavage were performed at 48 h post-infection. The mice treated with KRP-109

(KRP-109 mice) tended to have higher survival rate than those given saline. The

lung tissues of the KRP-109 mice had few neutrophils in the alveolar walls and

less inflammation. Furthermore, KRP-109 decreased significantly total cell and

neutrophil counts, and cytokine levels (interleukin 1β and macrophage

inflammatory protein 2) in bronchoalveolar lavage fluid.-Viable bacterial

numbers in lung were not influenced by treatment of KRP-109. The present

results indicate that KRP-109 reduces lung inflammation in a murine model, and

that KRP-109 may be useful for the treatment of patients with severe

pneumonia.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is one of the most common

pathogens, and causes community-acquired pneumonia that is accompanied by

high rates of morbidity and mortality [1]. The mortality rate associated with *S.*

pneumoniae pneumonia is more than 20%, despite the appropriate use of

antibiotics and aggressive intensive care support [2]. Therapies that are currently

being developed for treatment of this pneumonia include the development of

effective vaccines, the strengthening of immune responses in

immunocompromised hosts and the control of excessive inflammatory reactions.

Neutrophil elastase (NE) functions as a powerful physiological host defense

against microbial pathogens in severe pneumonia. However, excessive NE can

contribute to lung tissue damage, with effects including direct cytotoxicity to

endothelial and epithelial cells and degradation of adhesion molecules [3]. NE

plays an important role in the progression of acute lung injury (ALI). A specific

neutrophil elastase inhibitor, sivelestat, has been developed in Japan [4]. This

agent prevented subsequent progression of ALI in an animal model [5]. The

beneficial effects of sivelestat have also been reported in several other animal

models, including lipopolysaccharide (LPS)-induced lung inflammation and

ozone-induced airway response [6, 7]. We previously reported that this NE

inhibitor was effective in mouse models of *S. pneumoniae* and *Legionella*

pneumophila [8, 9]. In a Japanese phase III study, sivelestat was shown to be

effective for the treatment of ALI patients [10]. Taken together, these results

suggest that NE inhibitors may be useful for the treatment of ALI caused by

severe pneumonia. However, satisfactory outcomes with NE inhibitors in ALI

caused by severe pneumonia are still limited even now, and development of new

convincing therapeutic option is still desired in the clinical practice.

KRP-109 (Fig. 1) is a novel specific inhibitor of NE that was discovered by

Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). A distinct feature of KRP-109

is that it accumulates to a high level in lung tissue, which enables it to inhibit

NE activity that is released from infiltrated neutrophils under inflammatory

conditions in lung [11, 12]. The aim of this study was to evaluate the efficacy of

KRP-109 including pharmacological profile using a murine model of severe

pneumonia induced by *S. pneumoniae*. In addition to our past analysis in the

experimental bacterial pneumonia model [8, 9], we evaluated effects of

KRP-109 on cytokine release in lung using BALF in the presented study.

2. Materials and Methods

2.1. Agents

KRP-109 was kindly provided by Kyorin Pharmaceutical Co., Ltd. No antibiotic effect was observed when using KRP-109. The agent was dissolved in saline.

2.2. Bacteria strains

Penicillin-sensitive *S. pneumoniae* ATCC49619 was used in this study. The bacteria   were stored at -80°C in a Microbank system (Pro-Lab Diagnostics, Ontario, Canada) until use.

2.3. Laboratory animals

Five-week-old female CBA/J specific-pathogen-free mice (body weight, 16-20 g) were purchased from Charles River Japan. The CBA/J mouse model of pneumococcal pneumonia has been described previously (2). 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). The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

2.4. Experimental murine model of pneumococcal pneumonia

S. pneumoniae was cultured on horse blood agar for 20 h at 37°C under 5% CO_2 and was then scraped and suspended in brain-heart infusion broth (Becton Dickinson, Sparks, MD) mixed with horse serum, followed by culture with agitation for 6 h at 37°C at 250 rpm. Bacteria were then harvested by centrifugation ($3000 \times g$, 10 min). The organisms were resuspended in normal saline. The final number of bacteria was approximately 5×10^9 CFU/ml, as determined using the optical density method. Infection was induced by intranasal inoculation of 0.05 ml of bacterial suspension (2.5×10^8 CFU/mouse) into mice under anesthesia using pentobarbital sodium (40 mg/kg intraperitoneally) [13]. As a non-infected control, intranasal inoculation was conducted with saline as the same manner.

2.5. Study design

Treatment of KRP-109 was initiated at 8 h post-infection. KRP-109 (30 or 50 mg/kg) or physiological saline was administered intraperitoneally every 8 h. Survival was estimated to the death of the mouse or 7 days after infection. At 48 h post-infection, mice were anesthetized with pentobarbital and sacrificed. The lungs were dissected out, and were used bacteriological and

histopathological assays. To analysis of bronchoalveolar lavage fluid (BALF),

bronchoalveolar lavage was performed 48 h after infection as described

previously [14]. Briefly, bronchial cannula was inserted through the trachea

and bronchoalveolar lavage was sequentially performed 3 times using 1 ml of

saline each time. The recovered fluid fractions were pooled for each animal.

2.6. Bacteriological and histopathological analysis

The lungs were suspended in 1 ml of saline and the organs were

homogenized using a homogenizer (AS One Co., Osaka, Japan). Serial

dilutions were inoculated onto horse blood agar plates and incubated at 37°C

for 20 h. For histopathological examination, lung specimens were fixed in 10%

buffered formalin and stained with haematoxylin-eosin.

2.7. Analysis of bronchoalveolar lavage fluid (BALF)

The total cell number in the BALF was determined using a hemocytometer and

a differential cell count of 100 cells was done on slides stained with Diff-Quik

(Sysmex Corp., Kobe, Japan). The concentrations of macrophage inflammatory

protein 2 (MIP-2) and interleukin1β (IL-1β) in BALF were assayed using mouse

cytokine enzyme-linked immunosorbent assay (ELISA) test kits (R&D Systems,

Minneapolis, MN). Neutrophil elastase activity in BALF was measured using

the fluorogenic substrate Meo-Suc-Ala-Pro-Val-AMC [11]. Briefly, an aliquot

(80 µl) of BALF was incubated with 20 µl of 1 mM Meo-Suc-Ala-Pro-Val AMC

in assay buffer (2.5 M NaCl, 500 mM HEPES, pH 7.5) at 37°C for 18 h. After

incubation, the level of released AMC (7-amino-4-methyl-coumarin) was

determined by measurement of fluorescence using an excitation wavelength of

380 nm and an emission wavelength of 460 nm.

2.8. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences

between groups were evaluated by analysis of variance. Survival analysis was

made using the log rank test and survival rate was calculated by the

Kaplan-Meier method. *P* values of <0.05 denoted the presence of a statistically

significant difference.

3. Results

3.1. Survival study

Figure 2 shows the effect of KRP-109 treatment on survival rate of mice infected with *S. pneumoniae* over 7 days following infection. The survival rates for the 30 and 50 mg/kg KRP-109 treatment groups were higher than those for control group, but this increased survival was not significant when analyzed by the log rank test (P=0.12; KRP-109 50 mg/kg group versus control group).

3.2. Bacteriological examination of the lungs

The level of respiratory infection of inoculated mice in the KRP-109 or control groups was analyzed by bacteriological examination of the lungs. Respiratory infection, which was calculated as log_{10}CFU/ml, occurred in all inoculated mice. There were no significant differences between viable bacteria numbers of the control group and the KRP-109 groups (control, 7.32±0.17 log_{10}CFU/ml; 30 mg/kg KRP-109, 6.86±0.14 log_{10}CFU/ml; 50 mg/kg KRP-109, 6.88±0.15 log_{10}CFU/ml) (Fig. 3).

3.3. Histopathological examination

Light microscopy of the hematoxylin-eosin-stained lungs of the control group at 48 h after bacterial inoculation revealed large numbers of inflammatory cells, particularly neutrophils, infiltrating the alveolar spaces (Fig. 4A). In the

KRP-109 groups, mild inflammatory changes were evident at 30 mg/kg (Fig. 4B). Furthermore, there was less inflammation at 50 mg/kg (Fig. 4C).

3.4. BALF analysis

S. pneumoniae induced increase in total cells and neutrophils numbers in BALF. Numbers of total cell (Fig. 5A) and neutrophil (Fig. 5B) in BALF were significantly lower in the KRP-109 30 mg/kg group, and even lower in the KRP-109 50 mg/kg group as compared to the control group. Numbers of the total cell and neutrophil were decreased 54% and 52% by treatment of 50 mg/kg KRP-109. To examine the further effects of KRP-109, inflammatory cytokine levels in BALF were analyzed. The levels of IL-1β (Fig. 6A) and MIP-2 (Fig. 6B) were detected in BALF of the control group. Decreases in these cytokine levels were recognized in KRP-109 30 mg/kg group, however, were not significant. Treatment of 50 mg/kg KRP-109 decreased significantly the levels of IL-1β and MIP-2 values by 46% and 24%, respectively. NE activities were also detected in BALF of the infected control group. The NE activities of the KRP-109 groups were lower than those of the control group, although these differences were not significant (Fig. 7).

4. Discussion

KRP-109 is a potent and competitive inhibitor of NE, with a Ki of 5.1 nM. A distinct feature of KRP-109 is its high accumulation in lung tissue. In a rat model, the highest concentration of KRP-109, with a lung tissue/plasma concentration ratio exceeding 20, was observed in lung among other tissues after intravenous administration. Furthermore, KRP-109 alleviated lung damage induced by human NE and LPS in rat models [11, 12]. However, there have been no reports which have evaluated the efficacy of KRP-109 in a severe pneumonia model. In the present study, we therefore evaluated the efficacy of KRP-109 using a murine model of severe pneumonia induced by *S. pneumoniae*.

KRP-109-treated groups showed improved histopathological findings (Fig. 4) and decreased the number of inflammatory cells in BALF in a dose-dependent manner (Fig. 5). In addition, KRP-109 treatment significantly decreased the levels of IL-1β and MIP-2 in BALF (Fig. 6).

MIP-2, which is the functional homolog of the human IL-8, exhibits potent neutrophil chemotactic activity and may be a key mediator of neutrophil recruitment. In addition, it has been reported that NE induces the release of IL-8

from cells [15]. Furthermore, NE inhibitor has been reported to induce a

significant reduction in serum IL-1β in rats with relevant sepsis [16]. IL-1β acts

locally to stimulate chemotaxis and activate neutrophils in ALI and also

stimulates the production of extracellular matrix by fibroblasts [17]. Sivelestat

was also previously reported to significantly decrease MIP-2 levels in hamsters

infected with *S. pneumoniae* [18] and in a murine model of ventilator-induced

lung injury (VILI) [19]. Therefore, these cytokines are considered to play

important roles in ALI.

KRP-109 reduced NE activity in comparison with the control group, although

this reduction was not statistically significant (Fig. 7).In addition, KRP-109

improved survival rates in a dose-dependent manner, but this increased survival

was not statistically significant (Fig. 2). Although KRP-109 is a potent inhibitor

of human NE (IC_{50}=7 nM, Ki of 5.1 nM) [11], KRP-109 shows species

differences for inhibition of neutrophil elastase with mouse IC_{50} of 274 nM

(in-house data by Kyorin). These marginal effects of KRP-109 on NE activity

and the mouse survival rate may be due to species difference of neutrophil

elastase inhibition.

Also, continuous infusion of KRP-109 has been shown to decrease NE

activity in a bleomycin-induced lung injury model [20]. Therefore, the marginal effectivity of KRP-109 in this study may be due to the difference in drug administration. Even so, inhibitory effect on inflammatory changes in pulmonary histopathology finding was observed; therefore, we rather consider inhibition of initial neutrophils infiltration into lung and consequent lung damage, which should have been caused by elastase inhibition by KRP-109, led to subsequent reduction of neutrophil counts in BALF and inhibition of inflammatory histopathology changes of lung in the present study.

On the other hand, NE is a key molecule of the innate immune system and is required for effective killing of phagocytosed bacterial and fungal pathogens [21, 22]. Our results, however, did not show any significant differences between the level of respiratory infection in the control and the KRP-109-treated groups following bacteriological examination (Fig. 3). This result indicates that NE inhibitors do not aggravate infection.

Interestingly, Ishiyama et al. reported KRP-109 alleviated lung damage induced by human NE and LPS in rat models [11, 12]. Its effects consisted of inhibition of lung edema and lung hemorrhage. Furthermore, it was reported KRP-109 decreased TGF-β related genes expression and TGF-β1 release

induced by human NE in rat [20]. Based on present study and previous studies

on KRP-109 to date, it is considered that KRP-109 demonstrates

anti-inflammatory pharmacological effects, all of which are considered to occur

in the inflammation site of lung tissue, in ALI experimental conditions probably

contributed by its distinctive characteristics, i.e., high distribution in lung

together with specific inhibitory activity of human NE, and that KRP-109

possesses potential effects on ALI consequent to severe pneumonia. Future

studies should investigate the serum and tissue concentration of KRP-109 and

the efficacy of KRP-109 in combination with antibiotics in a model of severe

pneumonia.   In conclusion, KRP-109 improved lung inflammation in a murine

model of severe pneumococcal pneumonia. The present results suggest that

KRP-109 may be useful for the treatment of patients with severe pneumonia.

Acknowledgements

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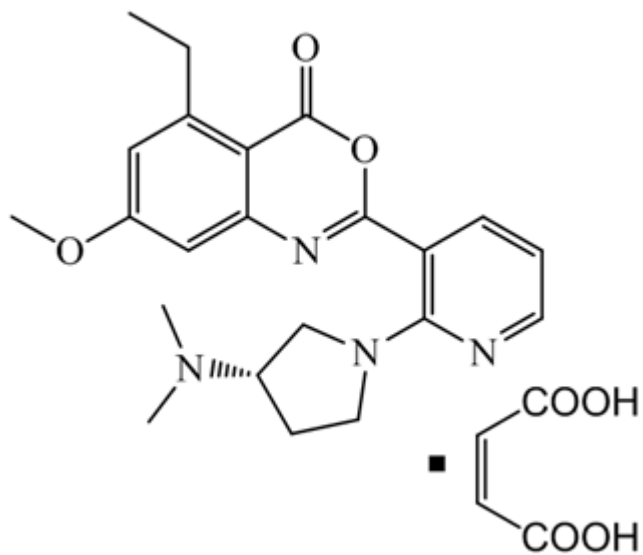
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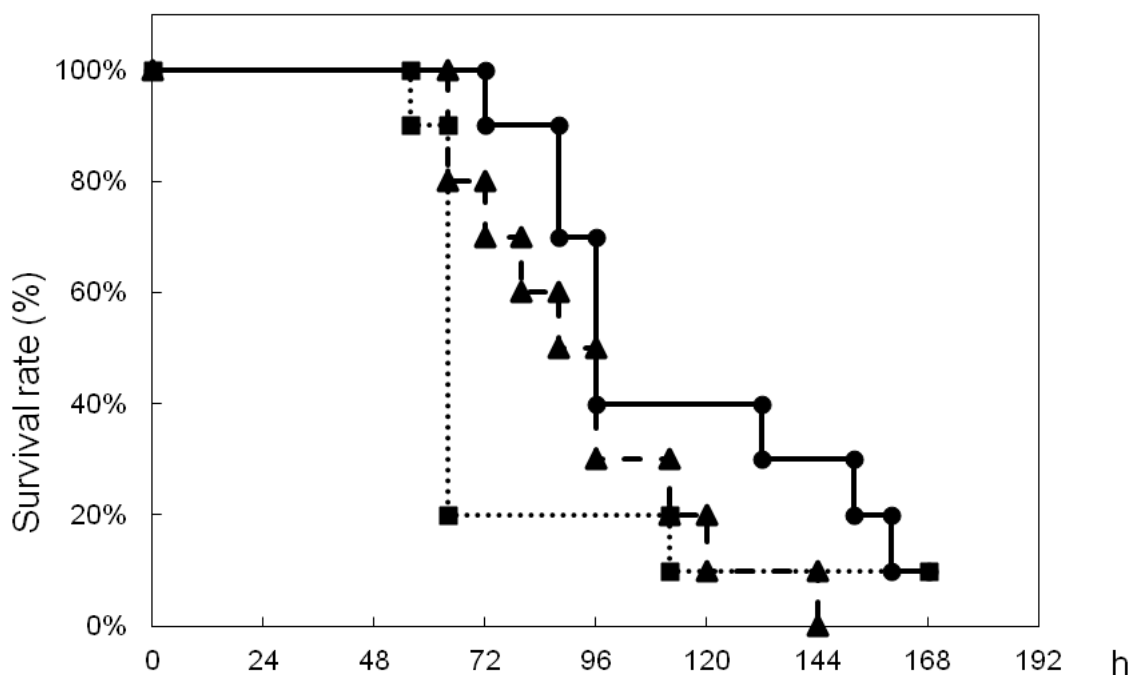

Fig. 1. Chemical structure of KRP-109.

Fig. 2. Survival study of mice with pneumococcal pneumonia after treatment with KRP-109. Ten mice in each group were treated with KRP-109 at a dose of 30 mg/kg (filled triangles) or 50 mg/kg (filled circles), or were treated with saline (filled squares). Survival was then estimated at the indicated times and the results are displayed as a Kaplan-Meier plot. The survival rates of KRP-109 groups were higher than that of control, but the increase in survival rate was not significant as assessed using the log rank test (P=0.12; KRP-109 50 mg/kg group versus control group).
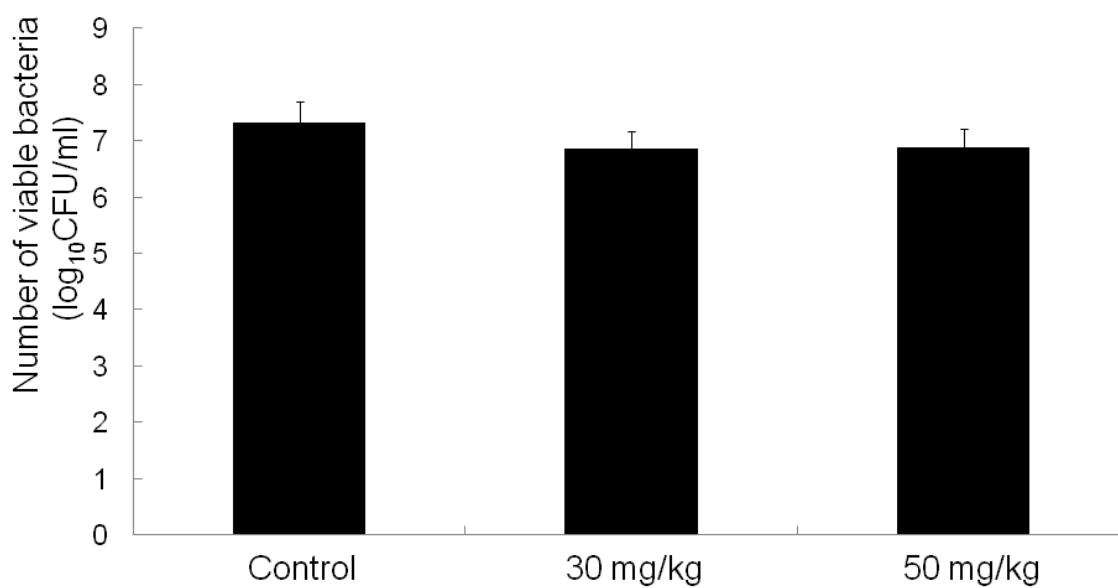
Fig. 3. Effect of KRP-109 on the number of viable bacteria in the lungs of mice.

Mice were inoculated with 2.5×10^8 CFU of *S. pneumoniae*. Treatment with

KRP-109 did not affect the number of viable bacteria in comparison with
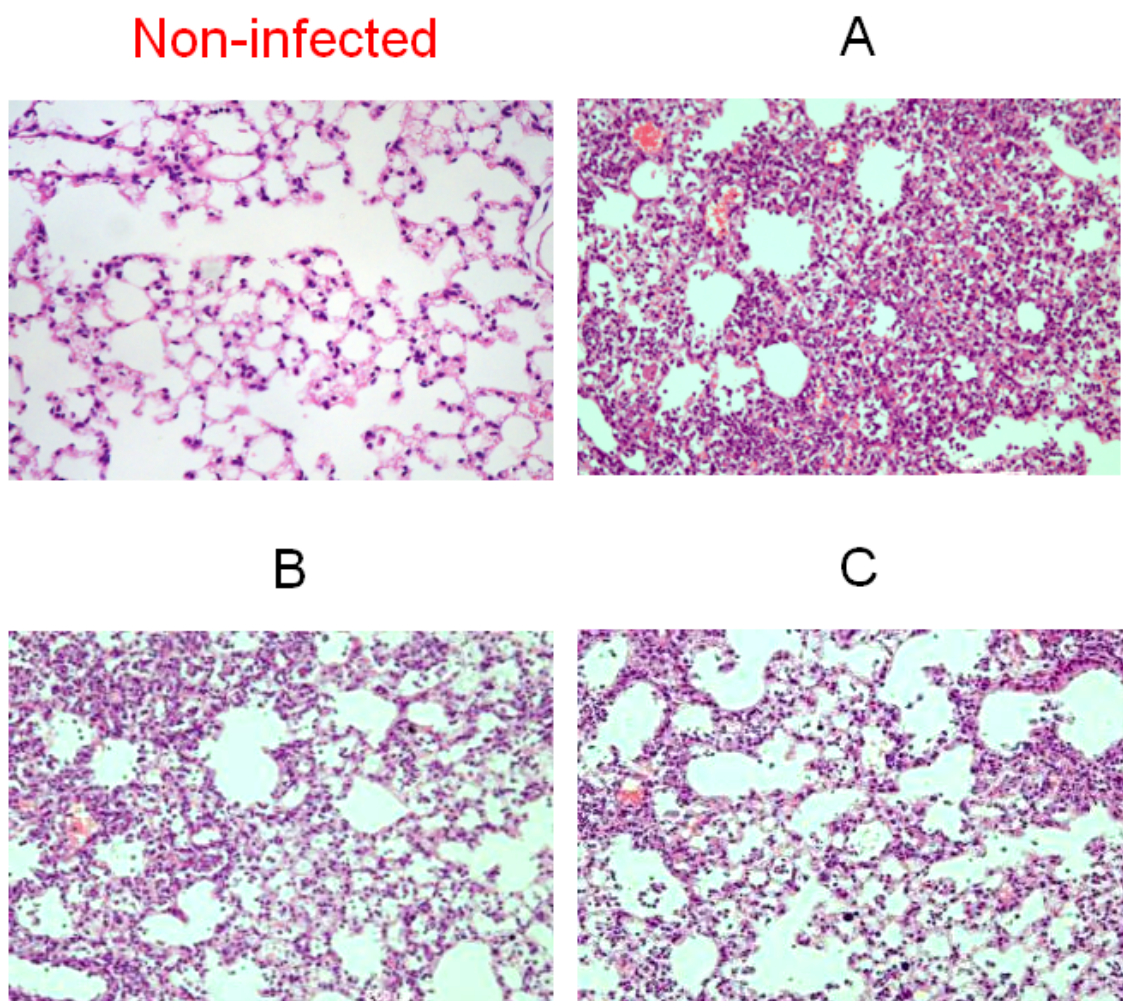
control (n=5 in each group).

Fig. 4. Histochemical analysis of the lungs of infected mice following KRP-109 treatment**.** High-power magnification (×100; hematoxylin and eosin) of the lung at 48 h post-infection. Photomicrographs exist lung tissues of control group (A), KRP-109 30 mg/kg group (B) and KRP-109 50 mg/kg group (C). KRP-109 treatment, especially at a dose of 50 mg/kg, inhibited lung inflammation due to infection(n=5 in each group).
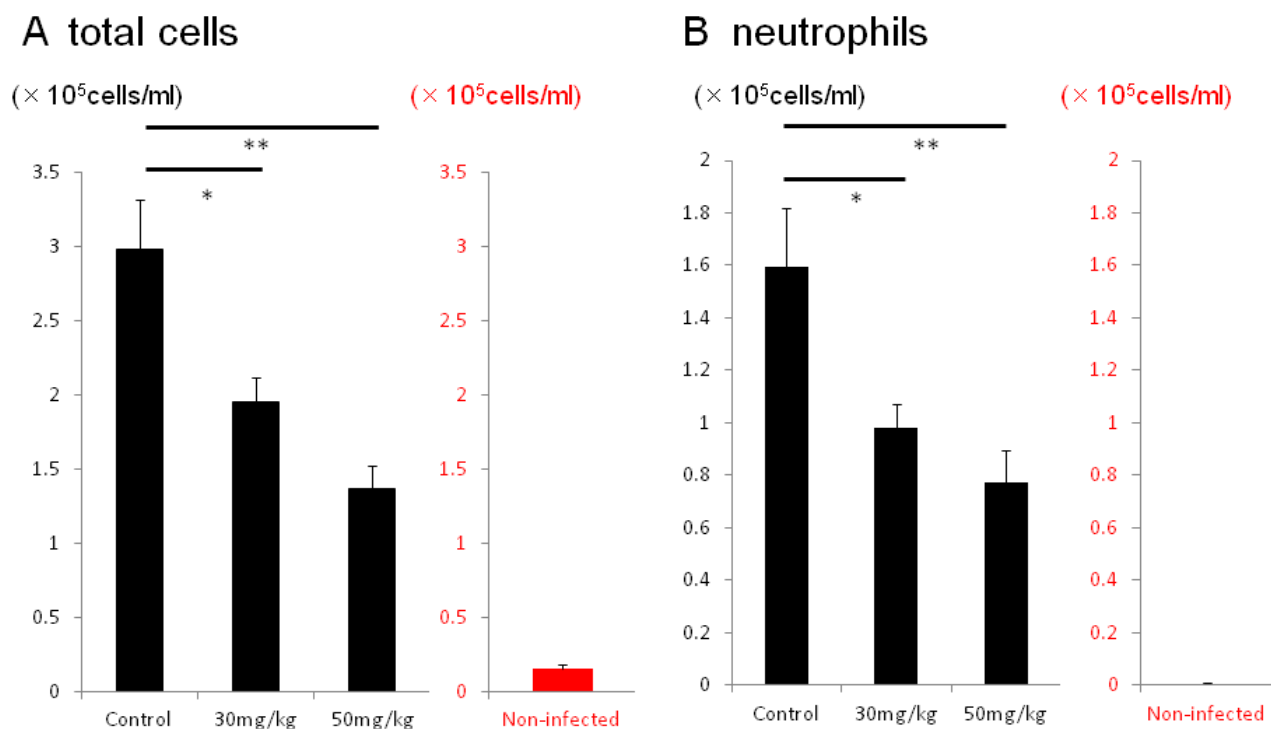
Fig. 5. Effect of KRP-109 on number of inflammatory cells in BALF of infected

mice. Numbers of total cells (A) and neutrophils (B) in BALF were compared

with control group and KRP-109 group (30 or 50 mg/kg). The total cell numbers

were determined using a hemocytometer with Turk stain and neutrophil

numbers were done on slides stained with Diff-Quik. The data are expressed as

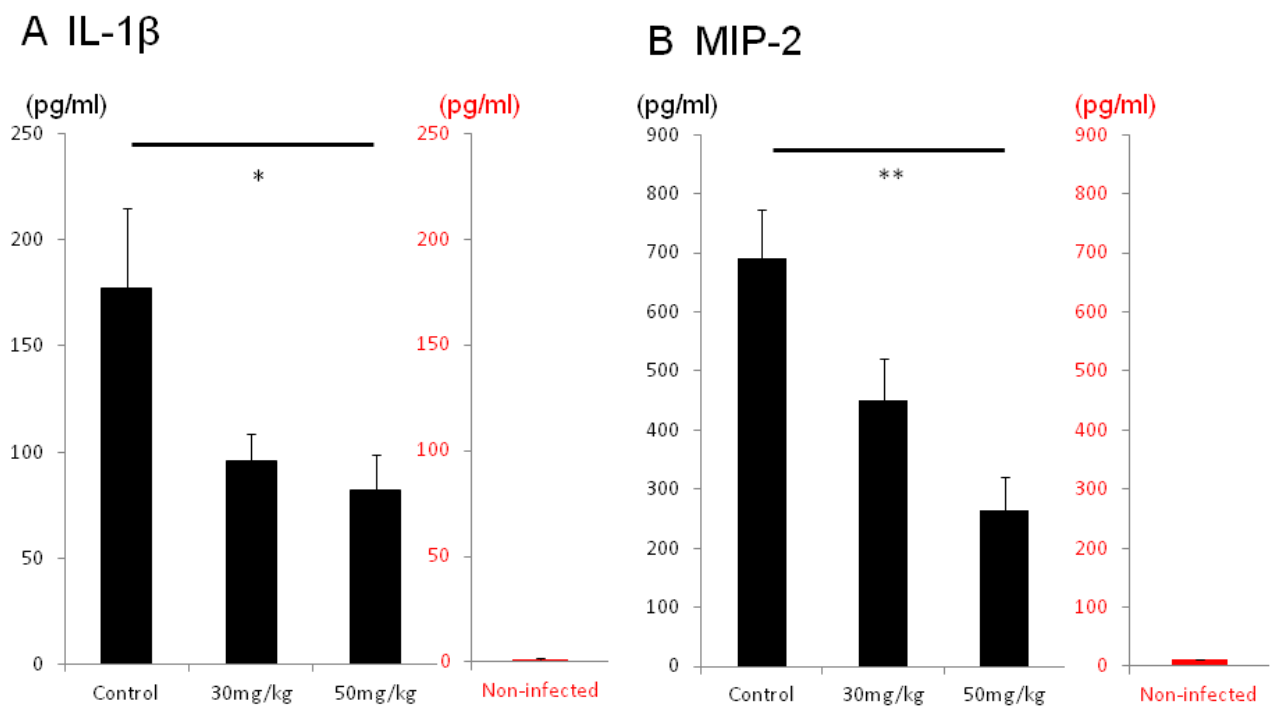means ± SEM. *, P<0.05, **, P<0.01 versus control group (n=8 in each group).

Fig. 6. Effect of KRP-109 on inflammatory cytokines in BALF of infected mice.

The cytokine levels in BALF on control and KRP-109 groups (30 and 50

mg/kg) were detected using ELISAs: IL-1β (A) and MIP-2 (B). The data are

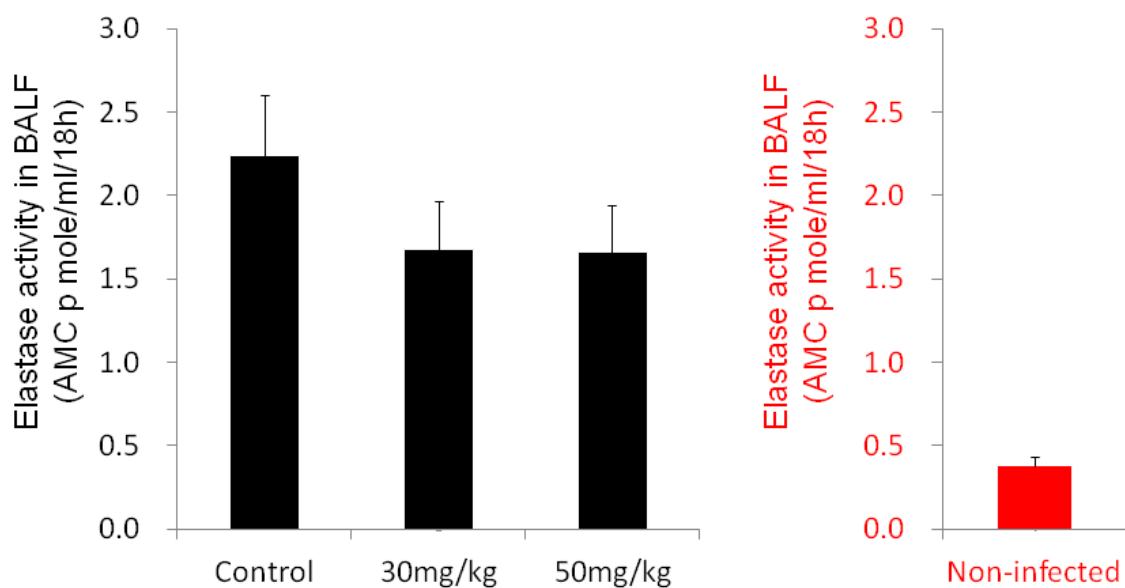expressed as means ± SEM. *, P<0.05, **, P<0.01 versus control group (n=8 in

each group).

Fig. 7. Effect of KRP-109 on NE activity in BALF. NE activity in BALF was

measured using the fluorogenic substrate Meo-Suc-Ala-Pro-Val-AMC (AMC).

KRP-109 reduced NE activity in comparison with the control group, but this

difference was not statistically significant (n=5 in each group).