Premedication with Clarithromycin Is Effective against Secondary Bacterial Pneumonia during Influenza Virus Infection in a Pulmonary Emphysema Mouse Model

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ABSTRACT

Secondary bacterial pneumonia (SBP) during influenza increases the severity of chronic obstructive pulmonary disease (COPD) and its associated mortality. Macrolide antibiotics, including clarithromycin (CAM), are potential treatments for a variety of chronic respiratory diseases owing to their pharmacological activities, in addition to antimicrobial action. We examined the efficacy of CAM for the treatment of SBP after influenza infection in COPD. Specifically, we evaluated the effect of CAM in elastase-induced emphysema mice that were inoculated with influenza virus (strain A/PR8/34) and subsequently infected with macrolide-resistant Streptococcus pneumoniae. CAM was administered to the emphysema mice 4 days prior to influenza virus inoculation. Premedication with CAM improved pathologic responses and bacterial load 2 days after S. pneumoniae inoculation. Survival rates were higher in emphysema mice than control mice. While CAM premedication did not affect viral titers or exert antibacterial activity against S. pneumoniae in the lungs, it enhanced host defense and reduced inflammation, as evidenced by the significant reductions in total cell and neutrophil counts and interferon (IFN)-γ levels in bronchoalveolar lavage fluid and lung homogenates. These results suggest that CAM protects against SBP during influenza in elastase-induced emphysema mice by reducing IFN-γ production, thus enhancing immunity to SBP, and by decreasing neutrophil infiltration into the lung to prevent injury. Accordingly, CAM may be an effective strategy to prevent secondary bacterial pneumonia in COPD patients in areas in which vaccines are inaccessible or limited.

Introduction

Chronic obstructive pulmonary disease (COPD) is the third-leading cause of death globally (World Health Organization, http://www.who.int/mediacentre/factsheets/fs310/en/ (Accessed March 14, 2016) and is of particular concern in middle- and low-income countries (Mathers and Loncar, 2006). Secondary infections by viral or bacterial pathogens often trigger acute exacerbations of COPD, resulting in morbidity and mortality (Bautista et al., 2010). The influenza virus is associated with 5–26% of cases of COPD exacerbation (Rohde et al., 2003; Kurai et al., 2013; Dai et al., 2015) and predisposes the host to secondary bacterial pneumonia (SBP). Streptococcus pneumoniae is associated with 10–25% of all cases of acute exacerbation of COPD (Sethi et al., 2002; Sapey and Stockley, 2006).

Coinfection with influenza virus and S. pneumoniae contributes substantially to the increased morbidity and mortality associated with seasonal and pandemic influenza (Murata et al., 2007; Morens et al., 2008; van der Sluijs et al., 2010; Cilloniz et al., 2012). In fatal cases of influenza virus infection, the time to death from the onset of illness is significantly shorter in patients with bacterial co-infections (Brundage and Shanks, 2008).

Owing to the significant increase in morbidity and mortality associated with influenza and secondary bacterial pneumonia infection in COPD patients, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) recommends the annual administration of the influenza vaccine and the pneumococcal polysaccharide vaccine to high-risk COPD patients. However, these vaccines are often not readily accessible in low-income countries and in low-resource settings. Accordingly, alternative preventive and prophylactic measures are necessary.

Long-term treatment with macrolide antibiotics decreases the risk of acute exacerbation of COPD according to some
studies (Albert et al., 2011; Yamaya et al., 2012; Donath et al., 2013; Spagnolo et al., 2013). A variety of pharmacological activities, including direct antibacterial, antiviral (e.g., against influenza virus and rhinovirus), anti-inflammatory, and immunomodulatory activity, as well as inhibitory effects on mucus secretion, bacterial virulence, and biofilm formation (Kanoh and Rubin, 2010; Zarogoulidis et al., 2012; Spagnolo et al., 2013) are thought to contribute to this protective effect, but the precise mechanisms of action and the effects of long-term macrolide therapy on influenza virus and S. pneumoniae coinfection in COPD patients have not been clarified.

In the present study, we investigated the effects and mechanism of action of clarithromycin (CAM) in a mouse model of COPD with secondary infection by influenza virus and subsequent coinfection by CAM-resistant S. pneumoniae.

Materials and Methods

Microorganisms. Influenza virus (strain A/PR8/34: H1N1 type) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The virus was cultured in Madin-Darby canine kidney cells for 3 days. Cells were then centrifuged at 2000 g for 15 minutes and the supernatant was stored at −80°C as a primary virus stock. A mouse-adapted influenza virus was prepared. Briefly, primary virus stock solutions were inoculated intranasally to anesthetized C57BL/6J mouse. After 3 days, lung homogenates were collected and centrifuged at 2000 g for 15 minutes. The supernatant was termed the first-generation mouse-adapted influenza virus; a third-generation mouse-adapted influenza virus was prepared by repeating the above methods and storing the stock solutions at −80°C. Viral stocks were thawed and diluted with phosphate-buffered saline (PBS) to the desired concentration prior to use.

The macrolide-susceptible S. pneumoniae strain ATCC49619 and a clinical isolate of macrolide-resistant S. pneumoniae strain NU4471 were used. ATCC49619 (minimum inhibitory concentration of clarithromycin, 0.125 μg/ml; serotype 19F) was purchased from ATCC. NU4471 (minimum inhibitory concentration of clarithromycin >256 μg/ml; serotype 19) was obtained from the Nagasaki University School of Medicine (Nagasaki, Japan). The presence of both erm B and mef E/A resistance genes in NU4471 was confirmed by polymerase chain reaction as previously reported (Fukuda et al., 2006). Bacteria were stored at −80°C until use.

Viral Titer and 50% Tissue Culture Infective Dose. The virus titer in Madin-Darby canine kidney cells was determined by measuring the 50% tissue culture infective dose (TCID₅₀) following the methods described by Reed and Muench (1938). Briefly, when cells reached 80% confluence in a 96-well plate, they were infected with 50 μl of 10-fold serial virus dilutions (four replicates). Minimal essential medium supplemented with 0.5% bovine serum albumin and 2.5 μg/ml trypsin was used for the virus dilutions. After 2 hours, the solutions were removed and the cells were incubated at 37°C. Seventy-two hours later, the cytopathic effect was monitored using an inverted microscope, and TCID₅₀ was determined.

Emphysema Mouse Model with Influenza and Secondary Bacterial Pneumonia. The mouse model is described in Fig. 1A. All experimental protocols and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and performed in compliance with Nagasaki University guidelines for animal experiments. Specific-pathogen-free male C57BL/6J mice

![Fig. 1. Experimental schedule of treatment.](image-url)

![Fig. 2. Survival curve of secondary bacterial pneumonia mice treated with or without CAM.](image-url)
8–9 weeks old; Charles River Laboratories, Japan, Inc., Yokohama, Japan) were anesthetized by intraperitoneal injection of 1.25% (w/v) pentobarbital. Pulmonary emphysema was induced by injecting 3 units of porcine pancreatic elastase (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 50 μl of sterile PBS into the trachea (Day –21). Pulmonary emphysema was verified by microscopic examination 14 days later in a preliminary evaluation. Twenty-one days after the porcine pancreatic elastase injection (Day 0), mice were inoculated intranasally with $1 \times 10^4$ TCID$_{50}$/mouse influenza virus. Four days later (Day 4), a subgroup of mice were also inoculated with $1 \times 10^6$ colony-forming units of $S$. pneumoniae NU4471 to induce secondary bacterial pneumonia. Eight to ten mice were used in each experimental group.

**Clarithromycin Medication and Survival Analysis.** CAM was obtained from Taisho Toyama Pharmaceutical (Tokyo, Japan). CAM powder was dissolved in 5% gum arabic solution and diluted to a concentration of 25 mg/ml. This solution was stored at 4°C for no more than 7 days. CAM was administered orally once daily (200 mg/kg per day) either from Day –4 (premedication group) or from Day 1 (postexposure medication group) until Day 14. In the premedication and postexposure medication groups, sets of mice were administered 5% gum arabic solution (200 μl) orally once daily as the vehicle control group. A survival analysis was performed using the log-rank test, and the Kaplan-Meier method was used to estimate short-term (14 days) survival for comparisons between the CAM premedication and postexposure medication groups and the respective vehicle controls.

**Pathologic and Bacteriological Examination of Lung Tissues.** On Day 6 (i.e., 6 days after inoculation with influenza virus and 2 days after coinfection with $S$. pneumoniae NU4471), a subset of mice from the CAM premedication group were sacrificed and lung tissue was taken for pathologic (n = 3 mice) and bacteriological examination. Bacterial counts in lung homogenates (n = 7 mice/group). Data are presented as means ± S.E. *P < 0.05, significant difference versus control.

**Fig. 3.** Representative hematoxylin and eosin-stained tissue sections of the vehicle control group (A, B) and CAM premedication group (C, D) showing pathologic inflammatory changes and bacterial proliferation on Day 6. Magnification: 40× (A, C) and 200× (B, D).

**Fig. 4.** Bacterial counts in lung homogenates (n = 7 mice/group). Data are presented as means ± S.E. *P < 0.05, significant difference versus control.

**Fig. 5.** Local antibacterial activity of CAM in the lung. Agar plates inoculated with (A) NU4471 (macrolide-resistant $S$. pneumoniae) and (B) ATCC49619 (macrolide-susceptible $S$. pneumoniae). Four paper discs were arranged as follows: upper left, lung homogenates of vehicle-treated mice; lower left, lung homogenates of CAM-treated mice; upper right, garenoxacin; and lower right, CAM.
bacteriological (n = 7 mice) examinations. For the pathologic examination, the tissue sample was fixed in 10% buffered formalin and stained with hematoxylin and eosin. For the bacteriological examination, the number of bacteria in the lungs was determined as described previously (Otsu et al., 2003). Briefly, the lungs were dissected under aseptic conditions and suspended in 1 ml of sterile saline at Day 6. Organs were homogenized, quantitatively inoculated onto blood agar plates by serial dilution, and incubated at 37°C for 24 hours. Colony numbers were counted and the number of bacteria was calculated as colony-forming units per milliliter.

**Local Antibacterial Activity and Viral Titer in Lung Tissue.** On Day 4 (i.e., 4 days after inoculation with influenza virus and immediately prior to coinfection with S. pneumoniae NU4471, a subset of mice from the CAM premedication group were sacrificed. Local antibacterial activity in the lung was determined using the paper disc agar diffusion method. Petri dishes containing blood agar (TSA II 5% Sheep Blood Agar M; Japan Becton, Dickinson and Company, Tokyo, Japan) were prepared and each plate was inoculated with cultures of macrolide-susceptible S. pneumoniae (ATCC49619) or macrolide-resistant S. pneumoniae (NU4471) adjusted to McFarland No. 0.5 by swabbing on the whole agar surface with cotton wool. Lungs were homogenized and centrifuged at 3000 rpm for 10 minutes, and the supernatant was evenly spread on both sides of filter paper discs (6-mm diameter paper disc for antibiotic assay; Toyo Roshi Kaisha, Ltd. Tokyo, Japan). The discs were aseptically placed on the plates. CAM discs (15 μg; Eiken Chemical Co., Ltd. Tokyo, Japan) and garenoxacin discs (5 μg; Eiken Chemical Co., Ltd.) were used as positive controls. The Petri dishes were incubated at 37.0°C for 24 hours. At the end of this period, inhibition zones that formed on the media were recorded.

The influenza viral titer in the lung was determined using the supernatant of the lung homogenates as viral solutions as described previously (50% Tissue Culture Infective Dose; TCID50/ml).

**Bronchoalveolar Lavage Cell Counts and Cytokine Analysis.** On Day 4 (i.e., 4 days after inoculation with influenza virus and immediately prior to coinfection with S. pneumoniae NU4471), a subset of mice from the CAM premedication group and vehicle control group were sacrificed for a bronchoalveolar lavage fluid (BALF) analysis. Additional control mice that were inoculated with PBS instead of influenza virus on Day 0 were also investigated. The effect of CAM premedication on inflammation in the lung was examined.

The anesthetized mice were sacrificed by incision of the axillary artery and vein. The lung blood was perfused by injection of 3 ml of sterile saline into the right ventricle. BAL was performed by washing the lungs and airways with 1 ml of sterile saline three times per mouse. The number of live cells in the BALF was determined using a LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, Inc., Annandale, VA). BALF was centrifuged at 1100 rpm for 2 minutes using the Cytospin III (Thermo Fisher Scientific K.K., Kanagawa, Japan), and differential cell counts were analyzed using Diff Quick staining (Sysmex, Kobe, Japan). The BALF supernatants were analyzed for cytokines and chemokines associated with the immune response to viral infections [interleukin (IL)-4, IL-10, IL-12, macrophage inflammatory protein-2 (MIP-2), IFN-α, IFN-β, and IFN-γ] by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions.

**Cytokine and Chemokine Analysis in Lung Homogenates and Serum.** On the basis of ELISA results from the BALF analysis, the levels of IL-10 and IFN-γ were examined in lung homogenates and serum on Day 4 using ELISA.

**Statistical Analysis.** Data were presented as means ± S.E. A survival analysis was performed using the log-rank test, and survival rates were calculated using the Kaplan-Meier method. Comparisons between two groups were performed using Mann-Whitney U tests. Comparisons among four groups were performed using the Tukey-Kramer method. A P-value of <0.05 was considered statistically significant.

**Fig. 6.** Influenza viral titers of lung homogenates at Day 4. Data are presented as means ± S.E., n = 4 mice. N.S., no significant difference between two groups; *P < 0.05, significant difference versus the vehicle control group.

**Fig. 7.** Anti-inflammatory effect of CAM on BALF on the basis of the number of (A) total cells and (B) neutrophils in each group. Data are presented as means ± S.E.; n = 5–9 mice. N.S., no significant difference between two groups; *P < 0.05, significant difference between two groups.
Results

Premedication with CAM Improved Survival Rates. Postexposure treatment with CAM did not improve the survival rate compared with that of the vehicle control. However, premedication with CAM resulted in a significantly higher survival rate than that of the vehicle control ($P$, 0.05, Fig. 2, A and B).

CAM Premedication Suppressed Lung Inflammation and Bacterial Counts. Figure 3 shows representative images of hematoxylin and eosin-stained lung sections from the premedication group at Day 6. Vehicle control mice exhibited bronchopneumonia, characterized by abundant neutrophils, particularly in peribronchiolar lesions as well as in airways, alveolar spaces, and the interstitium (Fig. 3, A and B). In contrast, the premedication group showed significantly less neutrophilic inflammation than that of the control group (Fig. 3, C and D) on the basis of a quantitative analysis of histologic images (control group: 102 ± 6 neutrophils/mm$^2$, premedication group: 65 ± 4 neutrophils/mm$^2$; $P < 0.0001$). The bacterial count was significantly lower in the lungs of the premedication group than those of the control group ($P < 0.05$, Fig. 4).

CAM Did Not Exhibit Antibacterial Activity against NU4471 in the Lungs. The discs with lung homogenates (Fig. 5A, lower left) and those with 15 μg of CAM (Fig. 5A, lower right) did not form inhibition zones on the macrolide-resistant NU4471 plate, but both discs formed inhibition zones on the macrolide-susceptible ATCC49619 plate (Fig. 5B).

CAM Did Not Affect Viral Titers at the Time of Inoculation with NU4471. There was no significant difference between the premedication and control groups in the viral titers of the lung homogenates on Day 4 (Fig. 6).

CAM Reduced the Number of Neutrophils in BALF. The total number of cells and neutrophils in BALF from the CAM-negative/influenza virus-positive group was significantly higher than that observed in the BALF from the CAM-negative/influenza virus-negative group. Although no significant differences in cell number were observed after CAM medication, there was a significant reduction in the number of neutrophils compared with the control group (Fig. 7). No significant differences between groups were observed for other cell subsets (lymphocytes, basophils, and eosinophils; data not shown).

Effects of CAM on Cytokines and Chemokines. The levels of IFN-α, IFN-β, and INF-γ were significantly higher than that observed in the BALF from the CAM-negative/influenza virus-negative group. Although no significant differences in cell number were observed after CAM medication, there was a significant reduction in the number of neutrophils compared with the control group (Fig. 7). No significant differences between groups were observed for other cell subsets (lymphocytes, basophils, and eosinophils; data not shown).

Fig. 8. Anti-inflammatory effect of CAM on cytokine levels in BALF: (A) IFN-α, (B) IFN-β, (C) IFN-γ, and (D) IL-10. Data are presented as means ± S.E., $n = 5–9$ mice. N.S, no significant difference between two groups; *$P < 0.05$, significant difference between two groups.
investigation of IFN-γ levels in the serum and lung revealed that INF-γ levels were significantly higher in the lung and serum during influenza infection than in the controls (Fig. 9). CAM medication significantly reduced IFN-γ levels in lung homogenates, but did not significantly influence its serum levels (Fig. 9).

**Discussion**

On the basis of our results, premedication with CAM initiated 4 days before influenza virus inoculation in COPD-model mice, but not postexposure medication, improved the survival of mice against subsequent coinfection with the influenza virus and *S. pneumoniae*. Mice with improved survival also had a less severe inflammatory response in lung tissues than those of mice in the control group. Although viral titers were not affected by CAM premedication, viable bacterial counts decreased in lung tissues, even though *S. pneumoniae* NU4471 is macrolide-resistant. CAM accumulates to higher concentrations in the bronchial epithelial fluid than in the serum (McCarty, 2000; Kikuchi et al., 2008); accordingly, we examined the local antimicrobial activity of CAM in lung tissues (Fig. 5) and confirmed that the lung homogenates did not possess antibacterial activity against *S. pneumoniae* NU4471. Further investigation revealed that CAM premedication significantly reduced the number of total inflammatory cells, including neutrophils, in BALF and significantly attenuated IFN-γ levels in BALF and lung homogenates. These results suggest that the improvement in survival rates associated with CAM premedication could be attributed to an anti-inflammatory effect exerted prior to the inoculation of mice with *S. pneumoniae*.

Taken together, CAM premedication reduced the susceptibility of COPD mice infected with influenza virus to subsequent *S. pneumoniae* infection by decreasing the overall inflammatory response and ultimately improved the survival of COPD model mice infected with both pathogens. Our results obtained using COPD-model mice are similar to previous reports demonstrating that macrolides offer clinical benefits in reducing acute exacerbations of COPD (Albert et al., 2011; Ni et al., 2015). Erythromycin or azithromycin therapy for 6–12 months could effectively reduce the frequency of exacerbations and improve the quality of life in patients with COPD (Albert et al., 2011; Ni et al., 2015). Unlike previous studies reporting that macrolides exert direct antiviral effects on the influenza virus (Miyamoto et al., 2008; Yamaya et al., 2010), CAM premedication did not influence viral titers in our study. Rather, CAM premedication appeared to act via a chemokine-mediated mechanism by disrupting the cycle of infection-inflammation and strengthening lung defenses, as reported previously (Spagnolo et al., 2013).

Interferons play critical and complex roles in host defense against viral and bacterial infections. For example, IFN-α and IFN-β (Type-1 IFNs) prevent the progression of local lung infection with *S. pneumoniae* (Trinchieri, 2010; LeMessurier et al., 2013), and IFN-γ (Type-2 IFN, Type-1 T-helper cell cytokine) combats the early stages of viral infection (Muller et al., 1994; Schroder et al., 2004). However, high levels of IFN-α, IFN-β, and IFN-γ induced by influenza virus infection enhance susceptibility to secondary pneumococcal infection (Sun and Metzger, 2008; Nakamura et al., 2011; Li et al., 2012; Lee et al., 2015) via various mechanisms, including IFN-γ-mediated inhibition of initial bacterial clearance from the lung by alveolar macrophages (Sun and Metzger, 2008). The trends toward reduced IFN-α and IFN-β and the significantly lower levels of IFN-γ observed after CAM premedication in our study and in previous studies (i.e., reduced IFN-γ levels were detected after macrolide therapy in mice with influenza virus–induced lung injury (Sato et al., 1998)) might have enhanced bacterial clearance from the lung and reduced the susceptibility of influenza-infected COPD mice to secondary pneumococcal pneumonia.

Influenza-infected COPD mice showed a more advanced pathology, characterized by significantly more neutrophils in the lung tissue and BALF compared with control mice. Excessive neutrophil infiltration into the lungs has been reported during influenza virus infection and disrupts the alveolar-capillary barrier, leading to acute lung injury and death (Seki et al., 2010; Galani and Andreakos, 2015; Herold et al., 2015). Conversely, reductions in neutrophils or neutrophil function increase the severity of influenza virus infection (Tate et al., 2009). CAM has been reported to enhance the nonphlogistic clearance of apoptotic neutrophils by alveolar macrophages (Yamaryo et al., 2003). Indeed, CAM premedication in our study reduced neutrophil numbers in the lung tissue and BALF of influenza-infected COPD mice and might have attenuated the potential for inflammation-induced lung damage.
While vaccination strategies are most efficient with respect to protection against influenza and pneumococcal infection in COPD patients, the global supply of influenza vaccines is inadequate during influenza pandemics and is of particular concern in low- and middle-income countries (Partridge and Kieny, 2013), where the incidence of COPD and the demand for influenza vaccinations are expected to increase. Our results suggest that macrolide premedication may be an effective strategy in COPD patients at a high risk of infection with influenza virus and S. pneumoniae to prevent severe or even fatal secondary bacterial pneumonia in these high-risk and low-resource settings. Additional studies are required to identify the ideal patient group and the optimal timing and duration of macrolide premedication to avoid in judicious administration over extended periods (Ni et al., 2015).

In conclusion, administration of CAM initiated 4 days prior to influenza virus infection in COPD mice reduced IFN-γ production and enhanced the immune response against secondary bacterial pneumonia to reduce bacterial counts and prolong survival rates. Future studies should evaluate the use of macrolide premedication in COPD patients at a high risk of infection with influenza virus and S. pneumoniae to prevent severe secondary bacterial pneumonia.

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Contributed new reagents or analytic tools: Kosai, Izumikawa, Yanagihara, Kohno.
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