Antimicrobial and immunomodulatory effect of clarithromycin on macrolide-resistant *Mycoplasma pneumoniae*

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Macrolide antibiotics are frequently administered to treat mycoplasmal pneumonia. However, macrolide-resistant *Mycoplasma pneumoniae* has recently been isolated from clinical specimens in Japan. Clarithromycin (CAM) is a 14-membered-ring macrolide that has host immunomodulatory activity. Here, we established a gnotobiotic mouse model that was monoassociated with macrolide-resistant *M. pneumoniae*, and pathologically and microbiologically analysed the effects of antibiotics against mycoplasmal pneumonia. We also examined the immunomodulatory activities of macrolide antibiotics in human lung carcinoma A549 cells *in vitro* and in a specific-pathogen-free (SPF) mouse model of pneumonia induced by *M. pneumoniae* antigen *in vivo*. CAM anti-mycoplasma antibiotics decreased the number of macrolide-sensitive and -resistant *M. pneumoniae* in the lungs of gnotobiotic mice. Thus, in SPF mice, CAM modulated pulmonary inflammation induced by *M. pneumoniae* antigens.

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INTRODUCTION

Mycoplasma pneumoniae is a common cause of community-acquired respiratory tract infection mainly in children and young adults, and the main symptoms are intractable dry cough, chest pain and fever. Convalescence is usually uneventful, but mycoplasmal pneumonia can cause various complications such as Guillain–Barré syndrome, Stevens–Johnson syndrome, hepatitis, myocarditis and arthritis (Ginestal *et al.*, 2004; Hakkarainen *et al.*, 1992; Stutman, 1987; Yamane & Kawai, 1978). This micro-organism adheres to bronchial epithelial cells, where it depresses ciliary movement and affects bronchial function. However, the pathogenic mechanisms of mycoplasmal pneumonia and extrapulmonary complications directly arising from *M. pneumoniae* virulence factors are difficult to explain as this micro-organism lacks lipopolysaccharide and does not produce toxin. Consequently, indirect tissue injury with a host immune response is related to the cause of mycoplasmal pneumonia (Mizutani *et al.*, 1971). A hamster model of *M. pneumoniae* infection has been created (Fernald *et al.*, 1972), but reagents necessary for

immunological analyses of hamsters remain insufficient. Furthermore, to induce mycoplasmal pneumonia by infecting specific-pathogen-free (SPF) mice that can be immunologically evaluated is difficult. We established a gnotobiotic model of mycoplasmal pneumonia using SPF mice and analysed the pathology of mycoplasmal pneumonia (Hayakawa *et al.*, 2002; Sekine *et al.*, 2009).

Macrolides are generally considered the first-choice of treatment for *M. pneumoniae* infection. Although tetracyclines and fluoroquinolones are effective against *M. pneumoniae*, these agents are toxic and thus not recommended for treating children. However, macrolide-resistant *M. pneumoniae* has been detected in clinical specimens since 2000 and almost all resistant strains have high MICs (Okazaki *et al.*, 2001). Macrolide-resistant *M. pneumoniae* can neutralize the effectiveness of treatment for mycoplasmal pneumonia (Matsuoka *et al.*, 2004).

Recent reports indicate that macrolide antibiotics have immunomodulatory activity such as evoking neutrophilic functions and cytokine production (Kawasaki *et al.*, 1998; Kikuchi *et al.*, 2002; Kohyama *et al.*, 1999). Here, we established a gnotobiotic model of *M. pneumoniae*

Abbreviations: CAM, clarithromycin; CPFX, ciprofloxacin; IL-8, interleukin-8; MINO, minocycline; SPF, specific-pathogen-free.

infection using germ-free mice. We then evaluated the antibiotic activities of clarithromycin (CAM) against mycoplasmal pneumonia using pathological and immunological methods. The immunomodulatory effects of CAM were analysed in SPF mice inoculated with sonicated crude *M. pneumoniae* antigen. We also examined the antimicrobial and immunomodulatory activities of anti-mycoplasma antibiotics against macrolide-resistant *M. pneumoniae* as well as the regulation of cytokine production by anti-mycoplasma antibiotics in an established cell line *in vitro*.

METHODS

Bacterial strains and culture. We used the following strains of *M. pneumoniae*. Type strain *M. pneumoniae* M129, against which the MIC of CAM is $0.0156 \mu\text{g ml}^{-1}$, was stocked at the Department of Infectious Diseases, Kyorin University School of Medicine. The clinically isolated *M. pneumoniae* strain 379, against which the MIC of CAM is $\geq 256 \mu\text{g ml}^{-1}$, was provided by the National Institute of Infectious Diseases. *M. pneumoniae* cells were cultured at 37°C under a 5% CO_2 atmosphere for 7–10 days in PPLO broth (Oxoid) containing mycoplasma supplement-G (Oxoid) and used for experimental infection and the preparation of soluble *M. pneumoniae* antigen.

Experimental animals and breeding conditions. We purchased female germ-free mice (IqI/Jic; 8 weeks of age) from Clea Japan and bred them in vinyl isolators that were sterilized with Expox (Alcide). The mice were fed with a cobalt 60-irradiated diet and sterilized water. We confirmed asepsis before experimental infection by aerobic and anaerobic cultures of caecal contents on GAM agar (Nissui) supplemented with 5% horse blood. We also used female SPF mice (BALB/cA Jcl; 8 weeks of age; Clea Japan) for sensitization studies.

Preparation of sonicated *M. pneumoniae* crude antigens. The *M. pneumoniae* strains M129 and 379 were cultured at 37°C for 7–10 days, and then harvested and washed twice by centrifugation at $10\,000\text{ g}$ for 20 min in Hanks' balanced salt solution (Gibco). The cells were sonicated 10 times for 1 min per burst at output 7 (Sonifier 250; Branson Ultrasonic). The supernatant decanted after centrifugation at $10\,000\text{ g}$ for 5 min served as crude antigen. The protein concentration of the suspension was measured using the Bio-Rad Protein Assay.

Experimental infection and inoculation. Animal experiments were approved by the Institutional Animal Care and Use Committee of Kyorin University School of Medicine (Approval No. 05-31-36). Mice were anaesthetized intraperitoneally with 25 mg sodium pentobarbital ($\text{kg body weight}^{-1}$) (Dainippon Sumitomo Pharma). Germ-free mice were intranasally infected with *M. pneumoniae* strains M129 and 379 prepared in PPLO broth without supplement yielding 10^5 – 10^6 c.f.u. ($40 \mu\text{l}$) $^{-1}$. The infection of the mice was repeated in the same manner at 4 weeks after the first challenge. The mice were sacrificed on day 7 after the last infection and lung tissues were obtained for bacteriological and pathological studies.

We intranasally inoculated SPF mice with $20 \mu\text{g}$ protein per $40 \mu\text{l}$ of sonicated crude antigens from *M. pneumoniae* strains M129 and 379. The inoculation of the mice was repeated in the same manner at 4 weeks after the first challenge and the mice were then sacrificed on

day 2 after the last inoculation. Lung tissues were collected for pathological studies.

Administration of anti-mycoplasma antibiotics. Gnotobiotic mice infected with *M. pneumoniae* were orally administered 100 and 200 mg CAM (Taisho Toyama Pharmaceutical) kg^{-1} , 5 and 50 mg minocycline (MINO; Sawai Pharmaceutical) kg^{-1} and 10 and 100 mg ciprofloxacin (CPFX; MP Biomedicals) kg^{-1} , which were all suspended in 0.2 ml 5% acacia, once daily for 7 days. Mice inoculated with *M. pneumoniae* antigen were also orally administered anti-mycoplasma antibiotics once daily for 2 days after the last inoculation.

Bacteriological assessments of infected mice. Lung specimens collected 7 days after the repeat infection were emulsified using a sterilized homogenizer (Ikemoto Scientific Technology), serially diluted and then inoculated on PPLO agar plates at 37°C for 7–10 days. The numbers of micro-organisms were determined from the numbers of colonies generated on the plates.

Pathological examination. The lower halves of right lungs of mice at 7 days after the repeat infection were fixed in 5% formalin. Sections of paraffin-embedded tissues were stained with haematoxylin and eosin and observed by light microscopy. On the second day after the last inoculation, the SPF mice were sacrificed in the same manner.

Cytokine production in alveolar epithelial cells induced by sonicated crude antigens from *M. pneumoniae* strain M129 (immunological examination *in vitro*). A549 human lung epithelial cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Gibco) in a 5% CO_2 humidified atmosphere at 37°C , and then 1.0×10^5 cells were seeded into 24-well plates (AGC Techno Glass) for up to 24 h. The medium was replenished and then the A549 cells were incubated with sonicated crude antigens from *M. pneumoniae* M129 (0, 0.1, 1.0, 5.0 and $10.0 \mu\text{g ml}^{-1}$) for up to 24 h. The specific immunoreactivity of interleukin-8 (IL-8) in culture supernatants was measured using an ELISA (R&D Systems).

Modulation of cytokine production in cultured cells induced by anti-mycoplasma antibiotics in the presence of sonicated crude antigens of *M. pneumoniae* M129. A549 cells (2.0×10^5) seeded in 24-well plates for up to 24 h were incubated with CAM, MINO and CPFX (0, 0.1, 1.0 and $10.0 \mu\text{g ml}^{-1}$). The medium and anti-mycoplasma antibiotics were replenished and then the A549 cells were incubated with sonicated crude antigens of *M. pneumoniae* strain M129 ($5.0 \mu\text{g ml}^{-1}$) for up to 24 h. The specific immunoreactivity of IL-8 in culture supernatants was measured using ELISA.

RESULTS

Effect of antibiotics on gnotobiotic mice infected with macrolide-sensitive and -resistant *M. pneumoniae* strains

Viable *M. pneumoniae* M129 (2.3×10^6 c.f.u. per lung) was isolated from gnotobiotic mice monoassociated with the M129 strain at 7 days after infection (Fig. 1a). Treatment with CAM, MINO and CPFX at 200, 50 and 100 mg kg^{-1} , respectively, significantly inhibited the growth of *M. pneumoniae* M129. Viable *M. pneumoniae* strain 379 (6.9×10^5 c.f.u. per lung) was isolated in gnotobiotic mice

Fig. 1. Effect of macrolide antibiotics on gnotobiotic mice infected with macrolide-sensitive M129 (a) and macrolide-resistant 379 (b) *M. pneumoniae* cells.

monoassociated with this strain at 7 days after infection (Fig. 1b). Each of CAM, MINO and CPFX dose-dependently reduced the numbers of strain 379.

Histopathological assessment of lungs

Fig. 2 shows a histological assessment of mouse lung tissue on day 7 after repeat infection with *M. pneumoniae* strain M129. We identified an inflammatory response with peribronchial lymphocytic infiltration in the lungs of gnotobiotic mice without CAM (Fig. 2a). Lymphocyte infiltration was slight in mice treated with CAM (100 mg kg⁻¹) (Fig. 2b). However, the lung tissues of gnotobiotic mice at day 7 treated with CAM (200 mg kg⁻¹) after repeat infection had not changed (Fig. 2c). Lung tissues from gnotobiotic mice treated with MINO (5 and 50 mg kg⁻¹) (Fig. 2d, e) and CPFX (100 mg kg⁻¹) (Fig. 2g) were similarly unchanged. However, mild, local inflammation was evident on day 7 in the lungs of gnotobiotic mice treated with 10 mg CPFX kg⁻¹ (Fig. 2f).

Fig. 3 shows histological staining of lung tissues from mice at day 7 after repeat infection with macrolide-resistant *M. pneumoniae* strain 379 and treatment with anti-mycoplasma antibiotics. The inflammatory response was mild in the lungs of repeatedly infected gnotobiotic mice without antibiotics (Fig. 3a), whereas lung tissues had not changed on day 7 after repeat infection among gnotobiotic mice that received anti-mycoplasma antibiotics (Fig. 3b–g).

Fig. 4 shows histological staining of mouse lung tissues on day 2 after repeat inoculation with sonicated antigens of *M. pneumoniae* strain M129. A severe inflammatory response with peribronchial neutrophilic infiltration was evident in the lungs of inoculated mice in the absence of antibiotics (Fig. 4a). However, CAM (100 and 200 mg kg⁻¹) moderated the severity of experimental pneumonia induced by sonicated antigens from *M. pneumoniae* strain M129 (Fig. 4b, c). Neither MINO (5 and 50 mg kg⁻¹) nor CPFX (100 and 200 mg kg⁻¹) decreased lung inflammation (Fig. 4d–g).

Fig. 5 shows histological findings of mouse lung tissues at day 2 after repeat inoculation with sonicated antigens of *M. pneumoniae* strain 379. Inflammation was obvious in the lungs of untreated inoculated mice (Fig. 5a), and none of the anti-mycoplasma antibiotics could decrease this response (Fig. 5b–g).

Production of IL-8 by A549 cultured cells stimulated with sonicated crude antigens from *M. pneumoniae* M129

Sonicated crude antigens of *M. pneumoniae* M129 dose-dependently stimulated IL-8 production in A549 alveolar epithelial type II cells (Fig. 6).

Anti-mycoplasma antibiotics modulate cytokine production by cultured cells in the presence of sonicated crude antigens of *M. pneumoniae* strain M129

Production of IL-8 by A549 cells stimulated with *M. pneumoniae* M129 sonicated crude antigens was decreased by CAM (Fig. 7), but not by either MINO or CPFX.

DISCUSSION

Interactions between bacteria and hosts can be investigated using gnotobiotic animals because the effects of bacterial normal flora can be ruled out (Butterton *et al.*, 1996; Kamiya *et al.*, 1997). Here, we evaluated the effects of anti-mycoplasma antibiotics using a gnotobiotic mouse model monoassociated with macrolide-sensitive and -resistant *M. pneumoniae* strains. CAM, MINO and CPFX significantly reduced the numbers of viable macrolide-sensitive *M. pneumoniae* cells in the lungs of gnotobiotic mice (Fig. 1a). Viable cells in the lungs of gnotobiotic mice

Fig. 2. Histopathological examination of lungs of mice infected with *M. pneumoniae* strain M129 and treated with saline (control) (a), 100 mg CAM kg⁻¹ (b), 200 mg CAM kg⁻¹ (c), 5 mg MINO kg⁻¹ (d), 50 mg MINO kg⁻¹ (e), 10 mg CPFY kg⁻¹ (f) and 100 mg CPFY kg⁻¹ (g). Magnification $\times 200$.

infected with macrolide-resistant *M. pneumoniae* were remarkably reduced by CAM; however, it was microbiologically ineffective (Fig. 1b). One report has indicated that bacterial growth can be suppressed by antibiotic concentrations below MICs (subMIC) (Odenholt-Tornqvist *et al.*, 1995).

Moreover, many reports have described the effects of new 14-membered-ring macrolide compounds that do not have antibacterial activity. Kudoh *et al.* (1987, 1998) have accumulated information about the mechanisms of macrolide antibiotic action from administering low-dose

Fig. 3. Histopathological examination of lungs of mice infected with *M. pneumoniae* strain 379 and treated with saline (control) (a), 100 mg CAM kg⁻¹ (b), 200 mg CAM kg⁻¹ (c), 5 mg MINO kg⁻¹ (d), 50 mg MINO kg⁻¹ (e), 10 mg CPFX kg⁻¹ (f) and 100 mg CPFX kg⁻¹ (g). Magnification ×200.

long-term erythromycin chemotherapy for diffuse pan-bronchiolitis. The 14-membered-ring macrolide antibiotics have an excellent transition to respiratory system organizations and phagocytes (Johnson *et al.*, 1980; Prokesch &

Hand, 1982). Intracellular CAM concentrations are 10–20-fold higher than extracellular concentrations. Macrolide antibiotics alter the migration and function of neutrophils, as well as the bactericidal ability of macrophages

Fig. 4. Histopathological examination of lungs of mice inoculated with sonicated antigens of *M. pneumoniae* strain M129 and treated with saline (control) (a), 100 mg CAM kg⁻¹ (b), 200 mg CAM kg⁻¹ (c), 5 mg MINO kg⁻¹ (d), 50 mg MINO kg⁻¹ (e), 10 mg CPFY kg⁻¹ (f) and 100 mg CPFY kg⁻¹ (g). Magnification $\times 200$.

(Anderson, 1989; Carlone *et al.*, 1989; Cuffini *et al.*, 1989). These results suggest that CAM decreased the growth rate of macrolide-resistant *M. pneumoniae* *in vivo* and that the CAM concentration was higher in phagocytes and in the respiratory system than in serum, even when the serum concentration was below the MIC.

Furthermore, CAM reduced the number of viable cells in the lungs of gnotobiotic mice and enhanced infiltration by polymorphonuclear leukocytes and macrophages. Macrolide antibiotics can exert long-term antimicrobial activity when slowly released to the lungs (Gladue *et al.*, 1989; Nightingale, 1997). These findings

Fig. 5. Histopathological examination of lungs of mice inoculated with sonicated antigens of *M. pneumoniae* strain 379 and treated with saline (control) (a), 100 mg CAM kg⁻¹ (b), 200 mg CAM kg⁻¹ (c), 5 mg MINO kg⁻¹ (d), 50 mg MINO kg⁻¹ (e), 10 mg CPFX kg⁻¹ (f) and 100 mg CPFX kg⁻¹ (g). Magnification ×200.

suggest that CAM reduced lung inflammation in *M. pneumoniae*-associated gnotobiotic mice by decreasing the number of bacteria through antimicrobial activity (Fig. 2).

Although *M. pneumoniae* lacks a cell wall and lipopolysaccharide, the cell components induce a powerful host immunoreponse. We examined the effects of antimycoplasmal antibiotics in SPF BALB/c mice that were

Fig. 6. Production of IL-8 by A549 cells cultured with sonicated crude antigens from *M. pneumoniae* strain M129. * $P=0.134$, ** $P=0.037$, *** $P<0.001$, **** $P<0.001$ (Student's *t*-test).

repeatedly inoculated with sonicated crude antigens of *M. pneumoniae*. Neither MINO nor CPFX moderated the induced experimental pneumonia but CAM improved histopathological lung inflammation (Fig. 4). Macrolide antibiotics affect not only phagocytic functions but also cytokine and NO production without antibacterial activity (Amsden, 2005; Ianaro *et al.*, 2000; Labro, 1998). The present results suggested that the anti-inflammatory activity of CAM is independent of antibacterial activity in the mouse model of pneumonia induced by sonicated antigens from macrolide-sensitive *M. pneumoniae*. However, none of the tested anti-mycoplasma antibiotics improved lung inflammation in a similar mouse model of pneumonia induced by macrolide-resistant sonicated antigens from *M. pneumoniae* (Fig. 5). The model mice were inoculated with the same amounts of *M. pneumoniae* antigens, but CAM did not exert anti-inflammatory activity against pneumonia induced by the macrolide-

resistant strain. These results imply that variable host immunoresponses evoked by macrolide-sensitive and -resistant strains caused differences in CAM immunomodulation between the two experimental pneumonia models. These findings raise the notion that immune control levels of CAM are altered by antigenic differences among *M. pneumoniae* strains responsible for lung inflammation.

Components of *M. pneumoniae* evoke a powerful IL-8 response in epithelial cells (Chmura *et al.*, 2003; Sohn *et al.*, 2005). Besides various inflammatory mediators, IL-8 activates the migration of inflammatory cells such as neutrophils and alveolar macrophages, and this response is thought to play a crucial role in the pathogenesis of pulmonary diseases caused by *M. pneumoniae*.

Incubating A549 cells with sonicated antigens of *M. pneumoniae* M129 *in vitro* resulted in their significant production of IL-8 (Fig. 6), which was dose-dependently reduced by CAM, but not by the other tested antibiotics (Fig. 7). These results suggest that the suppression of IL-8 production by CAM represents one mechanism of anti-inflammatory activity in the lungs of mouse models of *M. pneumoniae* infection.

In conclusion, CAM reduced the numbers of macrolide-sensitive and -resistant *M. pneumoniae* cells in the lungs of the gnotobiotic mouse model. Furthermore, CAM has adequate effects on *M. pneumoniae* infection through palliating the excessive inflammation that arises from the host immunoresponse.

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Fig. 7. Effect of anti-mycoplasma antibiotics on IL-8 production by A549 cells cultured with sonicated crude antigens of *M. pneumoniae* strain M129. * $P=0.143$, ** $P=0.046$, *** $P=0.036$, † $P=0.056$, †† $P=0.209$, ††† $P<0.001$, ‡ $P=0.401$, ‡‡ $P=0.291$, ‡‡‡ $P=0.002$ (Student's *t*-test).

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