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Efficacy of clarithromycin against H5N1 and H7N9 avian influenza a virus infection in cynomolgus monkeys

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ABSTRACT

Clarithromycin (CAM), a 14-membered ring macrolide, has anti-inflammatory and immunomodulatory actions and antiviral effects in seasonal influenza virus infection. We examined the prophylactic and therapeutic efficacy of CAM against H5N1 highly pathogenic and H7N9 low pathogenic avian influenza virus infections in cynomolgus monkeys. CAM suppressed H5N1 virus-induced severe signs of disease in the treated monkeys and inhibited virus propagation in tracheal samples and the production of inflammatory cytokines in the lungs of monkeys infected with H5N1 and H7N9 viruses. The prophylactic administration of CAM showed more suppressive effects on clinical signs of disease and viral titers than did therapeutic administration. Thus, since administration of CAM alone showed a tendency to ameliorate clinical sings and to reduce levels of inflammatory cytokines, the macrolides are expected to have effects in combination with the other antiviral drugs on the prophylactic and treatment of patients with severe avian influenza virus infection, which should be further investigated.

Avian influenza virus (AIV) infections spread in poultry and humans. H5N1 highly pathogenic avian influenza A virus (HPAIV) infection in humans was first reported in 1997. Bermejo-Martin et al., 2009 and 2019, 861 confirmed human cases of H5N1 HPAIV infection have been reported to the World Health Organization (WHO) from 16 countries, with 455 deaths. H7N9 low pathogenic avian influenza A virus (LPAIV) infection in humans was first reported in 2013, and until 2017, 1567 confirmed cases, including at least 615 deaths, have been reported to WHO (Gao et al., 2013; Watanabe et al., 2013). Although the transmission of the viruses among humans is limited at present, we need to develop new anti-viral drugs to reduce the mortality of AIV infection. Neuraminidase inhibitors (NAIs) have been widely used for the treatment of influenza virus infection. However, use of NAIs allowed the emergence of viruses less sensitive to NAIs even at a low frequency (Kiso et al., 2004; Dharan et al., 2009; Okomo-Adhiambo et al., 2010; Tamura et al., 2011; Takashita et al., 2013; Kiso et al., 2010). Therefore, development of alternative anti-viral drugs is desirable before the spread of NAI -resistant strains in AIV infection.

Clarithromycin (CAM) and erythromycin (EM), 14-membered ring macrolides, and azithromycin (AZM), a 15-membered ring macrolide, have been widely used for the treatment of airway inflammation. These macrolides have anti-bacterial and anti-inflammatory actions, and low-dose, long-term treatment with CAM, EM, or AZM has been reported to be effective in patients with chronic airway diseases such as diffuse panbronchiolitis (Kudoh et al., 1998), cystic fibrosis (Jaffe et al., 1998), and chronic rhinosinusitis (Shimizu et al., 2016). The mechanism of these drugs on clinical efficacy depends on their anti-inflammatory action rather than their anti-bacterial action, including (1) an immunomodulatory effect on inflammatory cells, fibroblasts, and epithelial cells, (2) modulation of cytokine/chemokine production, (3)

Abbreviations: CAM, Clarithromycin; AIV, avian influenza virus; HPAIV, highly pathogenic avian influenza A virus; LPAIV, low pathogenic avian influenza A virus; NAI, neuraminidase inhibitor; EM, erythromycin; AZM, azithromycin; SAα2, 3Gal; sialic acids with α2, 3-linkages to galactose

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inhibition of mucus hypersecretion, (4) suppression of transcription factors and inflammatory cytokine gene expression, and (5) inhibition of bacterial functions such as quorum-sensing and biofilm formation (Kanoh and Rubin, 2010; Shimizu and Suzaki, 2016; Shimizu et al., 2013). Recently, anti-viral actions of macrolides have been reported, and combination therapy consisting of the NAI oseltamivir and CAM or AZM showed earlier resolution of seasonal influenza virus infection (Ninomiya et al., 2002; Sawabuchi et al., 2009; Higashi et al., 2014; Kakeya et al., 2014). The clinical effects of macrolides were also reported in the pandemic influenza virus infection (Bermejo-Martin et al., 2009; Azuma et al., 2013; Martin-Loeches et al., 2013). However, the efficacy of macrolides for H5N1 HPAIV and H7N9 LPAIV infections has not been evaluated.

We previously found that these virus strains caused severe to moderate signs of disease in cynomolgus monkeys (Muramoto et al., 2014; Shichinohe et al., 2016) and examined the efficacies of anti-viral drugs, including NAIs and an anti-hemagglutinin monoclonal antibody, against these viruses (Itoh et al., 2014; Kitano et al., 2014). Using this monkey model, the efficacy of intragastric CAM against H5N1 HPAIV A/Vietnam/UT3040/2004 (H5N1) (VN3040) and H7N9 LPAIV A/Anhui/1/20134 (H7N9) (Anhui1) infection and its effects on clinical signs of disease, virus propagation, and cytokine responses in the lung tissues, which are hardly assessed in human patients, were examined in the present study.

First, the body temperature changes of monkeys after infection with VN3040 or Anhui1 were compared to those before virus inoculation (detailed materials and methods are described in the Supplemental information). The results of three untreated monkeys infected with each virus from the previous studies (Nakayama et al., 2013; Itoh et al., 2015) were added to the results of one untreated monkey in the present study to calculate the averages of four monkeys as untreated controls. VN3040 or Anhui1 infection caused a temperature rise by the next morning after virus inoculation, and a higher temperature than before infection was observed until day 7 (an endpoint in the present study) (Fig. 1A and B, and Table S1). No significant inhibition of temperature rise was observed in the groups intragastrically administered CAM from day 0 to day 6 (CAM0), compared with the control groups (ctl) infected with VN3040 or Anhui1 viruses without CAM administration. When monkeys infected with VN3040 were administered CAM from day −3 to day 6 for prophylaxis and treatment (CAM-3), the average temperatures on CAM-3 were lower than those of the control group, although significant differences were not calculated due to the death of one monkey (monkey #10 in Table S1) in the CAM-3 group on day 3 (Fig. 1A).

Clinical signs in infected monkeys were diagnosed according to Table S2 (fever, posture, respiration, appetite, and skin condition). The averages of clinical scores in the CAM0 group were lower on days 6 and 7 than those in the control group infected with VN3040, whereas no significant amelioration of clinical scores in monkeys treated with CAM was observed in Anhui1 infection (Fig. 1C and D, Fig. S1A and Table S3).

Viral titers in tracheal swab samples of monkeys infected with VN3040 or Anhui1 viruses were examined. The average viral titers in the tracheal samples of monkeys infected with VN3040 in the CAM0 and CAM-3 groups and monkeys infected with Anhui1 in the CAM0 group on days 5 and 6 were lower than those in the tracheal samples of the control groups. In particular, on day 6, the average viral titers in the tracheal samples of monkeys infected with the VN3040 and Anhui1 viruses were both lower than those of monkeys in the control groups, although no significant differences in total virus titers for 7 days were observed (Fig. 2A and B, Fig. S1B, and Table S4).

The effects of CAM on the production of inflammatory cytokines were examined in lung tissues 7 days after virus infection. Although no significant differences were found, the levels of interleukin (IL)-6, IL-1β, and IL-8 in lung tissues of monkeys infected with VN3040 and treated with CAM (CAM0) and administered CAM for prophylaxis and treatment (CAM-3) were lower than those in lungs of monkeys without treatment (Fig. 3 and Table S5). A level of interferon (IFN)-γ in lung tissues were lower in monkeys infected with Anhui1 and treated with CAM than in monkeys without treatment. Thus, CAM administration showed a tendency to reduce the production of inflammatory cytokines in the lungs after infection with either virus (Fig. 3).

The expression of SAα2,3Gal in the presence of CAM was examined in the cultured cells, using a monoclonal antibody against sialic acids with α2,3-linkages to galactose (SAα2,3Gal), which preferentially bind to hemagglutinin of AIV as a viral entry receptor. The expression levels

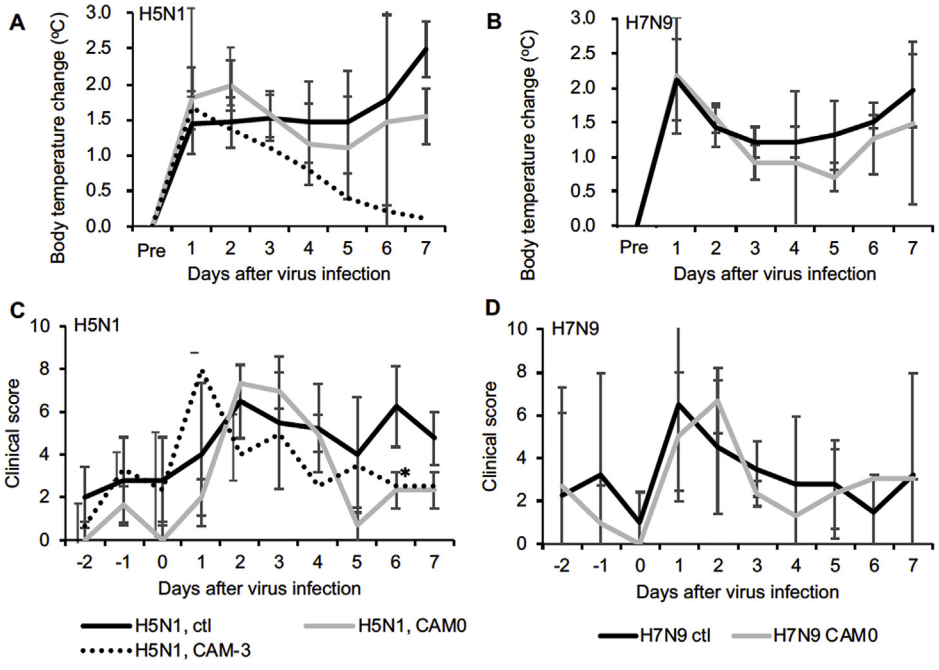

Fig. 1. Body temperature and clinical scores of cynomolgus monkeys treated with CAM. (A, B) Body temperature changes of cynomolgus monkeys infected with virus. Average temperatures of individual monkeys from 8 p.m. to 8 a.m. every night were calculated from temperatures recorded every 15 min (e.g., 'pre' indicates average temperatures from 8 p.m. to 8 a.m. on day −2 to day 0 before virus inoculation, and averages on day 1 indicate average temperatures from 8 p.m. on day 0–8 a.m. on day 1 after virus inoculation). Body temperature changes of individual monkeys each day after virus inoculation were compared with average body temperatures before virus inoculation (Table S1). Thereafter, averages and standard deviations of body temperature changes of monkeys are indicated in the graphs. (C, D) Averages of clinical scores of infected monkeys with or without CAM treatment. Clinical signs of disease on indicated days after virus infection were diagnosed on the basis of Table S2. The scores for individual monkeys are shown in Table S3. Averages and standard deviations of clinical scores in each group are shown. (A, C) Cynomolgus monkeys infected with H5N1 VN3040 virus (ctl: n = 4, CAM0: n = 3, CAM-3: n = 3 until day 3, n = 2 after day 4), (B, D) Cynomolgus monkeys infected with H7N9 Anhui1 virus (ctl: n = 4, CAM0: n = 3). *: Significant differences between control groups and treated groups on Student's *t*-test ($P < 0.05$). However, there is no significant difference on Mann-Whitney *U* test.

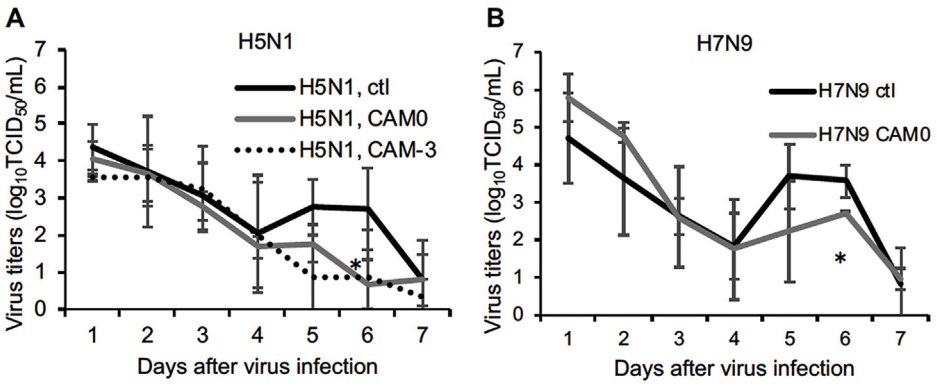

Fig. 2. Viral titers in tracheal swab samples of infected cynomolgus monkeys. Viral titers in the tracheal swab samples collected on the indicated days after virus infection were determined (Table S4). The averages and standard deviations of viral titers were calculated on each day. (A) Cynomolgus monkeys infected with H5N1 VN3040 virus, (B) monkeys infected with H7N9 Anhui1 virus. The detection limit of viral titer was 0.67 $\log_{10}$ $TCID_{50}$/mL. Values lower than the detection limit were assigned a value of 0. *: Significant differences between control groups and treated groups on Student's *t*-test ($P < 0.05$). However, there is no significant difference on Mann-Whitney *U* test.

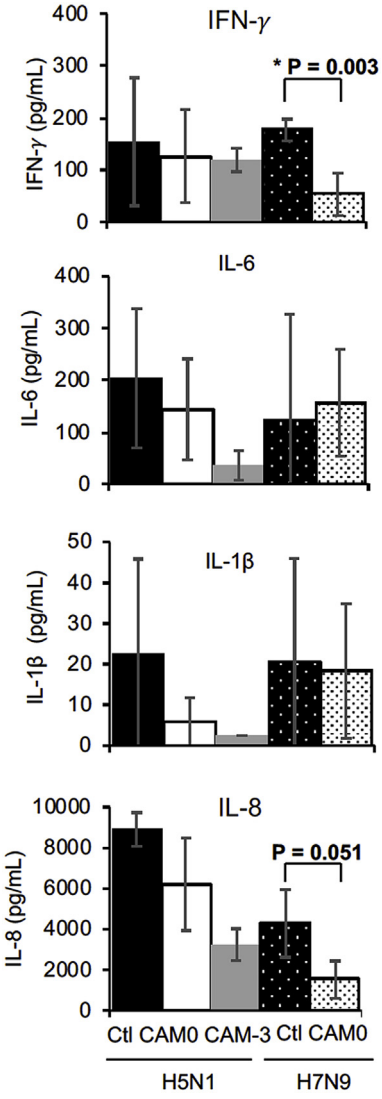

Fig. 3. Levels of cytokines in the lung of cynomolgus monkeys treated with CAM in H5N1 or H7N9 influenza virus infection. Lung tissues were collected from infected monkeys at autopsy. Levels of cytokines in tissue homogenates (10% w/v) were measured using a bead array assay (Table S5), and averages and standard deviations were calculated. (Left three columns) Cynomolgus monkeys infected with H5N1 VN3040 (ctl: n = 4, CAM0: n = 3, CAM-3: n = 2). (Right two columns) Monkeys infected with H7N9 Anhui1 (ctl: n = 4, CAM0: n = 3). No significant difference is detected with ANOVA among groups infected with VN3040. *: A significant difference between the control group and the treated group on Student's *t*-test ($P < 0.05$). However, there is no significant difference on Mann-Whitney *U* test ($P = 0.057$).

of SAα2,3Gal on the surface of Madin-Darby canine kidney (MDCK) cells and the percentage of MDCK cells positive for SAα2,3Gal were not affected by the presence of CAM (Fig. 4). This result suggests that CAM treatment inhibits viral titers in the tracheal samples of monkeys infected with VN3040 or Anhui1 viruses without inhibiting cell surface expression of their entry receptor, SAα2,3Gal, although CAM did not inhibit virus propagation in the MDCK cell culture (Fig. S2).

In the present study, intragastrically administered CAM improved clinical signs, partially inhibited virus propagation, and attenuated inflammatory cytokine responses in monkeys infected with AIV. In the monkeys infected with H5N1 HPAIV, CAM administration promoted the improvement of clinical scores and reduced the viral titers in tracheal samples 6 days after the infection. In H7N9 LPAIV infection, CAM administration showed no additional effects on clinical signs of disease because of the virus's lower pathogenicity. However, viral titers in tracheal samples and levels of IFN-γ and IL-8 in the lung tissues were reduced in H7N9 LPAIV infection. Therefore, CAM ameliorated clinical signs and inflammation caused by AIV infection in the monkey model.

CAM and EM, 14-membered ring macrolides, have been shown to inhibit virus attachment, infection, and propagation. *In vitro* experiments showed that CAM inhibits influenza virus and respiratory virus infection and that EM inhibits rhinovirus infection in human tracheal epithelial cells by reducing the expression of viral receptors and preventing viral RNA entry into cells (Asada et al., 2009; Yamaya et al., 2010; Hidari et al., 2013). CAM suppressed the expression of the influenza virus receptor, SAα2,3Gal, in airway epithelial cells (Hidari et al., 2013). In the present study, CAM administration inhibited virus propagation as evaluated by the viral titer in tracheal samples 6 days after infection with H5N1 or H7N9 virus, although CAM did not affect the expression of SAα2,3Gal, which binds to hemagglutinin of VN3040 and Anhui1 viruses as a viral entry receptor (Suzuki et al., 2001; Le et al., 2010; Watanabe et al., 2013). On the other hand, prophylactic CAM administration showed stronger inhibitory effects on body temperature, clinical scores, viral titers, and inflammatory cytokine responses in H5N1 HPAIV infection. It has been reported that CAM administration enhanced mucosal secretory IgA responses in mice and in children with influenza virus infection (Sawabuchi et al., 2009; Takahashi et al., 2012; Shinahara et al., 2013). These results indicate that part of the anti-viral actions of macrolides may be mediated through the modulation of host immune responses.

Anti-inflammatory and immunomodulatory actions of CAM, EM, and AZM include inhibition of mucus hypersecretion, modulation of cytokine and chemokine production, suppression of transcription factors, and suppression of gene expression of inflammatory cytokines, which were partly concordant with cytokine and chemokine responses in macaques in the present study (Kanoh and Rubin, 2010; Shimizu et al., 2013; Shimizu and Suzaki, 2016; Lee et al., 2017). CAM and EM suppress virus-induced cytokine production in airway epithelial cells. Treatment with EM or CAM increased the survival rate of mice infected with seasonal influenza virus (Sato et al., 1998). This effect was

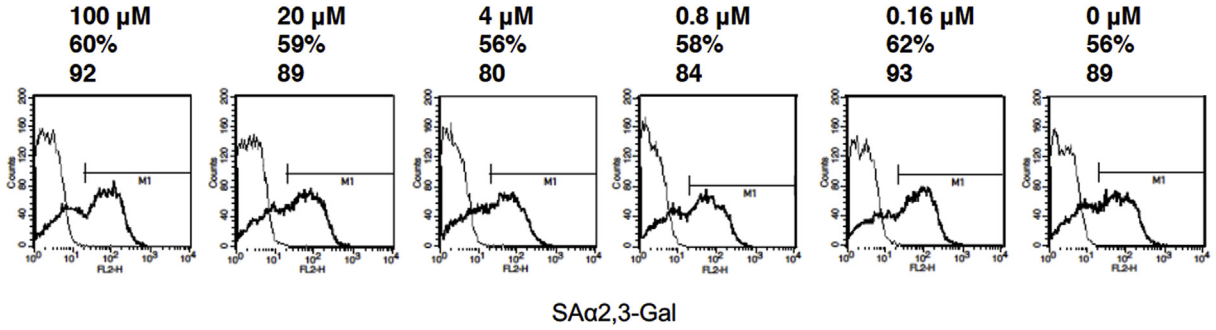

Fig. 4. Expression of SAα2,3Gal on the cell surface cultured with CAM. MDCK cells were cultured with CAM at the indicated concentrations overnight. SAα2,3Gal was stained with the anti-SAα2,3Gal monoclonal antibody. The percentage of SAα2,3Gal-positive cells and the mean fluorescence intensity of the cells in M1 gates are indicated at the indicated concentrations of CAM. One of the four experiments is shown for representative results.

associated with the suppression of lung injury and reduced production of reactive oxygen species and IFN-γ. In the present study, CAM administration suppressed the inflammatory cytokine responses in the lungs 7 days after infection with H5N1 or H7N9 virus. These results indicate that the therapeutic effects of macrolides in severe airway inflammation may be caused by the inhibition of the hypercytokinemia associated with the dysregulation of host immune responses.

Recently, a number of clinical benefits of macrolides against influenza virus infection have been reported in patients with severe symptoms. The clinical efficacy of combination therapy with macrolides has been reported in severe airway inflammation such as critical H1N1 pandemic influenza virus infection (Bermejo-Martin et al., 2009; Martin-Loeches et al., 2013) and sepsis with community-acquired pneumonia or ventilator-associated pneumonia, even in patients with macrolide-resistant pathogens (Rodríguez et al., 2007; Giamarellos-Bourboulis et al., 2008; Restrepo et al., 2009). However, CAM administration showed limited effects, especially in non-elderly, non-severely ill patients (Ishii et al., 2012), and the additional effects of macrolides were not remarkable in seasonal influenza virus infection (Sawabuchi et al., 2009; Ninomiya et al., 2002; Higashi et al., 2014; Kakeya et al., 2014). In the present study, the inhibitory effects on clinical scores were only observed in monkeys with H5N1 HPAIV infection. This might be due to the lower pathogenicity of Anhui1 virus than that of VN3040 virus in the monkeys (Muramoto et al., 2014; Itoh et al., 2015). Therefore, CAM treatment may have effects on amelioration of clinical scores in infection with H7N9 HPAIV since H7N9 HPAIV caused more severe signs of diseases in macaques (Yang et al., 2017; Imai et al., 2017; Suzuki et al., manuscript in preparation). These results suggest that the clinical inhibitory effects of CAM are significant in patients with severe symptoms and that combination therapy with macrolides is effective to suppress the systemic inflammatory responses and a fatal outcome in avian influenza virus infection.

Inhibitory effects of CAM administration on viral titers were observed for a short time in H5N1 HPAIV and H7N9 LPAIV infections in the present study. This might be partly due to administration of CAM to macaques once a day instead of twice a day in humans (Lee et al., 2017) since it is difficult to anesthetize macaques twice a day for intragastric administration of CAM.

At present, human-to-human transmission of H5N1 and H7N9 AIVs is limited in very close contact (Watanabe et al., 2013). Therefore, prophylactic use of CAM may be recommended to personnel who care patients with H5N1 and H7N9 AIV infection and workers in poultry if they do not obtain NAIs. When the infection with these viruses is confirmed in persons with the prophylactic use of CAM, addition of antiviral drugs such as oseltamivir and zanamivir may be possible as a combination therapy. The combination therapy of CAM with antiviral drugs such as NAIs against these viruses might have therapeutic effects since the monotherapy by the NAIs was effective in reduction of H5N1 and H7N9 virus titers in the macaque models (Itoh et al., 2014; Kitano et al., 2014; Hung et al., 2017; Lee et al., 2017; Lee et al., 2018), although the purpose in the present study was to evaluate the efficacy of CAM alone in H5N1 and H7N9 virus infection. The efficacy of the combination therapy would be examined using the macaque model in the future.

In conclusion, intragastrically administered CAM attenuated the severity of AIV (H5N1 and H7N9) infection in cynomolgus monkeys by inhibiting the virus propagation and by suppressing the cytokine responses of the infected lungs. Macrolides are relatively less expensive and easily accessible even in developing countries where AIV infection has been reported. Thus, since administration of CAM alone showed a tendency to ameliorate clinical sings and to reduce levels of inflammatory cytokines in the preclinical study using the macaque model, CAM is expected to have effects in combination with the other antiviral drugs on the prophylactic and the treatment of patients with severe AIV infection, which would be examined in the future.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104591.

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