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Doxycycline Attenuated *Mycobacterium avium* Induced Inflammation in Mice

Naoki Tashiro[1], Masaki Fujita[1,2*], Takemasa Matsumoto[1], Ryousuke Hirano[1], Junji Uchino[1], Eiji Harada[2], Satoshi Ikegame[2], Hiroshi Ouchi[2], Yoichi Nakanishi[2] and Kentaro Watanabe[1]

[1]*Department of Respiratory Medicine, Faculty of Medicine, Fukuoka University, Fukuoka 814-0180, Japan*

[2]*Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan*

[*]**Corresponding author:** Masaki Fujita, Department of Respiratory Medicine, Faculty of Medicine, Fukuoka University, Fukuoka 814-0180, Japan, Tel: 81-92-801-1011; E-mail: mfujita@fukuoka-u.ac.jp

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Abstract

Mycobacterium avium causes chronic and progressive respiratory infection. A therapeutic regimen including clarithromycin, rifampin and ethambutol has been commonly employed, however, the effect of such antibacterial therapy is often unsatisfactory. Doxycycline is an antibiotic known to have immuno-modulating effects as well as antibacterial activity. In this study, we investigated the effect of doxycycline administration on *M. avium* infection in mice. The administration of doxycycline attenuated lung inflammation caused by *M. avium* according to the results from a histology analysis and the number of inflammatory cells from BAL fluids. Moreover, doxycycline improved the survival rate in TNF-R1 KO mice infected with *M. avium*. However, doxycycline did not affect the colony number of *M. avium* in the lungs. These results suggest that doxycycline may have protective effects against *M. avium* induced inflammation in mice. The effects of doxycycline may be due to its biological effect apart from its antimicrobial function.

Keywords: MMPs; Mycobacterium avium-intracellulare; Inflammation; Cytokine

Introduction

Mycobacterium avium-intracellulare complex (MAC) is an acid-fast bacillus similar to *M. tuberculosis*. This pathogen causes chronic progressive respiratory infection as well as disseminated diseases in HIV patients [1,2]. Fortunately, the disease is not a transmissible disease. In 1997, the American Thoracic Society (ATS) recommended a four-drug regimen including clarithromycin or azithromycin, rifampin or rifabutin, ethambutol, and an initial aminoglycoside (streptomycin or amikacin) for pulmonary MAC disease [3]. Several studies have indicated that a clarithromycin-containing regimen demonstrated a 59%-92% response rate [4-6], however, relapses after the completion of medical therapy were frequent [7,8].Wallace and colleagues reported that more than half of their patients showed recurrence [7]. These results indicated that the treatment for pulmonary MAC disease remains insufficient.

Several antibiotics including macrolides are known to exert biological effects beyond their antibacterial activity. Macrolides are well known to have an inhibitory effect on inflammation through blocking IL-8 production [9] and suppressing the transcription factors involved in inflammation [10]. In addition to macrolides, tetracycline also has an anti-inflammatory effect. Tetracycline has been shown to decrease the release of neutrophil chemotactic factors or reactive oxygen species [11]. We previously demonstrated that doxycycline could modulate lung injury through the protection of lung structure by biological effects, namely gelatinase inhibitor [12]. We have hypothesized that the immunomodulatory effect of doxycycline could make a positive impact on the treatment of *M. avium* infection. In order to investigate whether or not doxycycline improves the *M. avium* infection, we utilized a murine model of *M. avium* infection.

Materials and Methods

Bacteria

A clinical isolate of *M. avium* from our hospital was used in this study. Bacteria were grown in Middlebrook 7H9 broth with Middlebrook ADC enrichment (Becton, Dickinson and Company, Sparks, MD) at 37°C with shaking or on Middlebrook 7H10 agar with Middlebrook OADC enrichment (Becton, Dickinson and Company, Sparks, MD) at 37°C for 14 days. The plates were incubated at 37°C in 90% humidity for 14 d and colonies were counted [13].

Animal model of *M. avium* infection

Eight-week-old C57Bl/6 mice and TNF receptor 1 knockout mice (TNFR1-KO) [14] from the C57Bl/6 background purchased from Jackson Laboratory were used in this study. TNFR1-KO mice were used because the mice are susceptible for M. avium more than wild type mice. These mice were provided sterile food and water in an environmentally-controlled room.

The intratracheal administration of *M. avium* (1×10^7 CFU/head) in 50 µl of sterile saline was done via tracheotomy to the mice under anesthesia [15]. The survival of the mice was observed daily for 60 days after infection. The mice were sacrificed on day 21 after infection. To measure the mycobacterial burden after inoculation, the left lung of the mice were incised and homogenized using a stainless mesh. Viable counts of mycobacterium in the homogenate were determined by plating on Middlebrook 7H10 agar plates for determining the number of colonies. After sacrifice, the right lungs of the mice were fixed with 10% formalin for 24 hours and embedded in paraffin. Sections were then obtained and stained with hematoxylin and eosin (H-E) or Ziehl-Neelsen (Z-N).

Doxycycline was purchased from Sigma-Aldrich (Tokyo, Japan). Doxycycline was added to the drinking water [12]. The rough assessment doses of doxycycline for one day were 2 mg/kg of body weight. A dose of 2 mg/kg doxycycline was chosen for this study since it closely correlated with the human dose. As a doxycycline control, water alone was supplied.

This study was approved by our Institutional Animal Care and Use Committee.

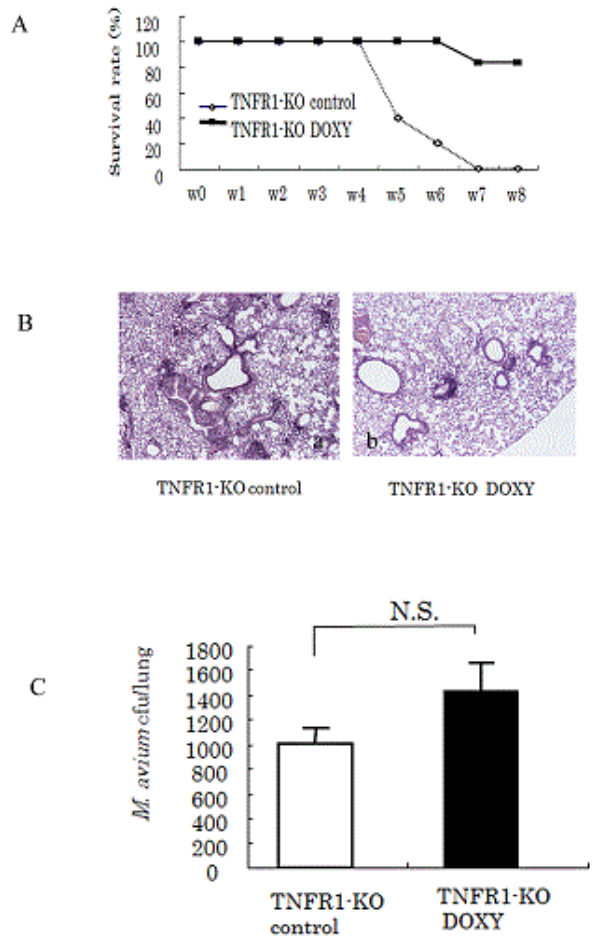

Figure 1: The effect of doxycycline on *M. avium* inoculation. (A) The mortality rate after *M. avium* inoculation in TNFR1-KO mice. DOXY indicates the doxycycline-supplied group. Control indicates the water-supplied mice. Doxycycline; n=6, control; n=5. p= 0.0018. (B) The histologic assessment of the lung after *M. avium* inoculation in TNFR-1 KO mice. (a) The lung from a *M. avium*-inoculated mouse supplied with doxycycline. (b) The lung tissue from a *M. avium*-inoculated mouse supplied without doxycycline (a and b; H-E staining, original magnification: 40X.). (C) The bacterial growth in the lung in TNFR-1 KO mice. Lane 1: *M. avium*-inoculated mice supplied with doxycycline; lane 2: *M. avium*-inoculated mice supplied with water. Each group consisted of five mice. N. S. indicates no significant differences.

Lung histology and morphometry

After sacrifice, the lungs were inflated at 25 cm H_2O static pressure by intratracheal instillation of 10% formalin for 24 hours and embedded in paraffin. Sections were then obtained and stained with hematoxylin and eosin.

Bronchoalveolar lavage (BAL) and gelatinase

BAL was performed on day 21 after intratracheal *M. avium* instillation. In the mice with a tracheal tube inserted, the lungs were lavaged with 0.5-ml aliquots of phosphate-buffered saline ten times for a total of 5 ml. The total cell counts were determined with a hemocytometer. Differential counts of BAL fluid were performed on 200 cells from smears stained with a modified Wright's stain (DiffQuik; American Scientific Products, McGas Park, IL). BAL fluid supernatants were analyzed using an ELISA kit according to the manufactures instructions (R&D Systems, Inc. Minneapolis, MN). Inflammatory cytokines/chemokines such as interferon (IFN)-γ, interleukin (IL)-12, keratinocyte-derived chemokine (KC), tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and IL-4 were measured. The gelatinolytic activity was detected by zymography as described previously [12].

Statistics

The data were expressed as the mean+standard error (SE). The Mann-Whitney test was used to compare the two groups. A value of $p<0.05$ was considered to indicate a significant difference. For comparing mortality, the Kaplan-Meier estimation for survival curves and the log-rank test was used. The statistical analyses were performed using the JMP 7.0 software program.

Results

To examine the influence of doxycycline, we studied the differences in the death rate, the histological observation of the lung, inflammatory cells in the alveoli, and the bacterial burden. In a preliminary experiment, we found that TNFR1-KO mice were more susceptible to *M. avium* than wild type mice. For example, wild type mice have never died of *M. avium* infection, on the other hand, TNFR1-KO mice frequently died after *M. avium* infection. Therefore, we initially used TNFR1-KO mice. Inoculation of 1×10^7 CFU of a clinical isolate of *M. avium* resulted in the death of TNFR1-KO mice without treatment. In contrast, nearly 80% of the mice survived for more than 60 days following doxycycline administration (Figure 1A). The lung pathology demonstrated severe lung damage, consisting of the infiltration of inflammatory cells and destruction of lung structures. Doxycycline treatment attenuated histologic changes (Figure 1B). However, doxycycline did not decrease the bacterial burden compared to the control treatment (Figure 1C).

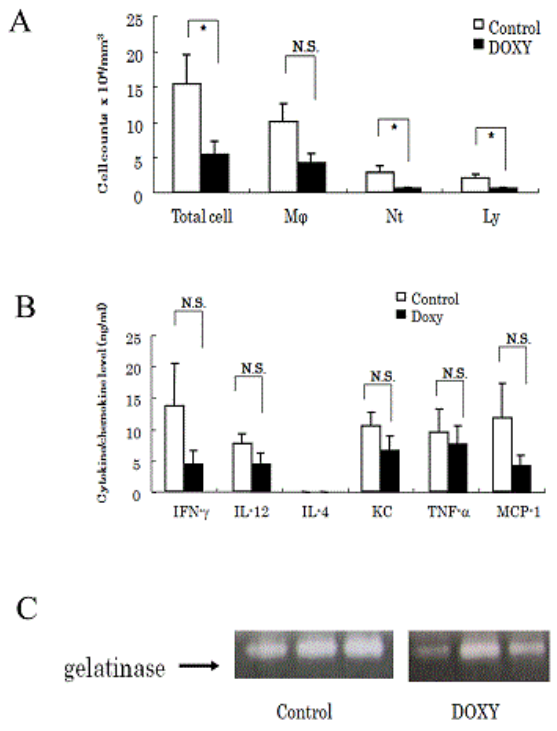

Figure 2: The effect of DOXY on the lung inflammation and MMPs. (A) The inflammatory cell accumulation in BAL fluids in wild type mice. Lane 1: *M. avium*-inoculated mice supplied with doxycycline; lane 2: *M. avium*-inoculated mice supplied with water. Each group consisted of five mice. *indicates a significant difference. N. S. indicates no significant differences. (B) The cytokine profile in BAL fluids. Cytokines in the BAL fluids were measured by the cytokine ELISA kit. The inhibition of the inflammatory cytokines was observed, however, it was not significant. Each group consisted of four mice. There was no significant difference between the two groups. (C) The gelatinolytic activity in the BAL fluids. Lane 1: *M. avium*-inoculated mice with water; lane 2: *M. avium*-inoculated mice with doxycycline. Each group consisted of three mice.

The number of inflammatory cells was decreased with doxycycline treatment. In particular, neutrophil and lymphocyte accumulation in the BAL fluid specimens significantly decreased after doxycycline administration (Figure 2A). In addition, we measured the inflammatory cytokines/chemokines profile after doxycycline treatment. IL-4 was not detected. The IFN- γ, IL-12, KC, TNF-α, MCP-1 levels all decreased with doxycycline treatment, however, the decrease was not significant. This tendency of inhibition was suggested due to doxycycline administration (Figure 2B). Gelatin zymography demonstrated the slight attenuation of gelatinase activity by doxycycline (Figure 2C).

Discussion

Doxycycline is recognized as a broad spectrum and effective agent against several pathogens, but not against acid-fast bacilli [16]. This study also indicated that doxycycline attenuated inflammation but did not decrease the bacterial burden. Therefore, the attenuation effect was attributed to biological effects beyond the normal antibacterial activity of this drug. Immunomodulatory effects of doxycycline treatment, similar to macrolide therapy [17], induced the phenomenon in the *M. avium* infection model. Recent randomized controlled trial have found improved outcomes in patients with pneumonia if their treatment regimens included a macrolide antibiotic and Cilloniz et al. reported that patients hospitalized for macrolide-resistant *S. pneumoniae* pneumonia did not have worse clinical outcomes if they were treated with macrolide including regimen [18]. We considered that he immunomodulation effect by doxycycline treatment resulted in the longer survival in this study.

Doxycycline was able to attenuate bleomycin-induced pulmonary fibrosis and lung injury induced by lipopolysaccharide (LPS) or *S. pneumoniae* [12,19] along with the reduction of gelatinase. In this study, a slight reduction in the gelatinase activity was also observed. Doxycycline is reported to inhibit gelatinase because doxycycline is a scavenger of zinc. We speculated that a possible mechanism of immunomodulation was through the inhibition of gelatinase. However, doxycycline is also known to inhibit reactive oxygen species release, apoptosis, and decrease neutrophil chemotaxis [11,20]. Indeed, we observed a decrease of inflammatory cells following doxycycline treatment. Moreover, doxycycline treatment had a tendency to inhibit the inflammatory cytokines/chemokines in this study. Further investigations are therefore necessary to clarify the precise mechanism of the biological effects of doxycycline.

Since doxycycline did not reduce the bacterial burden, it is difficult to include doxycycline in the conventional regimen. Some cases of pulmonary MAC disease demonstrate apparent lung inflammation and corticosteroids may be useful, although adverse effects are predicted [21,22]. Doxycycline may be administered instead of corticosteroids because the immunosuppression effect of doxycycline is negligible compared to corticosteroid therapy. Alternatively, doxycycline may be a candidate for the progression status of pulmonary MAC disease in addition to conventional therapy. Further clinical trials of conventional therapy plus doxycycline should be investigated in the future.

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