

Impaired host defence against *Mycobacterium avium* in mice with chronic granulomatous disease

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Introduction

Mycobacterium avium is a bacterium pathogenic for humans which proliferate intracellularly in phagocytes. The bacterium causes chronic progressive respiratory infection as well as disseminated diseases in immunocompromised hosts, such as human immunodeficiency virus (HIV) patients [1,2]. It has been reported that a clarithromycin-containing regimen demonstrated a 59–92% response rate to the infection [3–5]; however, relapses after medical therapy are common [6,7]. Investigation of the host defence mechanism against *M. avium* infection could lead to the development of a new remedy against *M. avium* infection.

Chronic granulomatous disease (CGD), an inherited disorder of phagocytic cells, results from an inability of phagocytes to kill certain types of bacteria, leading to recurrent life-threatening bacterial and fungal infections. CGD is due to a functional defect of the O₂-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytes. Generally, CGD patients are susceptible to catalase-positive, hydrogen peroxide-negative bacteria [8,9]. Phagocytes from CGD patients showed normal bacterial killing ability against non-catalase-producing bacteria,

Summary

Patients with chronic granulomatous disease (CGD), an inherited disorder of phagocytic cells, often contract recurrent life-threatening bacterial and fungal infections. CGD is considered to arise from a functional defect of the O₂-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in phagocytes. To determine whether or not NADPH oxidase is crucial to the host defence against *Mycobacterium avium*, we investigated the response against *M. avium* using CGD model mice (gp91-phox⁻) of C57BL/6 strain. A tracheal injection of 1 × 10⁷ colony-forming units (CFU)/head of *M. avium* strain FN into the CGD mice resulted in a pulmonary infection, while also increasing the mortality rate. In contrast, normal C57BL/6 mice injected with same dose of the organisms did not develop severe pulmonary infection and were able to survive through 2 months of observation. The macrophages obtained from the CGD mice were observed to have a higher burden of the bacterial growth than macrophages from normal C57BL/6 mice. These results suggest that the defect of the NADPH oxidase function impairs the host defence against *M. avium* infection.

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because phagocytes can generate hypochlorous acid (HOCl) using hydrogen peroxide produced by bacteria in phagosomes. Although *M. avium* produces catalase, there have so far been few clinical case reports of *M. avium* infection associated with CGD. The relationship between CGD, dysfunctional NADPH oxidase and mycobacterial infection therefore remains uncertain.

We used a mouse strain developed by Pollock *et al.* that has been established as a mouse model for an X-linked (gp91-phox⁻) form of CGD [10]. In this study, we injected *M. avium* into mice with CGD (CGD mice) and their wild-type C57BL/6 counterparts and then compared their susceptibility to the pathogen. We report herein that NADPH oxidase dysfunction results in an impaired host defence against *M. avium* infection.

Materials and methods

Bacteria

Strain FN of clinically isolated *M. avium* from our hospital was used in this study. All strains were grown in Middlebrook 7H9 broth with Middlebrook albumin dextrose

catalase (ADC) enrichment (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C with shaking or on Middlebrook 7H10 agar with Middlebrook oleic acid albumin dextrose catalase (OADC) enrichment (Becton, Dickinson and Company) at 37°C for 14 days. The plates were incubated at 37°C in 90% humidity and the colonies were counted after 14 days [11]. The *M. avium* strains produced catalase, but not hydrogen peroxide, as confirmed by our new agar to detect hydrogen peroxide-producing bacteria based on Prussian blue-forming reaction [12].

Mice

X-linked CGD mice, which were produced by a knock-out of gp91-phox from C57BL/6 mice, were donated by M. C. Dinauer (Riley Children's Hospital, Indianapolis, IN, USA) and A. Kume (Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan). These mice were provided with sterile food and water in an environmentally controlled room. Eight-week-old X-linked CGD mice were used in the experiment; C57BL/6 mice of the same age were used as the controls. The mice were given food and water *ad libitum* throughout the experiments.

Animal model of *M. avium* infection

A clinical strain FN of *M. avium* isolated from a patient in our hospital was used in this study. The intratracheal administration of *M. avium* [1×10^7 colony-forming units (CFU)/head] in 50 μ l of sterile saline was performed via a tracheotomy to the mice under anaesthesia. As a control, 50 μ l of saline was injected [13]. The survival of the mice was noted daily for 60 days after inoculation. Mice were killed on day 21 after infection. To measure the mycobacterial burden after inoculation, the left lungs of the mice were incised and homogenized using a stainless mesh. Viable counts of mycobacterium in the homogenate were determined by inoculation on Middlebrook 7H10 agar plates to count the number of colonies. After death, the right lungs were fixed with 10% formalin for 24 h and embedded in paraffin. Sections were obtained subsequently and stained with haematoxylin and eosin (H&E) and Ziehl–Neelsen (Z–N) staining.

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

Macrophage isolation and infection by *M. avium*

Bacterial proliferation in macrophages was assayed by methods described previously [14,15], with some modifications. Briefly, the peritoneal macrophages were obtained at 3 days after inoculation in 2 ml of 3% thioglycollate medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Macrophages were incubated in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan) without antibiotics using 12-well cell culture plates (Becton, Dickinson and

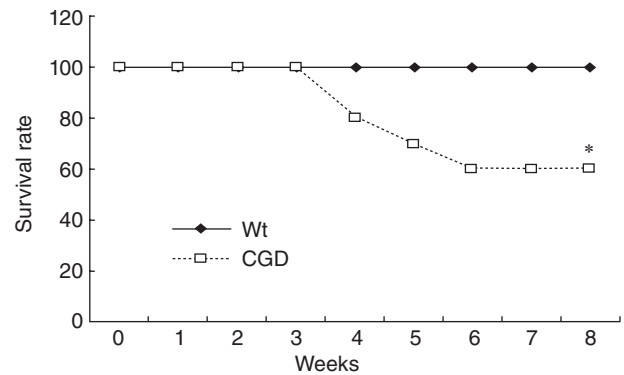


Fig. 1. The survival rate of mice infected intratracheally with *Mycobacterium avium*. *M. avium* strain FN was injected intratracheally at dose of 1×10^7 colony-forming units/head. WT: wild-type mice of C57BL/6; CGD: X-linked chronic granulomatous disease (CGD) mice. Each group consisted of 10 mice. *Significant difference ($P < 0.05$).

Company, Tokyo, Japan). Approximately 1×10^6 cells were incubated per well. *M. avium* was added to culture medium at multiplicity of infection (MOI) 10. Macrophages were collected and washed twice by phosphate-buffered saline (PBS) and lysed by the addition of sterilized water. The lysed medium was inoculated into Middlebrook 7H10 agar plates and the bacterial number was counted after 2 weeks of incubation at 37°C.

Statistics

Data were expressed as the mean \pm standard error (s.e.). The Mann–Whitney test was used to compare the two groups. A value of $P < 0.05$ was considered to indicate a significant difference. Statistical analyses were performed using a computer program (StatView version 5.0).

Results

To examine the influence of a defect in NADPH oxidase function on the susceptibility of mice to *M. avium*, we evaluated the differences in mortality rate, the histological features of the lung and bacterial burden in organs between C57BL/6 and CGD mice after intratracheal inoculation of the lung. Inoculation of 1×10^7 CFU of the clinically isolated strain resulted in the death of nearly half the CGD mice. In contrast, all C57BL/6 mice survived for more than 60 days of observation (Fig. 1). The right lungs were tested for lung pathology and the left lungs were tested for bacterial growth. In comparison to the wild-type mice, the lung pathology of the CGD mice demonstrated severe lung damage involving the infiltration of inflammatory cells and a large burden of lymphocytes and macrophages, as well as a small amount of neutrophils. In addition, destruction of the lung structures was observed (Fig. 2a). The wild-type mice demonstrated a small degree of inflammation in a very confined area of development at day 21. Inflammatory cells were mainly lymphocytes. To examine the relationship between lung

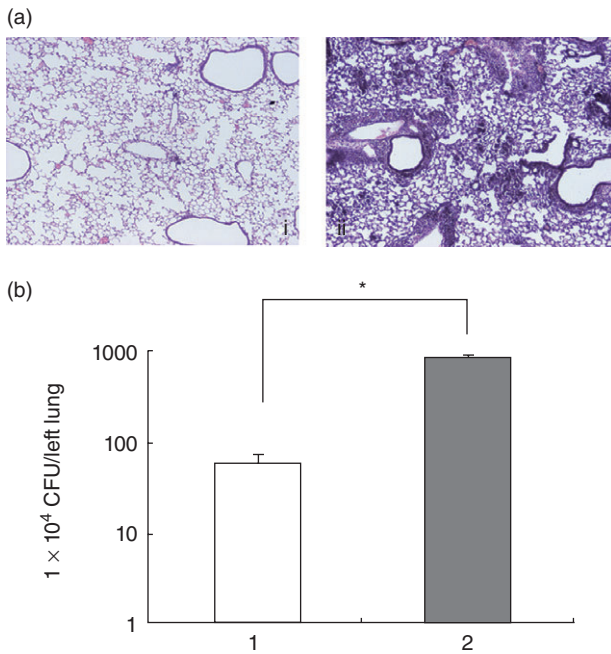


Fig. 2. The lung histology of the mice after *Mycobacterium avium* administration. (a) Lung histology was obtained 21 days after *M. avium* administration. (i) Wild-type mice that received *M. avium*; (ii) chronic granulomatous disease (CGD) mice that received *M. avium*. Note the marked inflammatory cell infiltrate in the lungs from CGD mice. All panels are haematoxylin and eosin-stained and viewed at the same magnification. Original magnification: 40 \times . We examined five mice in each group and all mice showed similar results. The figure represented the findings for one mouse. (b) Bacterial growth in the lungs was measured at 21 days after *M. avium* inoculation. Bacterial growth is shown by a log scale. Each group consisted of five mice. *Significant difference ($P < 0.05$).

pathological changes and the bacterial burden in the lungs of C57BL/6 and CGD mice, we determined the number of bacteria in the lung tissue (Fig. 2b). There was a greater number of bacteria in the lungs of the CGD mice in comparison to the lungs of the C57BL/6 mice at 21 days after infection. These results correlated well with the lung histology data. Although a defect in NADPH oxidase function impairs certain aspects of neutrophil activity, the proliferation of *M. avium* is considered to be related to the macrophage function. We therefore analysed the effect of NADPH oxidase impairment on the anti-mycobacterial activity of macrophages. Peritoneal macrophages from C57BL/6 and CGD mice were infected with *M. avium* and the intracellular growth of the pathogen in the macrophages was determined after 7 days of infection. There was a greater proliferation of *M. avium* in macrophages from the CGD mice than in those from the C57BL/6 mice (Fig. 3).

Discussion

Human defence mechanisms include both innate and adaptive immunity. Innate immunity involves phagocytosis and

intracellular killing by neutrophils and macrophages that act during the early stages of infection, while adaptive immunity involves T cell stimulation, macrophage activation and antibody production [16]. Neutrophils and macrophages kill microorganisms by generating microbicidal reactive oxygen species, such as superoxide anions, hydrogen peroxide, hydroxyl anions and hypochlorous acid [17].

CGD patients have a susceptibility to microorganisms that does not usually cause disease in people with normal immune systems. Bacteria are among the most common organisms that cause disease in CGD patients, particularly catalase-positive, hydrogen peroxide-negative bacteria such as *Staphylococcus aureus*, *Serratia marcescens* and *Aspergillus* species. However, susceptibility to mycobacteria remains controversial. In humans, mycobacterial infection has not been considered to be associated with CGD. For example, CGD neutrophils are as effective as normal neutrophils in destroying *M. tuberculosis* [18]. Recently, however, Lee *et al.* documented the cases of 17 patients with CGD living in a region endemic for tuberculosis [19]. In addition, Ohga published a case report of CGD complicated with *M. avium* [20]. Kusu-hara *et al.* also reported bacillus Calmette–Guérin (BCG) lymphadenitis in CGD patients [21]. These recent clinical reports suggested that the oxidative burst may be an important aspect of host defence against mycobacterial infections. To confirm the role of NADPH oxidase in host defence against *M. avium*, we performed an experiment using CGD model mice (gp91-phox⁻) infected with *M. avium*. We demonstrated that CGD mice (gp91-phox⁻) are susceptible to *M. avium* based on mortality, bacterial number, lung histology and proliferation in macrophages. These results suggest that a defect in the NADPH oxidase function causes lethal respiratory insufficiency; therefore, superoxide anion and its metabolites produced by phagocytes play an important role in host protection against *M. avium* infection.

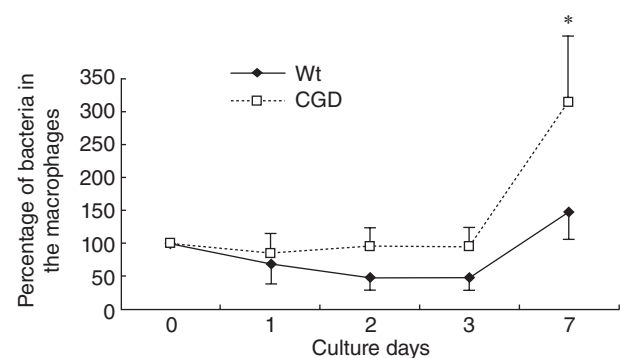


Fig. 3. Bacterial growth in the lungs and macrophages. Macrophages were obtained from either chronic granulomatous disease (CGD) mice or wild-type mice and infected with *Mycobacterium avium* at the dose of multiplicity of infection (MOI) 10. The bacterial growth was quantified and is shown by a log scale. Each group consisted of five mice. *Significant differences ($P < 0.05$). WT: wild-type mice of C57BL/6; CGD: CGD mice.

The *M. avium* strain used in this study was catalase-positive, hydrogen peroxide-negative. Another clinically isolated strain of *M. avium* from our university was also catalase-positive, hydrogen peroxide-negative. These strains are presumably virulent to CGD hosts. Segal *et al.* reported that the histopathology and cytokine expression data were similar between p47 $\text{phox}^{-/-}$ and wild-type mice, thus indicating that NADPH-derived reactive oxidants probably do not modulate the pulmonary inflammatory response to *M. avium* [22]. Furthermore, there was no significant difference in the spleen and lung *M. avium* burden between p47 $\text{phox}^{-/-}$ and wild-type mice. They concluded that NADPH oxidase-derived oxidants do not alter significantly the host response to *M. avium*. We do not understand fully the discrepancy between their data and our data. This discrepancy could be due to the difference in mouse strains, bacterial strains or route of infection. Adams *et al.* injected *M. tuberculosis* intravenously to CGD mice and observed that *M. tuberculosis* growth was enhanced markedly in the lungs of X-CGD mice compared with B6 mice, but was controlled in the spleen and liver [23].

In conclusion, our findings suggest that NADPH oxidase plays an important role in the innate immunity against *M. avium* in mice. In future, the susceptibility to mycobacteria with regard to human subjects should be investigated.

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Disclosure

The authors declare no conflicts of interest.

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