

Attenuation of Pulmonary *Mycobacterium Avium* Disease by Active Hexose Correlated Compound (AHCC) in Mice

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Abstract

Introduction: Pulmonary *Mycobacterium avium* disease is a chronic and progressive disease that is known to be difficult to treat. Active hexose correlated compound (AHCC) is an extract obtained by culturing mycelia of Basidiomycota. AHCC is reported to attenuate several experimental animal infection models. We hypothesized that AHCC could attenuate pulmonary *M. avium* disease in mice.

Methods: *Mycobacterium avium* (10^8 cfu/head) was administered intratracheally into mice (C57/BL6). Infected mice were supplied with 1,000 mg/kg/day of AHCC by oral administration until euthanasia. The mice were sacrificed at seven days later or 21 days later after *M. avium* infection. The lung homogenates were cultured on Middlebrook 7H10 agar plates for bacterial colony counts. Additionally, the number of inflammatory cells in the lungs was analyzed by FACS. Tissue sections of the lungs were stained by hematoxylin and eosin or Ziehl-Neelsen methods. In addition, the number of bacterial colonies in the macrophages was counted *in vitro*. Approximately 1×10^6 macrophages were incubated with *M. avium* at an MOI of 10 with or without 1 mg/ml of AHCC.

Results: The administration of AHCC improved lung inflammation caused by *M. avium* according to the histology results and decreased number of *M. avium* in the lungs. In the analysis of lung inflammatory cells, the number of TNFR1 cells and NK cells remained unchanged by AHCC administration, however, the number of TNFR2 cells slightly increased. There was no difference in the number of *M. avium* in macrophages treated with or without AHCC in the *in vitro* study.

Conclusion: AHCC plays a protective role in a murine model of pulmonary *M. avium* disease.

Keywords: Glucan; Mycobacteriosis; Biological response modifiers; Animal study

Introduction

Mycobacterium avium is a human pathogen which proliferates intracellularly in phagocytes. The bacterium causes chronic progressive respiratory infection as well as disseminated diseases in immunocompromised hosts such as HIV patients [1,2]. It has been reported that *M. avium* causes a chronic and progressive disease, pulmonary *Mycobacterium avium*-intracellulare complex (MAC) disease. Pulmonary MAC disease is known to be difficult to treat [3-7]. Therefore, the development of novel treatments for pulmonary MAC disease is needed.

Active hexose correlated compound (AHCC) is an extract obtained by culturing mycelia of Basidiomycota. The main component of AHCC is acetylated α -glucan. AHCC has been used as a dietary supplement in various countries. These glucans are thought to provide a carbohydrate that stimulates immune responses [8,9]. It has been previously reported that AHCC influences both natural immunity and acquisition immunity and is also effective against viral infection, Eumycota and other bacteria [10-12]. It has been reported that AHCC attenuated infection caused by *Candida albicans*, *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA) in a neutrophil depleted murine model by cyclophosphamide [13]. Since AHCC itself does not have any antibacterial ability against *C. albicans*, the attenuation effect depends on its immunological effect [14]. AHCC has also been demonstrated to have a protective effect against *Klebsiella pneumoniae* infection. AHCC could improve the immune defect induced by injury, infection or starvation [15]. Recent studies demonstrated that the activity of natural killer (NK) cells was improved by AHCC in an influenza virus model [16,17]. AHCC is thus considered to play a role as a biological response modifier (BRM).

We hypothesized that AHCC could act as a BRM and attenuate pulmonary *M. avium* disease. This study aimed to investigate the beneficial effect of AHCC in *M. avium* infection.

Materials and Methods

Bacterial strain

The clinically isolated *M. avium* strain FN from our hospital was used in this study. All isolates were grown in Middlebrook 7H9 broth with Middlebrook ADC enrichment (Becton Dickinson and Company, Sparks Glencoe, MD) at 37°C with shaking or on Middlebrook 7H10 agar with Middlebrook 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 [18,19].

Animal model of *M. avium* infection

C57BL/6 mice of the same age, three months, were used. The intratracheal administration of *M. avium* (1×10^8 CFU/head) in 50 μ l of sterile saline was performed via tracheotomy to the mice under

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anaesthesia [20]. AHCC was provided from Amino Up Chemical (Sapporo, Japan). Detail of AHCC was described previously [8-17]. Infected mice were supplied with 1,000 mg/kg/day of AHCC by oral administration until euthanasia. This dose of AHCC has been used previously and does not produce toxic effects in young mice [21]. AHCC was added to the drinking water [20] so that the dose is rough assessment doses. Infection with water (water group) and a non-infection control mice group were also set up. Mice were sacrificed at seven days after infection or 21 days after *M. avium* administration. The lung homogenates were cultured on Middlebrook 7H10 agar plates to determine the colony count. After sacrifice, the lungs were fixed with 10% formalin for 24 hours and embedded in paraffin. Tissue sections of the lungs were stained with hematoxylin and eosin (H-E) and Ziehl-Neelsen (Z-N) staining.

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).

This study was approved by our Institutional Animal Care and Use Committee (approved number; 0911352 by Fukuoka University).

Flow cytometric analysis (FACS)

Inflammatory cells in the lungs were also analyzed by FACS using the lung homogenates. After sacrifice, the individual lungs were dissociated in 5 ml PBS containing Collagenase II (2.5mg/ml, Sigma-Aldrich, St. Louis, MO) using the mouse lung dissociation program 1 on a GentleMACS tissue dissociator (Miltenyi Biotec Ltd., DE). This was followed by incubation in an incubator (37 °C, 30 min) and a final GentleMACS dissociation using the mouse lung dissociation program 2. Cells were pelleted by centrifugation at 300g for 10 min at 4°C. Pellets were washing twice with PBS, and lung mononuclear cells were purified by discontinuous 45/67.5% Percoll gradient centrifugation. Cells were dissolved with 45% Percoll (GE Healthcare UK Ltd, UK) and overlay the 45% Percoll suspension onto 67.5% Percoll centrifuged at 1000g for 30 min. Cells from the 45/67.5% interface were collected, washed, and resuspended in RPMI media. These cells were staining for analysis. Before staining, anti-CD16/32 antibody (clone 93; BioLegend, San Diego, CA) were added to the cells for block nonspecific staining and cells were incubated for 20 min at 4°C. Cells were stained with FITC-conjugated anti-NK mAb (clone DX5; eBioscience, San Diego, CA), APC-conjugated anti-CD120a mAb (clone55R-286; BioLegend) and PE-conjugated anti-CD120b mAb (cloneTR75-89; BioLegend) at 4°C for 30 min. After washing twice with PBS, cells were collected on a FACSCanto II (BD Biosciences, San Jose, CA) and data from 15,000 to 20000 individual cells were analyzed using BD FACSDiva software (BD Biosciences).

Macrophage isolation and infection by *M. avium*

Bacterial proliferation in murine macrophages was assayed by methods previously described elsewhere [19,22,23] with some modifications. Briefly, the peritoneal macrophages were obtained at three days after inoculation in 2 ml of 3% thioglycollate medium (Becton Dickinson and Company, Franklin Lakes, NJ).

The macrophages were incubated in RPMI1640 medium with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan) without antibiotics using 12-well cell culture plates (Becton

Dickinson and Company, Tokyo, Japan). RAW cells were also used; RAW cells were incubated with DMEM medium with 10% heat-inactivated fetal bovine serum without antibiotics. Approximately 1×10^6 cells were incubated per well and *M. avium* was added to the culture medium at a MOI of 10. AHCC (1 mg/ml) was subsequently added. After culturing, the macrophages were collected on day 1, day 2, and day 4. Collected macrophages were washed twice by PBS and lysed by the addition of sterilized water. The lysed medium was then plated onto Middlebrook 7H10 agar plates and the bacterial number was counted after two weeks of incubation at 37°C.

Statistical Analysis

The data are 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. All statistical analyses were performed using the StatView version 5.0 software program.

Results

First, we evaluated the safety of AHCC administration in mice. There was no apparent pathological change or change in the body weight after the administration of 1,000 mg/kg/day of AHCC for 21 days (data not shown). Then, *M. avium*-inoculated mice were supplied with 1,000 mg/kg/day of AHCC by oral administration until euthanasia. *M. avium*-inoculated mice administered water was also set up as the control group. In a previous study, doxycycline was successfully supplied in the water in the same manner [20]. The dose of AHCC was determined by previous reports [17]. Body weight was measured during AHCC administration. There was no apparent difference between AHCC treated mice and the controls (Figure 1). The lung histology was then evaluated. *M. avium*-inoculation resulted in the infiltration of inflammatory cells around the bronchus after 21 days, and AHCC treatment appeared to attenuate inflammatory cell infiltration (Figure 2). The colony number was also evaluated. AHCC treatment decreased the colony count of *M. avium* (Figure 3). The administration of AHCC attenuated lung inflammation caused by *M. avium* according to the histology results and decreased colony count of *M. avium* in the lungs.

We next attempted to determine the mechanism of AHCC responsible for the attenuation of *M. avium* infection. First, we performed an *in vitro* study. AHCC was added to macrophages infected with *M. avium*. As shown in (Figure 4), there was no difference

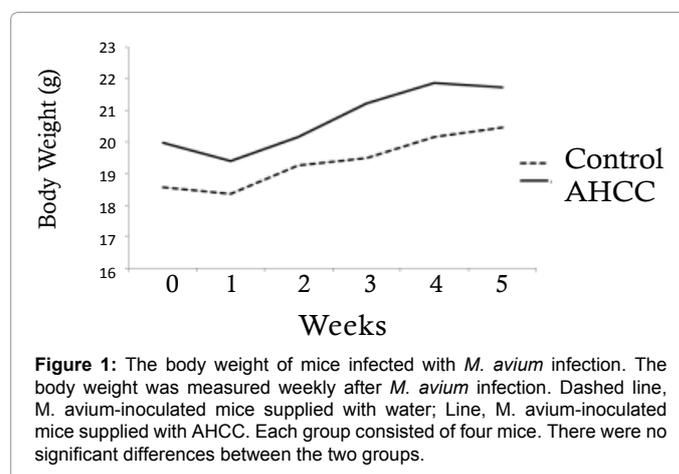


Figure 1: The body weight of mice infected with *M. avium* infection. The body weight was measured weekly after *M. avium* infection. Dashed line, *M. avium*-inoculated mice supplied with water; Line, *M. avium*-inoculated mice supplied with AHCC. Each group consisted of four mice. There were no significant differences between the two groups.

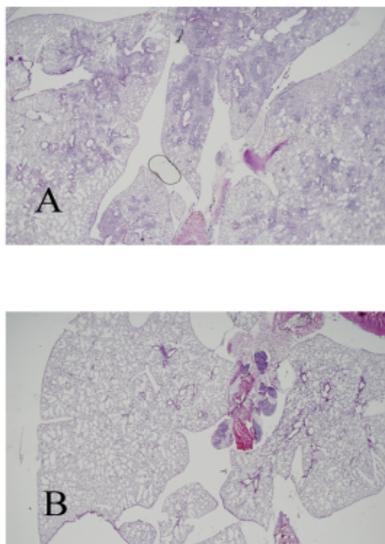


Figure 2: The histologic assessment of the lung after *M. avium* inoculation. (A) The lung from a *M. avium*-inoculated mouse supplied with water. (B) The lung tissue from a *M. avium*-inoculated mouse supplied with AHCC (H-E staining, original magnification: 20x).

a protective role against *Candida*, influenza virus, and *Klebsiella* infection. AHCC influences both the natural immunity and acquisition immunity [13,15,16]. Because an *in vitro* study did not demonstrate bactericidal activity, the effect of AHCC appears to depend on its role

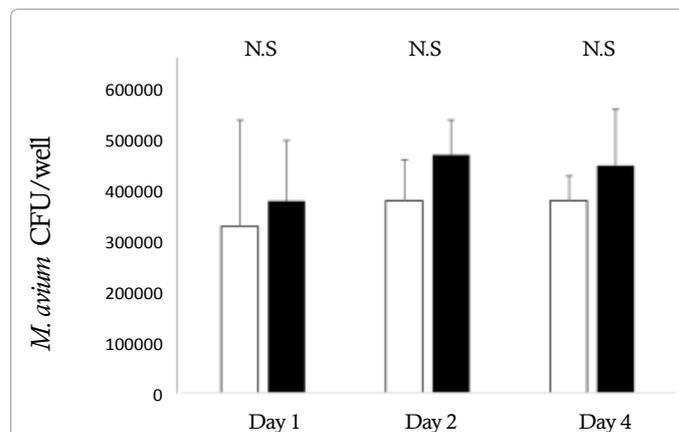


Figure 4: The bacterial growth in the macrophages. Lane 1, *M. avium*-inoculated mice supplied with water; lane 2, *M. avium*-inoculated mice supplied with AHCC. There were no significant differences (N.S.) between the two groups.

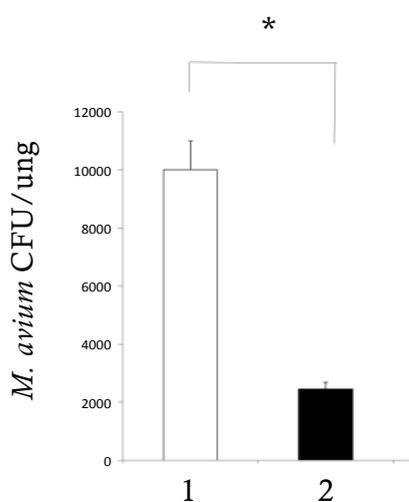


Figure 3: The bacterial growth in the lung. Lane 1, *M. avium*-inoculated mice supplied with water; lane 2, *M. avium*-inoculated mice supplied with AHCC. Each group consisted of four mice. * indicates significant differences between two groups.

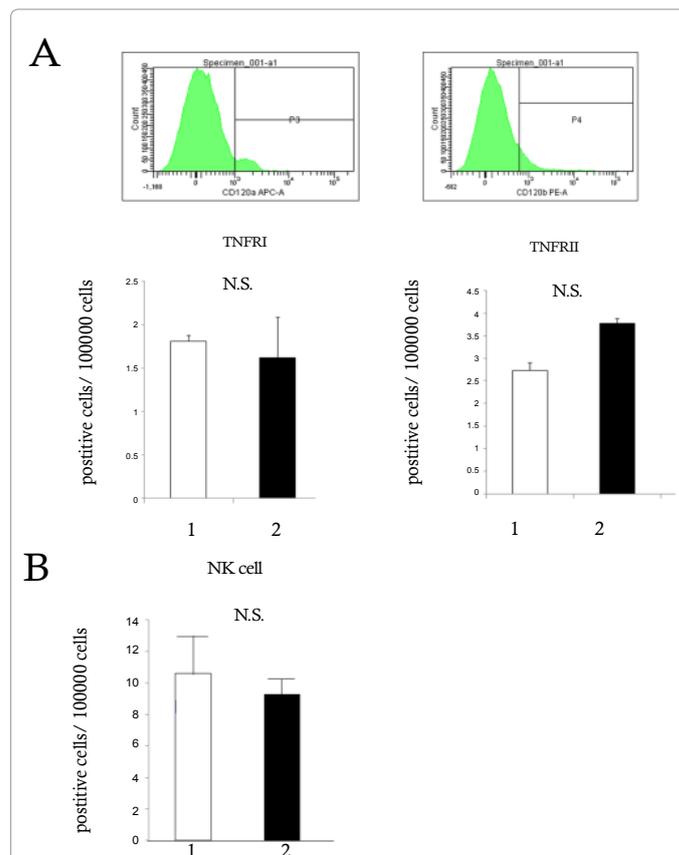
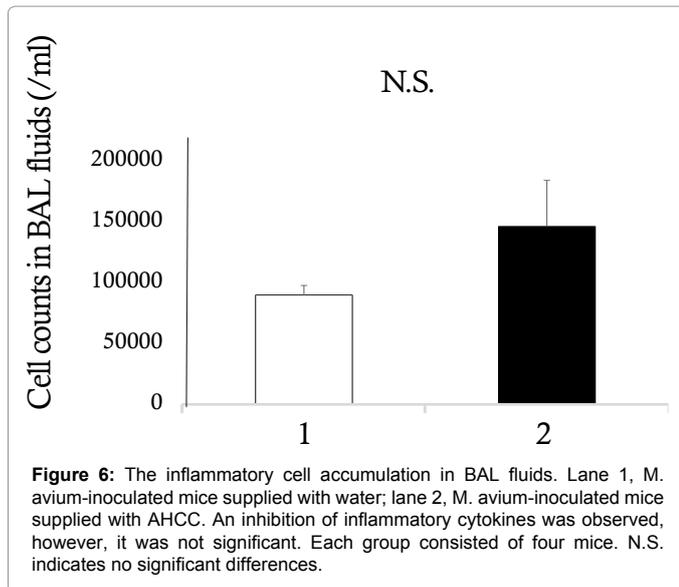


Figure 5: An analysis of the lung inflammatory cells by FACS. (A) Images of FACS for TNFR1 and II antibodies. Lane 1, *M. avium*-inoculated mice supplied with water; lane 2, *M. avium*-inoculated mice supplied with AHCC. (B) An analysis of the lung inflammatory cells for NK antibody. Lane 1, *M. avium*-inoculated mice supplied with water; lane 2, *M. avium*-inoculated mice supplied with AHCC. There were no significant differences (N.S.) between the two groups.

in the colony count of *M. avium* in RAW macrophages treated with or without AHCC *in vitro*. The study using peritoneal macrophages demonstrated similar results (data not shown). Lung inflammatory cells were subsequently analyzed by FACS. As a result, the number of TNFR1 and NK cells (Figure 5) remained unchanged following AHCC administration, however, the number of TNFR2 cells slightly increased. Interestingly, the number of BAL fluid cells, mainly macrophages, tended to be increased, although this increase was not significant (Figure 6).

Discussion

In this study, we demonstrated the attenuation effect of AHCC in *M. avium* infection in mice. AHCC has been reported to show



as a BRM. In fact, AHCC treatment tended to increase the number of macrophages in BAL fluids.

M. avium is an intracellular pathogen that grows in macrophage phagolysosomes. Th1 immune responses are thought to be important in protection against intracellular pathogens [24,25]. Specifically, the involvement of IFN- γ and IL-12 has been demonstrated in the protection against *M. avium* infection because patients with defects in IFN- γ /IL-12 signaling are more susceptible to disseminated and extrapulmonary *M. avium* diseases [26,27]. Additionally, the TNF signal is involved in immunoprotection. TNFR1 knockout mice are known to be susceptible to *M. avium* [28]. In this study, we measured the number of TNFR1-positive cells in the lung, which remained unchanged after AHCC treatment. We found a slight increase in the number of TNFR2 cells, however, the role of TNFR2 is unknown. A recent report revealed a novel role for NK cells, perforin, and granzysin in killing *Mycobacterium* and highlights a potential alternative defense mechanism that the immune system can use against mycobacterial infection [29]. AHCC has been previously reported to increase the number of NK cells in aged mice. α -1,4-glucans are recognized by C-type lectins, such as Dectin-1 on NK cells, thus initiating innate immunity [30]. The mechanism of attenuation was thought to be the activation of NK cells [17]. However, the number of NK cells remained unchanged in this study. The attenuation mechanism should be investigated in future studies.

In the present study, AHCC demonstrated a protective role against *M. avium* infection. However, the mechanism remains unclear. Recently, AHCC has been shown to play a potential role in the orchestration of immune responses and the maintenance of immune homeostasis, in part by priming the TLR-2 and TLR-4 gate at the intestinal epithelium. Such a response is likely due to the recognition of non-pathogenic food-associated molecular patterns (FAMPs) such as those found to be associated with other mushroom or yeast-derived compounds [31]. Further studies of the mechanism by which AHCC affects immunoprotection are required.

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Conflict of Interest

The authors declare no conflicts of interest.

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