

# Low-dose supplementation with active hexose correlated compound improves the immune response to acute influenza infection in C57BL/6 mice

Shoko Nogusa, Jeffrey Gerbino, Barry W. Ritz \*

*Department of Biology, Drexel University, Philadelphia, PA 19104, USA*

Received 4 December 2008; revised 19 January 2009; accepted 20 January 2009

## Abstract

Supplementation with mushroom-derived active hexose correlated compound (AHCC) modulates immunity and increases survival in response to a broad spectrum of acute infections, including influenza virus infection. However, dose-response data are nonexistent. Therefore, the aims of this study were to evaluate AHCC supplementation at various doses and determine the effects of low-dose supplementation on the immune response in a mouse model of influenza virus infection. We hypothesized that AHCC supplementation would influence the immune response to influenza infection in a dose-dependent manner. Male C57BL/6 mice were supplemented with AHCC at daily doses of 0.05, 0.1, 0.5, and 1 g/kg and infected intranasally with influenza A virus (H1N1, PR8). Supplemented mice demonstrated a dose-dependent increase in survival and reduction in the loss of body weight. To further evaluate the effects of low-dose AHCC supplementation on the immune response to influenza infection, mice were supplemented with 0.1 g/kg per day and infected with a sublethal dose of influenza virus. Supplemented mice exhibited enhanced virus clearance and decreased weight loss compared to controls. Low-dose supplementation did not influence total natural killer (NK) cell cytotoxicity, although lytic efficiency was increased in the spleens of AHCC-supplemented mice, indicating enhanced NK cell function per cell. In conclusion, these data suggest that the effects of AHCC on the immune response to influenza infection are dose dependent and that low-dose AHCC supplementation improves the response to influenza infection despite no effect on total NK cell cytotoxicity.

© 2009 Elsevier Inc. All rights reserved.

**Keywords:** Mushroom; Flu; Survival; Lung virus; Natural killer cell; Mice

**Abbreviations:** AHCC, active hexose correlated compound; CPM, counts per minute; NK, natural killer; SEM, standard error of the mean; TCID, tissue culture infectious dose.

## 1. Introduction

Active hexose correlated compound (AHCC) is an enzyme-fermented extract of the *Basidiomycetes* mushroom that is marketed as a dietary supplement or nutraceutical.

This complex compound contains a mixture of polysaccharides, amino acids, lipids, and minerals. The predominant components of this mixture are oligosaccharides, totaling approximately 74% of the total dry weight; of these, nearly 20% are partially acetylated  $\alpha$ -1,4-glucans with a mean molecular weight less than 5000 d.

Although the mechanism of action remains unknown, supplementation studies with AHCC have demonstrated positive effects on various parameters of immune function in

\* Corresponding author. Tel.: +1 215 895 6493.

E-mail address: [ritz@drexel.edu](mailto:ritz@drexel.edu) (B.W. Ritz).

both rodents [1–6] and humans [7–9]. Importantly, the results from several studies using in vivo models have demonstrated increased survival in AHCC-supplemented mice in response to a broad spectrum of acute infections. Collectively, these studies have evaluated the immunomodulatory effects of AHCC supplementation in response to both viruses and bacteria and have included both immunocompetent and immunosuppressed animals. For a review, see Ritz [10]. In our own study, we have previously established that supplementation with AHCC increased survival, enhanced natural killer (NK) cell activity, improved lung virus clearance, and lessened the severity of infection, including a reduction in weight loss and pulmonary inflammation, in young immunocompetent mice in response to acute infection with influenza virus [11].

A notable limitation in previous studies was the use of a single supplemental dose of 1 g of AHCC per kilogram of body weight per day, such that dose-response data are urgently needed to design future human clinical trials. Therefore, in this study, we examined the effects of multiple doses of AHCC on the immune response to influenza infection in mice. We hypothesized that AHCC supplementation would influence the immune response to influenza infection in a dose-dependent manner, as assessed by survival and lung virus clearance. We further established the lowest effective dose of AHCC in this model.

## 2. Methods and materials

### 2.1. Animals and diets

This study protocol was approved by the Drexel University Institutional Animal Care and Use Committee. Male C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Mass) and housed in microisolator cages in the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at Drexel University for a minimum of 1 week before use. Animals were fed an NIH-31 diet [12].

### 2.2. Supplementation with AHCC

The study agent was obtained from the manufacturer, Amino Up Chemical Co, Ltd (Saporro, Japan) and administered orally by pipette at the specified concentration in 25  $\mu$ L sterile distilled water. Mice were supplemented daily for 7 days before and throughout the course of infection, as previously described [11].

### 2.3. Influenza virus infection

Influenza A/Puerto Rico/8/34 was originally provided by Dr Walter Gerhardt, University of Pennsylvania. This H1N1 strain of mouse-adapted influenza A virus was propagated in specific pathogen-free eggs (B&E Eggs, York Springs, Pa), and cell-free supernatants were stored at  $-36^{\circ}\text{C}$  until use. At baseline (day 0), mice were anesthetized by intraperitoneal injection with Avertin (2,2,2-tribromoethanol, Sigma, St.

Louis, Mo) and infected intranasally with the specified dose of virus. Mice were monitored and weighed daily.

### 2.4. Lymphocyte isolation

The protocol for lymphocyte isolation has been described in detail [11,13]. Briefly, mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation, and spleens and lungs were aseptically removed. Spleens were homogenized, and mononuclear cells were isolated from cell suspensions layered on Histopaque-1083 (Sigma) and centrifuged at  $1400 \times g$  for 20 minutes. Lungs were incubated in 3 mg/mL Collagenase A and 80 Kuntz units of DNaseI/mL (Roche Diagnostics, Indianapolis, Ind) at  $37^{\circ}\text{C}$  for 2 hours. Lung homogenates were then passed through a 40- $\mu\text{m}$  nylon mesh, and mononuclear cells were isolated on Histopaque-1083, as described above.

### 2.5. Lung virus titers

Supernatants from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney (American Type Culture Collection [ATCC], Manassas, Va) cells, as described in Po et al [13] and Nogusa et al [14]. After incubation at  $37^{\circ}\text{C}$  for 24 hours, 0.002% L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added, followed by a 72-hour incubation. Chicken red blood cells were prepared at 1% in phosphate-buffered solution (Mediatech, Herndon, Va) and added to cultures. Virus titers were then calculated based on the hemagglutination pattern and reported as the 50% tissue culture infectious dose (TCID<sub>50</sub>).

### 2.6. Flow cytometry

The protocol for immunophenotyping of lymphocytes by flow cytometry has been described previously [11,13]. Briefly, cells from lung or spleen were resuspended in phosphate-buffered solution containing fluorochrome-conjugated antibodies (eBioscience, San Diego, Calif) to CD8 (APC or PE-Cy5) and NK1.1 (PE-Cy7) and incubated on ice in the dark for 30 minutes. Cells were then washed 3 times and fixed in 1% paraformaldehyde (Sigma). Samples were acquired on a FACS Canto (BD, San Jose, Calif) and analyzed using FlowJo software (Tree Star, Ashland, Ore).

### 2.7. Natural killer cell cytotoxicity

A standard  $^{51}\text{Cr}$  chromium-release assay was used to assess NK cell cytotoxicity, as previously described [11,15]. Briefly, YAC-1 target cells (ATCC) were incubated with 200  $\mu\text{Ci}$   $\text{Na}^{51}\text{CrO}_4$  (PerkinElmer, Waltham, Mass) for 2 hours with frequent agitation. The cells were then washed in RPMI-1640 with 10% fetal bovine serum (Mediatech) and plated in triplicate in V-bottom 96-well plates with lung or spleen cell preparations at an effector/target ratio of 50:1. After 4 hours at  $37^{\circ}\text{C}$ , supernatants were harvested onto UniFilter microplates (PerkinElmer), and radioactivity was recorded by a Packard TopCount  $\gamma$ -counter (PerkinElmer)

and reported as counts per minute (CPM). Spontaneous release was determined in medium alone and maximum release in 5% Triton X-100 (Sigma). Spontaneous release was always less than 5% of maximum release. Calculations were as follows:

$$\% \text{ cytotoxicity} = \frac{(\text{experimental CPM} - \text{spontaneous CPM})}{(\text{maximum CPM} - \text{spontaneous CPM})} \times 100$$

$$\text{Lytic efficiency} = \frac{\text{number of target cells killed}}{\text{number NK cells per well}}$$

### 2.8. Statistical analyses

Results are expressed as means  $\pm$  standard error of the mean (SEM), and statistical analyses were performed using GraphPad InStat 3 software (La Jolla, Calif). Comparisons between and within groups were analyzed by analysis of variance with Tukey-Kramer multiple comparisons, while Mann-Whitney *U* tests were used when data were not normally distributed. Significance was accepted at  $P < .05$ .

## 3. Results

### 3.1. The AHCC supplementation increased survival and body weight recovery in a dose-dependent manner

Male C57BL/6 mice were supplemented with AHCC at daily doses of 0.05, 0.1, 0.5, and 1.0 g/kg of body weight per day for 7 days before and throughout infection with influenza virus at a lethal dose of  $10^4$  TCID<sub>50</sub>. Supplemented mice demonstrated a dose-dependent increase in survival (Fig. 1) and reduction in the loss of body weight, an important indicator of the severity of infection (Fig. 2). Only mice supplemented with 0.1 g of AHCC/kg per day or greater recovered from infection.

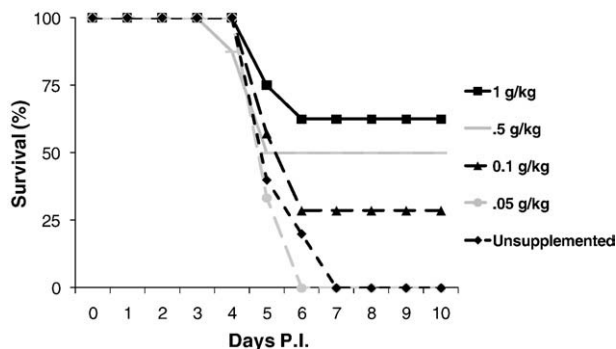


Fig. 1. Dose-Response analysis of survival in AHCC-supplemented mice after influenza infection,  $10^4$ TCID<sub>50</sub>, 6- to 8-week-old mice,  $n = 8$  mice per group at baseline.

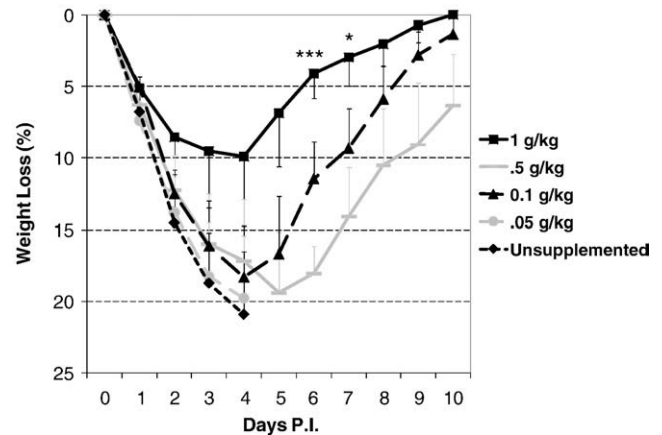


Fig. 2. Dose-Response analysis of weight loss and recovery in AHCC-supplemented mice after influenza infection,  $10^4$  TCID<sub>50</sub>, 6- to 8-week-old mice,  $n = 8$  mice per group at baseline. Values are means  $\pm$  SEM, \* $P < .05$ , \*\*\* $P < .001$  compared to 0.5 g/kg group.

### 3.2. Low-dose AHCC supplementation (0.1 g/kg per day) enhanced virus clearance from the lungs

To evaluate the effects of low-dose AHCC supplementation on the immune response to influenza infection, young adult (6 months) C57BL/6 mice were supplemented with AHCC at a dose of 0.1 g/kg per day for 7 days before and throughout infection with influenza at a sublethal dose of  $10^3$  TCID<sub>50</sub>. Lung virus was undetectable at baseline, as expected. Lung virus titers were similar in control and supplemented mice at day 3 postinfection (Table 1). However, lung virus remained elevated in control mice but was no longer detected in the lungs of AHCC-supplemented mice at day 7. These data were corroborated by the recovery of body weight beginning at day 3 in supplemented mice, whereas control mice continued to lose weight through day 7 (Fig. 3).

### 3.3. Low-dose AHCC supplementation increased splenic NK cell lytic efficiency but not total percent cytotoxicity

Both control and AHCC-supplemented mice demonstrated an influenza-induced increase in NK cell cytotoxicity at day 2 postinfection in lungs and spleens, with no significant difference between groups (Table 2). There were also no differences in the percentage or number of NK cells in the lungs or spleens of supplemented mice at

Table 1

Lung virus titers in AHCC-supplemented mice (0.1 g/kg per day) after influenza infection

	Day 3	Day 7
Control	$3.6 \pm 1.8$	$2.3 \pm 1.0$
AHCC supplemented	$2.8 \pm 1.5$	ND

Values represent means  $\pm$  SEM expressed as TCID<sub>50</sub>, log<sub>10</sub>,  $n = 3$  mice per group at day 3 and  $n = 5$  mice per group at day 7. ND indicates not detectable.

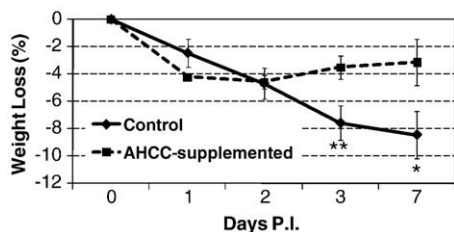


Fig. 3. Weight loss as a percent of initial body weight in AHCC-supplemented mice after influenza infection,  $10^3$  TCID<sub>50</sub>, 6-month-old mice,  $n = 8$  controls and  $n = 12$  AHCC-supplemented mice at baseline. Mice were randomly selected from each group for the determination of lung virus titers at days 3 and 7 postinfection (Table 1). Values are means  $\pm$  SEM, \* $P < .05$ , \*\* $P < .01$  compared to baseline.

baseline or postinfection, as compared to controls (data not shown). However, lytic efficiency, a calculation of NK cell cytotoxicity per cell, was elevated in the spleens of AHCC-supplemented mice at day 2.

#### 4. Discussion

As previously reported [10], supplementation with 1 g of AHCC per kilogram of body weight per day increases survival in mice in response to a broad range of infectious agents, including influenza virus, *Klebsiella pneumoniae*, and opportunistic pathogens. To our knowledge, however, this is the first report of a dose-response analysis of the effects of AHCC supplementation on the immune response to an acute virus infection. Supplementation with AHCC resulted in a dose-dependent increase in survival in mice challenged with a lethal dose of influenza virus. Further, those mice supplemented with AHCC at a dose of 0.1 g/kg per day or greater that survived the infection were able to recover from the infection, as indicated by the recovery of body weight through day 10 postinfection. Therefore, we have determined the lowest effective dose of AHCC in this model, 0.1 g of AHCC/kg per day, which protected approximately 25% of mice against acute, lethal influenza infection.

The potential application of this study is the design of a phase I/II clinical trial to evaluate the safety and efficacy of AHCC supplementation as an immunomodulatory agent in humans. Therefore, it is of interest to estimate the human equivalence dose from these data. The human equivalence dose calculation is based on the body surface area of the test animal and assumes a 60-kg human [16]. In this study, low-dose supplementation (0.1 g/kg per day), which is equivalent to approximately 500 mg/d in humans, improved virus clearance and NK cell lytic efficiency. Taken together, our studies in young and young adult mice suggest that AHCC supplementation induces immune-enhancing effects at doses that are equivalent to 500 mg to 5 g/d in humans. Importantly, supplementation with AHCC at these doses was well tolerated. The no observed adverse effect level was not determined; therefore, the maximum recommended

starting dose for human clinical trials cannot be obtained from these data.

The strengths of this study were the evaluation of the effects of multiple doses of AHCC supplementation on immune outcomes in an established model of murine influenza virus infection and the incorporation of both lethal and sublethal doses of virus. However, it remains unknown whether these results can be extended to other infection models. Although previous studies have reported positive effects of high-dose AHCC supplementation against a broad range of infectious agents in mice, it is unknown whether a similar dose-response would emerge using these other models.

An additional limitation of this and previous studies evaluating the effects of AHCC on immunity is a lack of mechanistic insight. We and others have clearly shown that AHCC alters the innate immune response to acute infection. In previous studies, AHCC supplementation increased influenza-induced NK cell activity and enhanced survival in response to a broad range of infectious threats, including both viruses and bacteria, supporting the hypothesis that AHCC may activate nonspecific, innate immune mechanisms [10]. This consistent outcome has generally been attributed to the unique concentration of  $\alpha$ -1,4-glucans generated during the fermentation process used to produce AHCC [1,6,7]. However, although the activities of related  $\beta$ -glucans derived from mushrooms and yeast cell walls have been studied and appear to activate innate immunity through innate pattern recognition receptors [17], it remains unknown whether  $\alpha$ -1,4-glucans may mediate immunity through a similar mechanism.

In summary, supplementation with AHCC resulted in a dose-dependent increase in survival in mice in response to acute influenza infection, and low-dose supplementation

Table 2  
Natural killer cell cytotoxicity and lytic efficiency in lungs and spleens of AHCC-supplemented mice (0.1 g/kg per day) after influenza infection

	Day 0	Day 2
% cytotoxicity <sup>a</sup>		
Lungs		
Control	6.8 $\pm$ 0.4	17.1 $\pm$ 3.0 <sup>b</sup>
AHCC supplemented		19.4 $\pm$ 3.5
Spleen		
Control	9.2 $\pm$ 1.4	12.1 $\pm$ 1.3
AHCC supplemented		11.6 $\pm$ 1.2
Lytic efficiency <sup>c</sup>		
Lungs		
Control	0.019 $\pm$ 0.004	0.041 $\pm$ 0.006
AHCC supplemented		0.046 $\pm$ 0.016
Spleen		
Control	0.027 $\pm$ 0.008	0.029 $\pm$ 0.003
AHCC supplemented		0.096 $\pm$ 0.022 <sup>d</sup>

<sup>a</sup> Effector:target = 50:1;  $n = 3$  mice per group per day.

<sup>b</sup>  $P < .05$  compared to day 0.

<sup>c</sup> Lytic efficiency = number of target cells killed / number of NK cells per well;  $n = 3$  mice per group per day.

<sup>d</sup>  $P < .05$  compared to control mice on day 2.



(0.1 g/kg per day) improved virus clearance and NK cell lytic efficiency on a per cell basis. Additional studies are now needed to determine the mechanism of action of AHCC as an immunomodulatory agent.

### Acknowledgment

This work was funded in part by a nonrestrictive grant from Amino Up Chemical Co, Ltd, Sapporo, Japan, the manufacturer of the agent that was studied.

### References

- [1] Aviles H, O'Donnell P, Orshal J, Fujii H, Sun B, Sonnenfeld G. Active hexose correlated compound activates immune function to decrease bacterial load in a murine model of intramuscular infection. *Am J Surg* 2008;195:537-45.
- [2] Aviles H, Belay T, Vance M, Sun B, Sonnenfeld G. Active hexose correlated compound enhances the immune function of mice in the hindlimb-unloading model of spaceflight conditions. *J Appl Physiol* 2004;97:1437-44.
- [3] Gao Y, Zhang D, Sun B, Fujii H, Kosuna K, Yin Z. Active hexose correlated compound enhances tumor surveillance through regulating both innate and adaptive immune responses. *Cancer Immunol Immunother* 2006;55(10):1258-66.
- [4] Wang S, Wakame K, Igarashi Y, et al. Beneficial effects of active hexose correlated compound (AHCC) on immobilization stress in rat. *Dokkyo J Med Sci* 2001;28:559-65.
- [5] Burikhanov RB, Wakame K, Igarashi Y, Wang S, Matsuzaki S. Suppressive effect of active hexose correlated compound (AHCC) on thymic apoptosis induced by dexamethasone in rat. *Endocr Regul* 2000;34:181-8.
- [6] Matsushita K, Kuramitsu Y, Ohiro Y, et al. Combination therapy of active hexose correlated compound plus UFT significantly reduces the metastasis of rat mammary adenocarcinoma. *Anticancer Drugs* 1998;9: 343-50.
- [7] Matsui Y, Uhara J, Satoi S, et al. Improved prognosis of postoperative hepatocellular carcinoma patients when treated with functional foods: a prospective cohort study. *J Hepatol* 2002;37:78-86.
- [8] Uno K, Kosuna K, Sun B, et al. Active hexose correlated compound (AHCC) improves immunological parameters and performance status of patients with solid tumors. *Biotherapy (Japan)* 2000;14(3):303-9.
- [9] Ghoneum M, Wimbley M, Salem F, McKlain A, Attallah N, Gill G. Immunomodulatory and anticancer effects of active hexose correlated compound (AHCC). *Int J Immunother* 1995;11:23-8.
- [10] Ritz BW. Supplementation with active hexose correlated compound increases survival following infectious challenge in mice. *Nutr Rev* 2008;66(9):526-31.
- [11] Ritz BW, Nogusa S, Ackerman EA, Gardner EM. Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection. *J Nutr* 2006; 136:2868-73.
- [12] Ritz BW, Aktan I, Nogusa S, Gardner EM. Energy restriction impairs natural killer cell function and increases the severity of influenza infection in young adult male C57BL/6 mice. *J Nutr* 2008;138:2269-75.
- [13] Po JL, Gardner EM, Anaraki F, Katsikis PD, Murasko DM. Age-associated decrease in virus-specific CD8+ T lymphocytes during primary influenza infection. *Mech Ageing Dev* 2002;123:1167-81.
- [14] Nogusa S, Ritz BW, Kassim SH, Jennings SR, Gardner EM. Characterization of age-related changes in natural killer cells during primary influenza infection in mice. *Mech Ageing Dev* 2008;129: 223-30.
- [15] Plett PA, Gardner EM, Murasko DM. Age-related changes in interferon- $\alpha/\beta$  receptor expression, binding, and induction of apoptosis in natural killer cells from C57BL/6 mice. *Mech Ageing Dev* 2000; 118:129-44.
- [16] Guidance for Industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. Rockville (Md): U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER); 2005. Available at: [www.fda.gov/cder/guidance/index.htm](http://www.fda.gov/cder/guidance/index.htm).
- [17] Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S. Dectin-1 mediates the biological effects of  $\beta$ -glucans. *J Exp Med* 2003;197(9):1119-24.