

Active Hexose Correlated Compound (AHCC) Enhances Resistance to Infection in a Mouse Model of Surgical Wound Infection

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ABSTRACT

Background: Infection is the most common postoperative complication within the surgical wound and during severe trauma. In spite of the use of modern sterile techniques and prophylaxis, infection continues to be a leading cause of death in these patients. Therefore, it has become crucial to develop new alternatives to prevent the effects of trauma and other complications on the immune system and improve resistance to infection. The objective of this study was to test the prophylactic effects of oral administration of active hexose correlated compound (AHCC), a natural immunoenhancer, on survival in a mouse model of surgical soft tissue infection.

Methods: The model involves the intramuscular administration of a 50% lethal dose (LD₅₀) of *K. pneumoniae* to mice that have restricted food intake for 24 hours prior to and six hours after infection and simulates local infection and food deprivation that often occur during trauma or surgical procedures. In the present study, AHCC was administered orally to Swiss Webster mice for eight days prior to and during the infection period. Survival, time of death, LD₅₀, and clearance of bacteria of this group were compared with those control mice receiving the excipient alone.

Results: Survival and mean time to death were increased significantly in the AHCC-treated group; the LD₅₀ was greater in mice receiving AHCC than in mice receiving the excipient. Mice receiving AHCC were better able to clear bacteria from their systems than were control animals.

Conclusions: The results suggest that AHCC protects mice in this model by restoring the immune and other systems negatively affected by trauma, infection, and food deprivation. More studies are necessary to determine the intrinsic mechanisms involved in this model and whether AHCC can prevent infection or improve survival in human beings with severe trauma or undergoing surgical procedures.

INFECTIONS ARE COMMON in patients sustaining severe trauma and surgical wounds. The use of aseptic techniques, antibiotics, and other modern approaches has proved to be inefficient in the prevention and control of infection [1].

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Infection has always been a characteristic of human life, and infection after modern surgery continues to be a major problem for healthcare professionals worldwide. Until the middle of the 19th Century, when Ignaz Semmelweis and Joseph Lister introduced the concept of antiseptic surgery, most wounds became infected, and severe infections carried a mortality rate of 70–80% [2]. Developments in microbiology and immunology have helped to make surgery safer. However, the incidence of healthcare-associated infections remains high and represents a substantial burden of disease [3]. In spite of the use of prophylactic antibiotics, infection remains the leading cause of late death after trauma [4]. Infection also still constitutes a real risk of surgery and a substantial burden of disease for both patients and healthcare services in terms of morbidity, mortality, and economic cost [3,5].

The immune system is affected in individuals with severe trauma [6–9]. Functional alterations of the immune system include reduced phagocytic capacity and decreased expression of the class II histocompatibility complex antigens on monocytes [7,9]. Immunosuppression induced by trauma could be transient, with recovery from injury without complications, or could be prolonged, with the risk of serious infections [6–9]. Another factor to be considered in patients requiring surgery is nutritional status. In animal studies, acute starvation induces changes in immune parameters [10–13] and loss of 33% of circulating lymphocytes [13]. Cats deprived of food for several days showed a decrease in the total number of lymphocytes, a lower proliferative response to mitogens, and a decrease in the CD4:CD8 ratio [10]. The development of an agent that could prevent the immunosuppression induced by trauma, food deprivation, and other conditions characteristic of the perioperative period would be of great value. The main goal would be to restore the immune function of individuals subjected to these conditions and consequently to increase innate resistance to infection.

Cytokine therapy and other immunostimulatory agents have failed to provide protection against infection [14–19]. The lack of success is probably multifactorial, but it is plausible that

the action of these agents did not have a sufficiently broad effect on immune function. Because of the complexity of the mechanisms involved in infection and consequent mortality, it appears that most, if not all, immune cells need to be functioning normally to enhance resistance to infection.

In the present study, we tested the hypothesis that oral administration of active hexose correlated compound (AHCC) would enhance immune function and increase survival in a mouse model of surgical soft tissue infection. This compound is an extract prepared from cultured mycelium of a *Basidiomycetes* mushroom [20] whose enhancing effects on immunity have been shown extensively [21–35]. This compound enhances several aspects of the immune response, including natural killer cell activity and cytokine production [21,22]. Previous studies have shown the beneficial effects of AHCC on the function of the immune system [26] and resistance to infection [27] using a rodent model that simulates space flight conditions.

MATERIALS AND METHODS

Bacteria

Klebsiella pneumoniae strain ATCC 43816 was obtained from the American Type Culture Collection (Bethesda, MD). Stock cultures were maintained in tryptic soy broth (TSB) plus 50% glycerol and stored at -80°C until use.

Animals

Specific pathogen-free female Swiss/Webster mice aged 9–11 weeks, each weighing 21–25 g, were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). Animals were housed in a quiet, isolated room with a controlled temperature and light cycle and with access to food and water *ad libitum*. Experimental procedures commenced after one-week acclimation. All experimental manipulations were approved by the Institutional Animal Care and Use Committee of State University of New York at Binghamton and were carried out under the supervision of a veterinarian.

Administration of AHCC

Mice in the AHCC group received the compound for one week prior to and daily throughout the infection period by gavage to ensure delivery of the entire dose of 1 g/kg, the reported maximum effective dose of the compound in animals [26]. Control mice received water, the excipient used for AHCC preparation, by the same route.

Intramuscular surgical soft tissue infection model

Mice receiving AHCC and the control mice receiving the excipient were inoculated intramuscularly in the right thigh with 100 microliters of phosphate-buffered saline (PBS) containing one 50% lethal dose (LD₅₀) of *K. pneumoniae*. Mice were deprived of food but allowed free access to water 24 h prior and 6 h after infection. Under these conditions, this model simulates systemic infection conditions that often occur after trauma or surgical procedures, including systemic contamination as a result of the infection [36].

Experimental groups and evaluation of course of infection

The following experimental groups were utilized for each course of infection: Infected mice treated with AHCC (AHCC) and infected mice treated with the excipient alone (control). After infection, mice were followed for 15 days and observed four times a day, seven days a week. Mice showing signs of terminal illness, including lethargic behavior and ruffled fur, were euthanized by cervical dislocation. Analyses of survival, mean time of death, LD₅₀ doses, and bacterial load in the blood were assessed. Eight to twelve mice in each group were used for each experiment to allow statistical analyses. Experiments were repeated at least twice using the same experimental conditions.

Inocula preparation

An isolated colony from a freshly thawed bacterial aliquot was inoculated into 20 mL of TSB and incubated overnight at 37°C with shaking for aeration. A fresh culture for log-phase growth was prepared in the ratio of 1:200 overnight culture in TSB and incubated at 37°C

with shaking for aeration. Cultures were harvested when the A₆₀₀ = 0.5 (DU530 spectrophotometer; Beckman Coulter, Fullerton, CA), which correlates with bacterial counts. Bacteria were washed by pelleting at 1,900 × g, resuspended in PBS, pH 7.4, and diluted to the appropriate dose in PBS. Bacterial density was confirmed by plating 100 microliters of appropriate 10-fold serial dilutions of cultures on tryptic soy agar (TSA) plates. The density was recorded as colony-forming units (CFU)/mL.

For the LD₅₀ determinations, bacteria were grown to mid-log phase in TSB for approximately 3.3 h at 37°C with gentle shaking. Cells were harvested and washed twice with PBS by spinning at 1,900 × g for 10 min. Cell pellets were subsequently resuspended in 10 mL of PBS and diluted serially to the desired concentrations. Mice were inoculated intramuscularly with 100 microliters of PBS containing doses ranging from 1 × 10⁷ to 1 × 10² CFU/mL, and the LD₅₀ was determined using the Reed-Muench estimation [37]. Concentrations were confirmed by plating three consecutive 10-fold dilutions of the suspension on TSA.

Bacterial load studies

Mice that survived the infection were euthanized by cervical dislocation. Whole blood obtained by cardiac puncture with sodium citrate anticoagulant was diluted serially, and 100 microliters (three 1:10 serial dilutions) was plated on TSA and incubated for 18–24 h at 37°C. Colonies were counted on the three plates, and the CFL/mL was calculated. Plasma was obtained after centrifugation of citrated blood at 1,000 × g for 10 min and stored at –20°C until use.

K. pneumoniae antigen preparation

An isolated colony of *K. pneumoniae* was grown in 250 mL of TSB for 4 h at 37°C with shaking. Bacterial cells were washed twice in PBS at 1,900 × g for 10 min, resuspended in 10 mL of distilled water, and sonicated with 10 repeated 30-sec pulses at high intensity using an ultrasonic cell disruptor (Heat Systems, Farmingdale, NY). Cellular debris and unlysed cells were removed by centrifugation at 1,900 × g for 40 min at 4°C. The supernatant liquid con-

taining the antigen was filtered (0.22 micrometer, Sigma, St Louis, MO), aliquoted, and stored at -80°C until use. A sample was removed for protein determination using a standard Pierce bicinchoninic acid assay (BCA; Pierce, Rockford, IL).

Enzyme-linked immunosorbent assay for detection of IgG and IgM antibodies to K. pneumoniae

Specific immunoglobulin (Ig) G and IgM anti-*K. pneumoniae* antibodies were detected as described previously with some modifications [38] using an enzyme-linked immunosorbent assay (ELISA) in plasma collected from mice that survived the infection. Briefly, 96-well Nunc-Immuno MaxiSorp Surface microtiter plates (BioWorld Laboratory Essentials, Dublin, OH) were coated with 100 microliters of *K. pneumoniae* antigen 5 mcg/mL in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6; Sigma). Plates were kept overnight at $2-8^{\circ}\text{C}$. After washing $3\times$ (PBS, pH 7.4–0.05% Tween 20), nonspecific sites were blocked with 275 microliters of blocking buffer (1% bovine serum albumin [BSA], 5% sucrose in PBS, pH 7.4) for 1 h at room temperature and washed as above. Plasma samples were diluted in 1% BSA in PBS at 1:200 for IgG and 1:100 for IgM, 100 microliters of this dilution was added, and the mixture was incubated at 37°C for 2 h. Secondary antibodies conjugated to horseradish peroxidase (HRP) were diluted in 1% BSA in PBS pH 7.4 (reagent diluent); 100 microliters of a 1:20,000 dilution of goat anti-mouse IgM (Sigma) and 100 microliters of a 1:40,000 dilution of rabbit anti-mouse IgG (Sigma) were plated and incubated at 37°C for 2 h; 100 microliters of substrate, tetramethylbenzidine (R&D Systems, Minneapolis, MN) was added to washed wells and developed at room temperature for 20 minutes. The reaction was stopped with 50 microliters of 2N H_2SO_4 , and optical density at 450 nm was determined using an Elx808 Ultra Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Statistical analysis

At least two experiments were performed for each determination. Data were analyzed using Statview 5.0.1 with alpha set *a priori* at 0.05. Re-

sults were expressed as percent survival at each time point, as determined by the Kaplan-Meier method. Differences in survival between the groups were compared using the log rank (Mantel-Cox), Breslow-Gehan-Wilcoxon, Tarone-Ware, Peto-Peto-Wilcoxon, and Harrington-Fleming tests. The Student *t*-test was used to test the statistical significance of differences between any two groups.

RESULTS

Oral administration of AHCC enhances survival and prolongs the time to death of mice infected with K. pneumoniae

Mice receiving AHCC survived longer than mice receiving the excipient (Fig. 1). At fifteen days, 15% of the mice survived in the control group compared with 55% in the AHCC group. The differences were statistically significant with all survival tests: Log rank ($p = 0.0018$), Breslow-Gehan-Wilcoxon ($p = 0.0058$), Tarone-Ware ($p = 0.0031$), Peto-Peto-Wilcoxon ($p = 0.01$), and Harrington-Fleming ($p = 0.0031$).

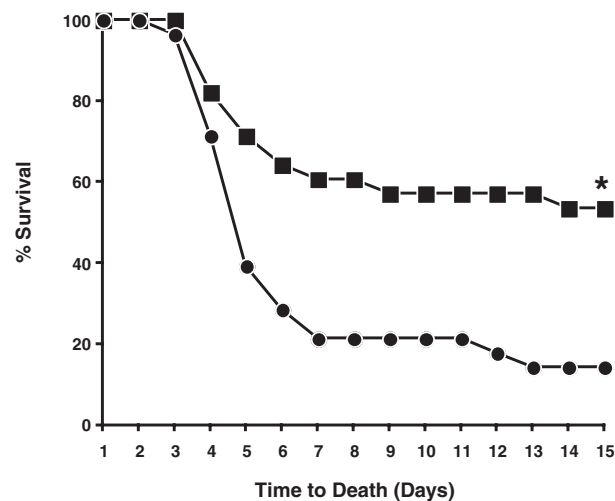


FIG. 1. Effect of oral AHCC on survival of food-deprived mice infected with *K. pneumoniae* (10^2-10^4 CFU/mL) by intramuscular injection. Data are expressed as percent survival at each time point determined by Kaplan-Meier method. Differences in survival between experimental groups were compared using the log rank (Mantel-Cox) ($p = 0.0018$), Breslow-Gehan-Wilcoxon ($p = 0.0058$), Tarone-Ware ($p = 0.0031$), Peto-Peto-Wilcoxon ($p = 0.0050$), and Harrington-Fleming ($p = 0.0031$) tests. (●) Food-deprived and infected mice receiving excipient. (■) Food-deprived and infected mice receiving AHCC. (N = 28 for both groups.)

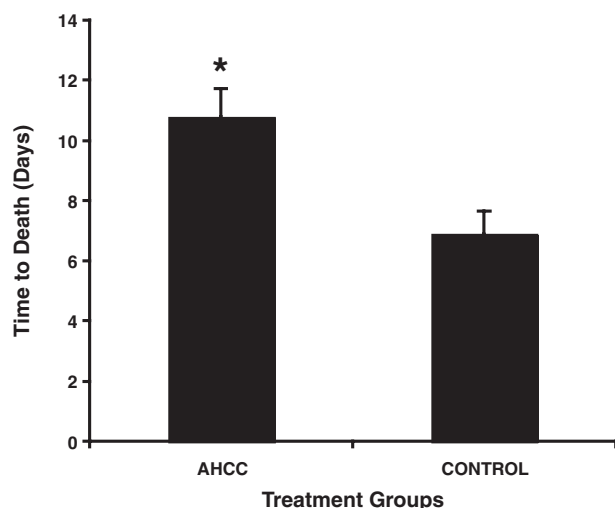


FIG. 2. Effect of oral AHCC on time to death of food-deprived mice infected with *K. pneumoniae* (10^2 – 10^4 CFU/mL) in intramuscular injection model. Values are means of time to death \pm SE in days (* $p < 0.05$).

Similar results were found when analyzing the mean time to death (Fig. 2), in that the AHCC group had a significantly ($p < 0.01$) longer mean time to death (10.8 ± 0.9 days) than the control group (6.9 ± 0.8 days). These results suggest that AHCC overcame the effects of acute food deprivation and infection.

Mice that received AHCC had a greater LD₅₀ of bacteria than mice receiving the excipient

The LD₅₀ was increased about ten-fold in the AHCC group ($3.1 \pm 0.4 \times 10^3$) compared with the control group ($0.24 \pm 0.05 \times 10^3$) (Fig. 3). Administration of AHCC restored the conditions of the host to levels previously reported in normal mice not subjected to acute food deprivation [13].

Mice receiving AHCC were more likely to clear bacteria from their systems

At fifteen days, bacteria were present in only 23% of the surviving mice receiving AHCC (3/13) compared with 60% of the surviving mice receiving the excipient (3/5) (Fig. 4).

*No differences in specific anti-*K. pneumoniae* antibody production in mice that survived infection*

To ensure that survivors were actually infected and to assess the effect of AHCC on an-

tibody production, specific IgG and IgM antibodies against *K. pneumoniae* antigen were measured by ELISA in plasma samples collected from survivors (Table 1). All but one surviving mouse in the AHCC group were positive for *K. pneumoniae* infection, as assessed by the concentrations of both IgG and IgM antibodies. There were no statistical differences in the concentrations of IgG or IgM between food-deprived mice receiving AHCC and food-deprived mice receiving the excipient ($p > 0.05$).

DISCUSSION

Surgical soft tissue infections occur when a microorganism has the opportunity to proliferate in tissue and the body's defenses cannot combat the organism or its proliferation. Among other factors, malnutrition has been associated with a higher incidence of postoperative soft tissue and nosocomial infections in patients with trauma or undergoing major surgery [13,39,40]. In fact, malnourished patients have a three-fold higher risk of postoperative surgical site infection than well-nourished patients [13]. In recent years, the goal has been to keep bacterial contamination of the incision to a minimum and to prevent those organisms from proliferating [13]. However, modern approaches, including the use of an-

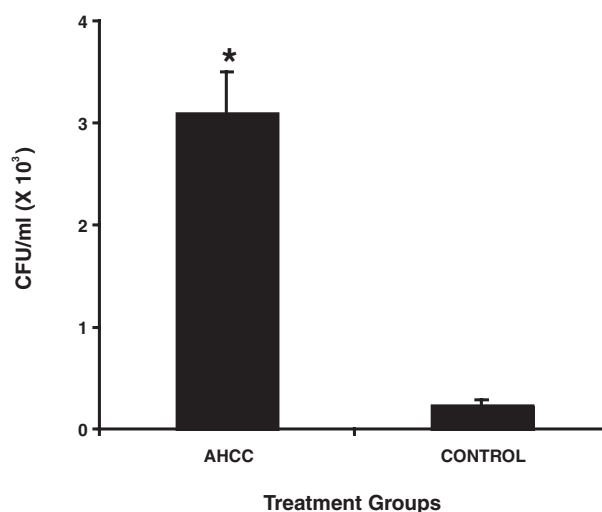


FIG. 3. Effects of oral AHCC on LD₅₀ in food-deprived mice infected with *K. pneumoniae*. Data are expressed as means \pm SE of CFU $\times 10^3$ per mouse (* $p < 0.05$).

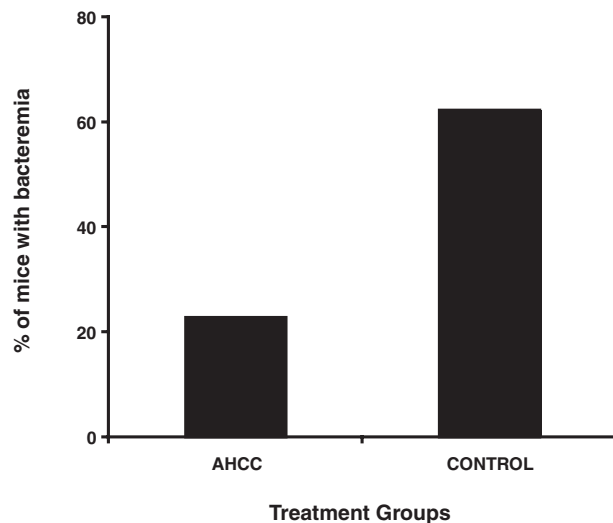


FIG. 4. Bacteremia in food-deprived mice that survived *K. pneumoniae* infection after treatment with either AHCC or excipient. Mice were euthanized fifteen days after infection, and blood was obtained by cardiac puncture. Data are expressed as percentage of mice with detectable bacteria in blood.

tibiotics, have had limited success [1]. Similarly, the use of stimulants of the host's own defenses (Freund's adjuvant, *Mycobacterium bovis* strain bacille Calmette Guerin, and *Corynebacterium parvum*) has been disappointing because of the reported toxic side effects and the high risk of inducing autoimmune disease [13].

Food deprivation is a common event used to avoid complications during and after surgical procedures and anesthesia recovery. It is well documented that acute starvation induces a transient impairment of the immune system with full recovery after the refeeding period [10–13]. In the present study, we tested the hypothesis that AHCC would enhance the resistance to infection of mice subjected to trauma, likely by restoring the function of the immune system. To test this hypothesis, we used a

mouse model of surgical wound infection that simulates conditions that follow trauma or surgery such as local contamination and food deprivation. The results of this study supported the hypothesis and showed that AHCC was effective at improving the conditions of food-deprived mice, as shown by the significant increase in survival and time to death. It was also clear that food-deprived mice receiving the excipient were not able to overcome the effects of food deprivation, with the mortality continuing to increase even after refeeding. It appears that the conditions of the host at the time of infection are crucial to the final outcome of infection. The LD₅₀ decreased about ten-fold in food-deprived mice receiving the excipient, but was restored to normal in mice that received AHCC. A similar reduction was reported in Swiss Webster mice deprived of food for 48 hours before *K. pneumoniae* intramuscular infection and refed four hours after challenge [13].

Our results are consistent with previous studies in which AHCC restored the conditions of mice affected by exposure to space flight conditions in a ground-based model [26,27]. Exposure to spaceflight conditions increased the susceptibility to bacterial infections [41,42] by suppressing immune function [43]; AHCC not only restored immune function to normal [27], but also improved survival [26]. From previous and present results, it appears that AHCC is effective when the immune system is compromised, but AHCC did not significantly improve the immune function or survival of normal mice infected with bacteria [26,27].

The mechanisms involved in the modulation of infection in animal models of trauma, including food deprivation, remain unclear. It is likely that alterations of the immune system

TABLE 1. ANTI-KLEBSIELLA PNEUMONIAE ANTIBODIES IN PLASMA FROM FOOD-DEPRIVED MICE THAT SURVIVED INFECTION^a

| | N | IgG | IgM |
|--------------------------|----|-------------|------------|
| Mice receiving AHCC | 11 | 2.02 ± 0.15 | 1.8 ± 0.10 |
| Mice receiving excipient | 4 | 2.36 ± 0.23 | 2.1 ± 0.14 |

^aValues are means ± SE, given as optical density.

Plasma samples obtained from survivors were tested for specific immunoglobulins G and M antibodies using ELISA. Concentrations were compared with those in citrated plasma from uninfected mice.

induced by food deprivation [10–13] contributed to the higher mortality rate seen in the control group. Timing of food deprivation appears to be a crucial determinant as well. Resistance to fungal infection was profoundly diminished when the period of food deprivation ranged from 48 h before to 24 h after infection, whereas food deprivation initiated immediately, or at 24 h or 48 h, after infection, resulted in moderate or no change in resistance to infection [45]. Similarly, no changes in mortality occurred when food deprivation was initiated 72 h before infection but finished at the time of infection [44]. These results suggest that in order to induce changes in mortality, food deprivation must occur some time prior to infection and continue after infection. It appears that refeeding mice immediately after infection restores the physiology of the host to normal rapidly. Our results are consistent with these previous studies; resistance to infection was severely impaired in mice deprived of food 24 h prior and continuing 6 h after infection.

The mechanisms by which AHCC increases survival in food-deprived mice remain unclear. It appears that improvement in resistance is the result of enhancement of the innate immune response by AHCC rather than a direct cytotoxic effect on bacteria. This hypothesis is supported by *in vitro* studies in which AHCC enhanced the function of natural killer cells [30] and induced interleukin (IL)-12 production [35]. Studies in peritoneal cells have shown that AHCC significantly increases production of NO and cytokines such as tumor necrosis factor- α , IL-1- β , and IL-6 [27]. Innate immunity, therefore, appears to be greatly affected in this model and enhanced by AHCC.

Additionally, other mechanisms appear to be linked with the effects of food restriction on the function of the hypothalamic-pituitary-adrenal axis [45,46] and adrenal cortex [46]. Food restriction increases the production of corticosterone [47] and affects the production of catecholamines [48]. Circulating corticosteroid concentrations are elevated in response to environmental stress, including food deprivation, extreme weather, exercise, and crowding. The function of corticosteroids is to increase the

metabolic rate and mobilize fuel stores. These events are believed to enhance performance during emergency or stressful situations [47]. The effects of food deprivation on corticosterone and catecholamines are enhanced in humans by stress-related trauma and the imminence of surgical procedures. These could be key factors for suppression of the immune system and reduced resistance to infection. It is possible that neurohormones such as catecholamines, released during food deprivation and trauma, exert direct effects on bacterial growth [49–52], and AHCC may modulate, not only the function of the immune system, but also the secretion of these hormones. Proof of this postulate will require additional experimentation.

Active hexose correlated compound may be taken as a nutritional supplement [21–35]. Therefore, it is possible that the direct effects of AHCC on the nutritional status of the host contributed to the enhanced resistance to infection of the food-deprived and infected mice.

In conclusion, it is clear that in this model, treatment followed by continued treatment with AHCC protects mice from death when infected with *K. pneumoniae*. The success of the treatment appears to be related to the status of mice prior to and after infection. Overall, the data suggest that AHCC may be more effective when there are intrinsic defects in the immune system of the host. Mice treated with AHCC were able to clear infection much more effectively than were mice receiving the excipient. This finding suggests that AHCC may be useful in helping to clear bacteria in patients with trauma or undergoing surgical procedures. However, more work is required to define the mechanisms involved in AHCC-induced protection of mice.

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