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Active hexose correlated compound enhances resistance to *Klebsiella pneumoniae* infection in mice in the hindlimb-unloading model of spaceflight conditions

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Aviles, Hernan, Tesfaye Belay, Kimberly Fountain, Monique Vance, Buxiang Sun, and Gerald Sonnenfeld. Active hexose correlated compound enhances resistance to *Klebsiella pneumoniae* infection in mice in the hindlimb-unloading model of spaceflight conditions. *J Appl Physiol* 95: 491–496, 2003. First published April 11, 2003; 10.1152/japplphysiol.00259.2003.—Previous studies have demonstrated that resistance to infection is decreased in Swiss Webster female mice maintained in the hindlimb-unloading model (Aviles H, Belay T, Fountain K, Vance M, and Sonnenfeld G. *J Appl Physiol* 95: 73–80, 2003; Belay T, Aviles H, Vance M, Fountain K, and Sonnenfeld G. *J Allergy Clin Immunol* 110: 262–268, 2002). This is a model of some of the aspects of spaceflight conditions, including lack of load bearing on hindlimbs and a fluid shift to the head. Active hexose correlated compound (AHCC), extracted from *Basidiomycete* mushrooms, has been shown to induce enhancement of immune responses, including enhanced natural killer activity. In the present study, AHCC was orally administered to mice to determine whether the treatment could decrease immunosuppression and mortality of mice maintained in the hindlimb-unloaded model and infected with *Klebsiella pneumoniae*. The results of the present study showed that administration of AHCC by gavage for 1 wk (1 g/kg body wt) before suspension and throughout the 10-day suspension period yielded significant beneficial effects for the hindlimb-unloaded group, including *1*) decreased mortality, *2*) increased time to death, and *3*) increased ability to clear bacteria. The results suggest that AHCC can decrease the deleterious effects of the hindlimb-unloading model on immunity and resistance to infection.

antiorthostatic; stress; rodents

THE IMMUNE SYSTEM IS ONE OF the important regulatory mechanisms affected by spaceflight (12, 19, 22, 36–40, 44). Alterations of immunity could impair the ability of the host to deal with infections and tumors. Hindlimb unloading of rodents is a ground-based model for some of the effects of spaceflight on the immune system (11, 20, 25, 26, 41). Maintenance of rodents in this model induces muscle and bone loss and a fluid shift to the head, which are similar to changes induced by spaceflight (16, 29–31, 33). Previous studies in our laboratory have shown that hindlimb unloading results in altered immune responses and resistance to infection with pathogens (3, 5, 14). As plans for long-term missions and flight opportunities continue to develop, it is important to develop countermeasures to prevent or ameliorate any compromised resistance to infection, thereby ensuring the safety of potential space travelers.

Immunotherapy, a means by which the impaired immune system can be revitalized to carry out its natural functions, has been used successfully especially in cancer patients to enhance the immune system (17, 23). Immunotherapy can include the use of naturally occurring substances. Potential agents include natural substances having immunotherapeutic efficacy when taken orally (17). More than 50 compounds have yielded immunoenhancing potential for anticancer activity as shown in in vitro and in vivo studies (7, 27, 35, 47). Six have been investigated in human cancers, and all have proven to be nontoxic and very well tolerated. Active hexose correlated compound (AHCC) is an extract prepared from cocultured mycelia of several species of *Basidiomycete* mushrooms (17). It contains polysaccharides, amino acids, and minerals and is orally bioavailable. It has been shown to have a positive effect on the immune system of humans (23) and rodents (8, 24, 46, 48, 49). AHCC administered to rats reduced the growth and metastasis of mammary adenocarcinoma cells (24), prevented the thymic apoptosis induced by dexamethasone (8), inhibited the onset of diabetes induced by drugs (48), increased natural killer cell activity and induced the production of IL-12 (48, 49).

There are a limited number of well-controlled studies on the effect of AHCC on the immune function (8, 24, 46, 48, 49) and no studies focusing on the role of AHCC on resistance to infection in hosts subjected to adverse conditions such as microgravity. In the present study, we tested the hypothesis that oral administration of AHCC would decrease the level of immunosuppression and mortality observed in mice maintained in the hind-

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limb-unloading model and infected with *Klebsiella pneumoniae.*

MATERIALS AND METHODS

Bacteria. A clinical isolate of *K. pneumoniae* was obtained from the Clinical Microbiology Laboratory, Carolinas Medical Center (Charlotte, NC). Stock cultures of the bacterium were maintained in tryptic soy broth (TSB) medium plus 50% glycerol and stored at −80°C until use.

Animals. Specific pathogen-free female Swiss Webster mice, 9–11 wk old and each weighing 21–25 g, were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). These are outbred mice that have been used for our previous studies (3, 5). Animals were housed in a quiet, isolated room with controlled temperature and light cycle, and they had access to food and water ad libitum. Experimental procedures commenced after 1-wk acclimation. All experimental manipulations were approved by the Atlanta University Center Institutional Animal Care and Use Committee and were carried out under the supervision of a veterinarian.

Hindlimb-unloading procedure. Hindlimb unloading was carried out as previously reported (3, 5). Briefly, hindlimb-unloaded mice were suspended via the tail with no load bearing on hindlimbs and a 15–20° head-down tilt.

Administration of AHCC. Mice in the AHCC group received the compound for 1 wk before the hindlimb-unloading procedure and daily throughout the suspension and infection period. AHCC was administered by gavage to ensure that the mice always received the entire dose. Gavage was performed by using a straight dosing needle (Ejay International, Glendora, CA), following the recommendations of the company for use in mice (22 gauge and 1.5-in. length). To ensure that stress due to the gavage procedure was not responsible for the observed changes in survival, a group of mice received the excipient (PBS) used for the AHCC preparation by gavage. The dose was 1 g/kg, which is the reported maximum effective dose of the compound in humans.

Experimental groups. The following experimental groups were utilized for each course of infection study: *1*) hindlimb-unloaded and normally caged mice receiving by gavage either AHCC or PBS and infected with *K. pneumoniae*, *2*) normally caged mice infected with *K. pneumoniae*, and *3*) normally caged mice not receiving any treatment. Groups of 12–16 mice in each group were used for each study to allow for statistical analyses. Experiments were repeated at least twice under the same experimental conditions. A restrained control group, used in our laboratory's previous studies, was not included because no differences between restrained and normally housed mice were observed in those studies (3, 5).

Inoculum preparation. A standard loop containing bacteria from a previously frozen stock culture of *K. pneumoniae* was inoculated into 5 ml of TSB for 5 h at 37°C. One loopful of the suspension was inoculated on tryptic soy agar (TSA) plates for colony isolation. After 24 h incubation at 37°C, one single colony was transferred to fresh TSB medium and incubated at 37°C, and standard growth curves were prepared by plotting absorbance readings at 595 nm vs. the corresponding bacterial counts at different time points. Counts were expressed as colony-forming units (CFU) per milliliter.

For the 50% lethal dose (LD_{50}) determinations, bacteria were grown to mid-log phase in TSB for 4 h at 37°C with gentle shaking. Cells were harvested and washed twice with PBS by spinning at 3,000 *g* for 10 min. Cell pellets were subsequently resuspended in 10 ml of PBS and serially diluted to the desired concentrations. Mice were intraperitoneally inoculated with 100 μl of PBS containing doses ranging from 1×10^9 to 1×10^4 CFU/ml, and the LD_{50} was determined by using the Reed-Muench estimation (34). Concentrations were confirmed by plating three consecutive 10-fold dilutions of the suspension on TSA solid medium.

Experimental infection with bacteria. Infections were undertaken 48 h after hindlimb-unloading treatment. To calculate the LD_{50} for the experimental groups, a total inoculum of 100 $\mu l \cdot mouse^{-1} \cdot injection^{-1}$ containing serial 10-fold dilutions was administered via the intraperitoneal route. To ensure the accuracy of doses of bacteria injected into each mouse, a sample of each *K. pneumoniae* suspension was serially diluted and plated on TSA solid medium and incubated at 37°C. The CFU/ml counts were obtained after 24 h. Animal survival was assessed four times a day for 15 days. If the mice showed signs of terminal stages of infection, including lethargic behavior and ruffled fur, they were euthanized.

Bacterial load studies. Mice that survived the infection were euthanized by cervical dislocation. Twenty microliters of citrated blood were serially diluted and plated on TSA for CFU counting, and the remaining volume was kept at 4°C for 24 h. One hundred microliters of the diluted blood were plated on TSA (three 1:10 serial dilutions) and incubated for 24 h at 37°C. The number of CFU was determined by averaging the number of colonies counted on the three serially diluted TSA plates. Plasma was obtained after centrifugation of citrated blood at 3,000 *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 medium for 4 h at 37°C with gentle shaking. Bacterial cells were washed twice in PBS at 3,000 *g* for 10 min and resuspended in 10 ml of distilled water. The bacterial suspension was sonicated with 10 repeated 30-s pulses at high intensity by using an ultrasonic cell disruptor (Heat Systems, Farmingdale, NY). Cellular debris and unlysed cells were removed by centrifugation at 3,000 *g* for 40 min at 4°C. The supernatant containing the antigen was filtered (Sigma Chemical, St. Louis, MO) and aliquoted at −80°C until use. One aliquot was removed for protein determination with a standard bicinchoninic acid assay (Pierce, Rockford, IL).

ELISA for detection of IgG and IgM antibodies to K. pneumoniae. Specific IgG and IgM anti-*K. pneumoniae* antibodies were detected in plasma collected from mice that survived by using an ELISA as previously described with some modifications (3, 4). Briefly, 96-well Nunc-Immuno MaxiSorp surface microtiter plates (BioWorld Laboratory Essentials, Dublin, OH) were coated with 100 μl containing 5 μg/ml of *K. pneumoniae* antigen in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6). Plates were kept overnight at room temperature. After the plates were washed three times (PBS, pH 7.2, and 0.05% Tween 20), nonspecific sites were blocked with 300 μl of blocking buffer (1% BSA, 5% sucrose in PBS, pH 7.2, and 0.05% NaN_3) for 1 h at 37°C. Plasma samples were diluted at 1:200 for IgG and 1:100 for IgM detection, and 100 μl of this dilution were plated and incubated at 37°C for 2 h. Secondary antibodies diluted in 1% BSA in PBS (pH 7.2) (reagent diluent) and conjugated to horseradish peroxidase were used; 100 μl of a 1:20,000 dilution of goat anti-mouse IgM (Sigma Chemical) and 100 μl of a 1:40,000 dilution of rabbit anti-mouse IgG (Sigma Chemical) were plated and incubated at 37°C for 2 h. Reactions were detected by using 100 μl of mixture preparation of the tetramethylbenzidine kit (R&D Systems, Minneapolis, MN). Plates were developed at room temperature for 20 min, and the reaction was stopped with 50 μl of 2 N H_2SO_4. Optical density was determined by

using a Spectramax 250 microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA) set to 450 nm.

Statistical analysis. At least two separate experiments for each determination were performed in this study. Data were analyzed by using Statview 5.0.1 with α set a priori at $P < 0.05$. Results were expressed as percentage of survival at each time point as determined by the Kaplan-Meier method. Differences in survival between the groups were compared by using the Mantel-Cox log-rank, Breslow-Gehan-Wilcoxon, Tarone-Ware, and Peto-Peto-Wilcoxon tests. Student's *t*-test was used to test statistical significance between any two groups, and ANOVA was used for differences between more than two groups.

RESULTS

Oral administration of AHCC results in increased survival and mean time to death of hindlimb-unloaded mice infected with K. pneumoniae. Figure 1 shows the rate of survival in hindlimb-unloaded mice. Mice receiving AHCC had dramatically increased survival compared with mice receiving PBS. Only 25% survived in the hindlimb-unloaded PBS-treated group compared with 75% in the hindlimb-unloaded AHCC-treated group ($P < 0.01$). The mean time to death was also significantly increased in the AHCC group (8.5 ± 0.5 days) compared with the PBS group (5.4 ± 0.8 Days) ($P < 0.05$; Fig. 2).

Normally housed mice receiving AHCC showed a trend toward enhanced survival compared with mice receiving the excipient (PBS). Sixty-seven percent of mice survived in the PBS group compared with 86% in the AHCC group. However, the differences were not statistically significant ($P > 0.2$). Similar results were found when the mean time to death was analyzed: the AHCC group had a trend toward increased mean time to death (9.1 ± 0.4 days) compared with the PBS group (7.7 ± 1.0 days), but no statistical differences could be found between those groups ($P > 0.05$). Infected mice not subjected to gavage had 70% survival and a mean time to death of 8.0 ± 0.5 days. No statistical differences were found compared with mice receiving gavage ($P > 0.05$).

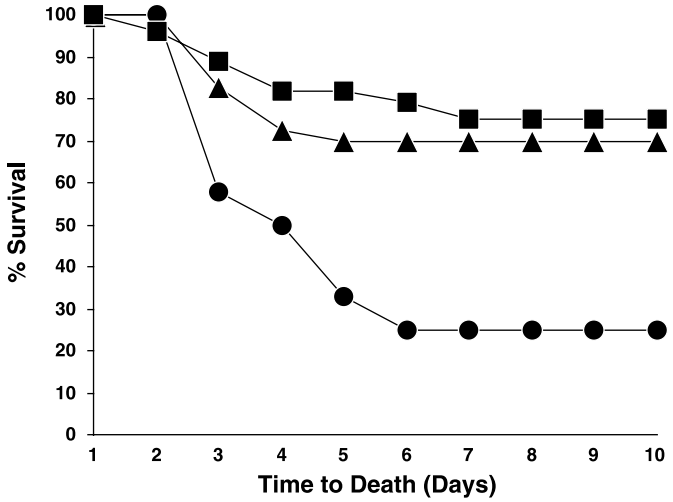

Fig. 1. Effect of active hexose correlated compound (AHCC) on survival of hindlimb-unloaded mice infected with serial dilutions of *Klebsiella pneumoniae*. Values are expressed as percent survival at each time point determined by the Kaplan-Meier method. Difference in survival between the 3 experimental groups of mice was compared by using the Mantel-Cox log-rank test ($P < 0.01$). ●, Hindlimb-unloaded mice receiving PBS; ■, hindlimb-unloaded mice receiving AHCC; ▲, normally caged mice nonsubjected to gavage.

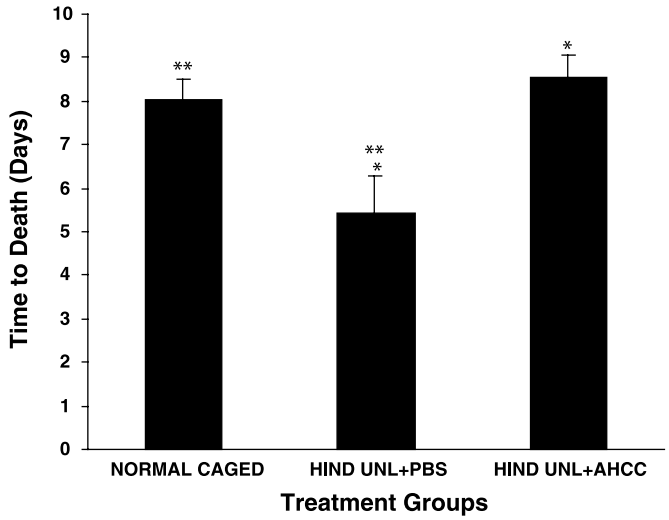

Fig. 2. Effect of AHCC on the mean time to death of hindlimb-unloaded mice receiving by gavage either PBS (Hind Unl+PBS) or AHCC (Hind Unl+AHCC) and no treatment by gavage (Normal Caged). Values are means ± SE expressed in days. *$P < 0.05$ hindlimb unloaded + PBS vs. hindlimb unloaded + AHCC. **$P < 0.05$ normal caged vs. hindlimb unloaded + PBS.

AHCC increases the compared with mice receiving the excipient. The LD_{50} (CFU/mouse) for normally housed mice increased in the AHCC group ($95.2 \pm 15.2 \times 10^6$) compared with PBS group ($63.8 \pm 1.25 \times 10^6$). However, the differences in the calculated LD_{50} values were not statistically significant ($P > 0.05$). Similar to the results for survival and mean time to death, the major differences were seen in mice under hindlimb-unloaded conditions. The number of CFU/mouse required to kill 50% of mice in the AHCC group were significantly increased ($73.5 \pm 1.5 \times 10^6$) compared with the PBS group ($21.0 \pm 1.0 \times 10^6$), $P < 0.01$, (Fig. 3).

Mice receiving AHCC were more likely to clear bacteria from the system. Mice that survived infection were subjected to euthanasia by cervical dislocation at the end of the experimental period. Quantitative culture of bacteria was performed by plating serial dilutions of blood samples on TSA solid medium. Bacteria were present in only 17.5% of mice receiving AHCC compared with 42% of mice receiving PBS (Fig. 4).

AHCC increases the production of IgG-specific anti-K. pneumoniae antibodies in mice that survived infection. To ensure that survivors were actually infected with *K. pneumoniae* and to asses the role of AHCC on antibody production, specific IgG and IgM antibodies against this bacterium were tested in plasma samples collected from survivors (Table 1). All tested mice were positive for *K. pneumoniae* infection as assessed by the levels of both IgG and IgM antibodies against *K. pneu-*

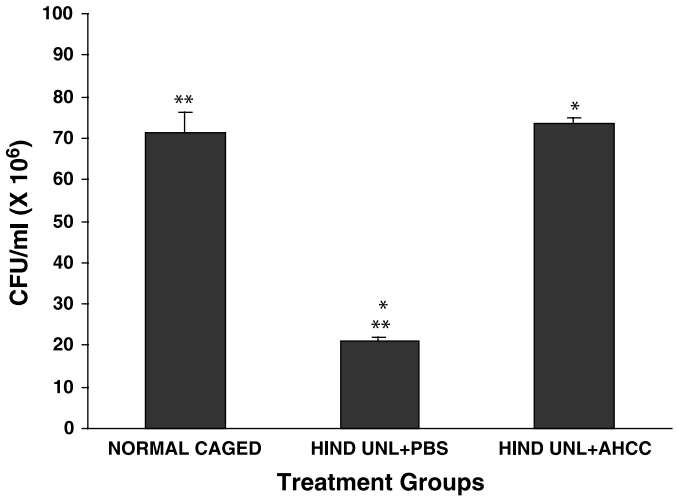

Fig. 3. Effects of oral administration of AHCC on 50% lethal dose in hindlimb-unloaded mice infected with serial dilutions of *K. pneumoniae*. Values are means ± SE expressed in colony-forming units (CFU × 10^6) per mouse. *$P < 0.01$ hindlimb unloaded + PBS vs. hindlimb unloaded + AHCC. **$P < 0.01$ normal caged vs. hindlimb unloaded + PBS.

moniae antigen. Levels of IgG antibodies were significantly decreased in hindlimb-unloaded mice receiving PBS by gavage [1.06 ± 0.23 optical density (OD)] compared with normally caged mice not receiving gavage (1.88 ± 0.15 OD) ($P < 0.05$). Mice in the AHCC group showed increased levels of this type of antibody in normally caged mice (1.98 ± 0.19 OD) compared with mice receiving PBS (1.29 ± 0.26 OD). Similarly, hindlimb-unloaded mice receiving AHCC showed increased levels of IgG antibodies (1.58 ± 0.21 OD) compared with mice receiving PBS (1.06 ± 0.23 OD). Overall, independent of the type of treatment (hindlimb-unloaded or normally caged), mice receiving AHCC significantly increased the production of IgG-specific antibodies (1.77 ± 0.15 OD) compared with mice receiving PBS (1.19 ± 0.15 OD) ($P < 0.05$). There were no statistical differences in IgM production .

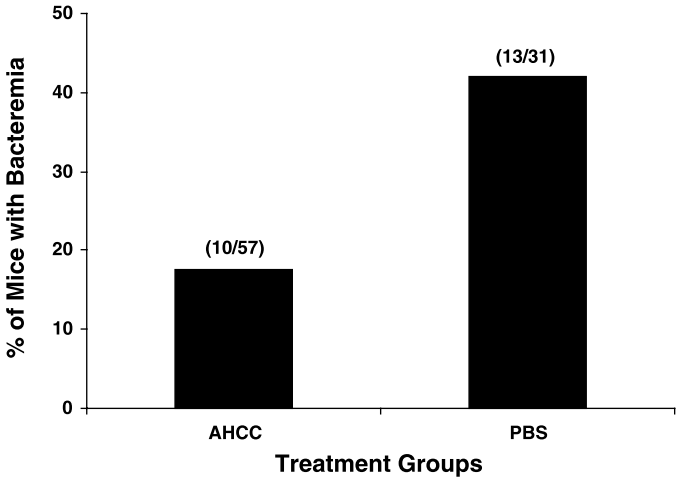

Fig. 4. Bacteremia detected in mice that survived *K. pneumoniae* infection treated with PBS or AHCC. Nos. in parentheses are the no. of mice that survived/total no. of mice. Values are expressed as the percentage of mice with bacterial counts in blood.

Table 1. *Anti-Klebsiella pneumoniae IgG and IgM antibody levels measured in plasma samples obtained from mice that survived infection*

Treatment Groups*	*n*	IgG, OD	IgM, OD
Normally caged	40	1.88 ± 0.15†	0.50 ± 0.05
Normally caged + PBS	12	1.29 ± 0.26	0.44 ± 0.04
Normally caged + AHCC	33	1.98 ± 0.19‡	0.42 ± 0.05
Hindlimb unloaded + PBS	8	1.06 ± 0.23†‡	0.39 ± 0.09
Hindlimb unloaded + AHCC	27	1.58 ± 0.21	0.37 ± 0.03

Values are means ± SE given as optical density (OD); *n*, no. of mice. OD values obtained from normal noninfected mice were subtracted from values obtained from infected mice. AHCC, active hexose correlated compound. *Plasma samples obtained from survivors were tested for specific IgG and IgM anti-*K. pneumoniae* using ELISA. †$P < 0.05$. ‡$P < 0.05$.

DISCUSSION

Hindlimb unloading of rodents has been shown to alter immunologic responses in a similar fashion to spaceflight (11, 20, 25, 26, 41). Several earlier studies suggest that hindlimb unloading could compromise resistance to infection (14, 25, 26). Previous studies in our laboratory using this model and pathogens with potential to cause problems for space travelers (15, 42–45), such as *K. pneumoniae* (5) and *Psuedomonas aeruginosa* (3), showed changes in resistance to infection. These studies demonstrated that hindlimb-unloading suspension increased significantly the susceptibility of mice to infection with these microorganisms. When mice were infected with 1 LD_{50} of bacteria, only 5–6% of mice survived in the hindlimb-unloaded group compared with normally housed and restrained controls (~50%) (3, 5).

AHCC is extracted from cocultured mycelia of several species of *Basidiomycete* mushrooms (17). It has been shown to have a positive effect on the immune system of humans (23) and rodents (8, 24, 46, 48, 49). AHCC is commercially available and utilized as a nutritional supplement. Humans have readily tolerated oral administration of AHCC without adverse effects (13).

The purpose of this study was to determine whether AHCC treatment can ameliorate or prevent the deleterious effects of hindlimb unloading on resistance to infection and immune response. Results of this study suggest that oral administration of AHCC improves the overall condition of the host, resulting in increased resistance to infection. The beneficial effects of this compound were more evident in mice under adverse conditions of hindlimb unloading. It has been reported that hindlimb unloading affects the immune system (11, 20, 25, 26, 41). Therefore, it is probable that AHCC can best enhance the impaired immune function. With the use of this model, mice that received AHCC showed significantly increased survival, mean time to death, and LD_{50}. Also, hindlimb-unloaded mice in the AHCC group were more able to clear bacteria from the sys-

tem. In normally housed mice not subjected to any adverse conditions, the trend was similar but the increase in survival was not as dramatic as seen in the hindlimb-unloaded group.

The mechanisms involved in the modulation of infection after hindlimb-unloading procedure remain unclear. Several factors, such as stress-induced alterations of the immune system and the unloading and fluid shift found in this the model, could have contributed to the increased mortality seen in the hindlimb-unloaded group receiving PBS. The mechanisms by which AHCC dramatically increases survival in the hindlimb-unloading group are not clear. It is probable that the antibacterial activity observed in this model is due to an indirect enhancement of the immune response rather than a direct cytotoxic effect on bacteria. The idea of enhancement of the immune system is supported by in vitro studies in cells (21) and IL-12 production (49). Macrophages and neutrophils are the first line of defense against microbial invasion. Recognition of microbial products such as LPS results in activation of several components of the innate immunity, including complement and acute-phase proteins (28). Activation of innate immunity induces the synthesis and release of proinflammatory cytokines, such as TNF, IL-1, IL-8, and IL-6. The activation of signaling pathways, transcription factors, and the balanced gene expression of pro- and anti-inflammatory cytokines function to decrease tissue injury and death by promoting clearance of bacteria and recovery of the host (28). The increased resistance seen in the AHCC group may be due to the enhancement of components of innate immunity. In fact, in vivo and in vitro studies that used peritoneal cells have shown that AHCC significantly increases nitric oxide production (24). Additional studies have shown that cytokine therapy reverses the immunosuppression seen in the hindlimb-unloading model (1, 2). It is probable also that AHCC plays a role in the regulation of nuclear factors. It has been shown that LPS activates NF-κB (31). Activation of this factor has been linked to production of TNF-α, IL-1-β, IL-6, and IL-8, among others cytokines (6). Humoral immunity was enhanced by AHCC, especially IgG antibodies. However, the early mortality observed in this model (3–6 days) and the time required for producing specific antibodies lead us to believe that it is very unlikely that antibodies play a critical role in survival. The increase of IgG antibodies in the AHCC group rather could play an important role in ameliorating later reinfections involving, perhaps, memory cell development.

Additionally, other mechanisms may involve stress hormones. Release of stress hormones, such as corticosterone, has been shown to inhibit immune responses and decrease resistance to infection (9, 10, 18). Innate immunity appears to be greatly affected by hindlimb unloading and is protected by AHCC; administration of AHCC may be effective by blocking immunosuppressive effects of stress hormones. This will be the subject of future studies.

In conclusion, it is clear that pretreatment followed by treatment with AHCC protects hindlimb-unloaded mice from death when infected with *K. pneumoniae*. Greater effects were found in mice that were profoundly immunosuppressed. Mice that were hindlimb unloaded and treated with AHCC were protected to a greater extent than were normally caged mice treated with AHCC. These 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 PBS. This suggests that AHCC may be useful in ameliorating microbial infections in immunosuppressed hosts. Future studies are required to define the potential and mechanism of AHCC as a countermeasure to minimize any detrimental effects not only during spaceflight but also during any condition where the function of the immune system is compromised.

DISCLOSURES

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