

Active hexose correlated compound enhances the immune function of mice in the hindlimb-unloading model of spaceflight conditions

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Submitted 12 March 2004; accepted in final form 6 June 2004

Aviles, Hernan, Tesfaye Belay, Monique Vance, Buxiang Sun, and Gerald Sonnenfeld. Active hexose correlated compound enhances the immune function of mice in the hindlimb-unloading model of spaceflight conditions. *J Appl Physiol* 97: 1437–1444, 2004. First published June 11, 2004; 10.1152/jappphysiol.00259.2004.—Hindlimb unloading is a ground-based model that simulates some of the aspects of spaceflight conditions, including lack of load bearing on hindlimbs and a fluid shift to the head. It has been shown that treatment with active hexose correlated compound (AHCC) restores resistance to infection in mice maintained under hindlimb-unloading conditions. The present study was designed to clarify the mechanisms by which AHCC enhances resistance to infection in this model. We hypothesized that oral administration of AHCC will enhance the function of the immune system, which could lead to the increased resistance to infection observed in this model. AHCC or the excipient was orally administered to mice, and the function of the immune system was assessed in spleen and peritoneal cells isolated from those groups. The results of the present study showed that administration of AHCC for 1 wk before and throughout the second day of the hindlimb-unloading period enhanced the function of the immune system assessed by spleen cell proliferation and cytokine production in spleens and nitric oxide and cytokine production in peritoneal cells. These findings suggest that AHCC can be used as a potent immunoenhancer, especially in cases in which the immune system is suppressed by any condition, including diseases such as human immunodeficiency virus infection and cancer.

antiorthostatic; suspension; stress; mitogens; natural supplement; rodents

ONE OF THE MAJOR LIMITATIONS to studying human physiology during and after spaceflight has been the relative infrequency of opportunities and the cost of spaceflight missions. As a result, research in this area has been conducted by using ground-based models (1). Hindlimb unloading of rodents is a ground-based model that has been used successfully to reproduce many of the effects of spaceflight on the physiology of the host (1). Exposure to hindlimb-unloading conditions induces muscle and bone loss and a fluid shift to the head, which are similar to changes induced in that area by spaceflight (20, 33–36). The immune system is one of the important regulatory mechanisms affected by spaceflight (15, 23, 26, 40–44, 46) and has been shown to be dramatically affected in ground-based models (14, 24, 29, 30, 45).

Numerous substances have been used to revitalize the immune response of hosts with impaired function of the immune system (21, 27). Many of them are natural compounds that

have proven to be nontoxic for humans and with great potential for anticancer activity (9, 31, 39, 48). Active hexose correlated compound (AHCC), an extract prepared from cocultured mycelia of several species of *Basidiomycete* mushrooms, has received special attention in the past few years (21). AHCC is commercially used as a nutritional supplement and contain a mixture of polysaccharides, amino acids, and minerals. In addition, AHCC is orally bioavailable, well tolerated in humans, and free of adverse effects (16, 21). Chemical analysis has shown that ~74% of AHCC is oligosaccharides and ~20% is of the α -1, 4-glucan type. The acetylated forms of the last group are oligosaccharides with low molecular mass (5 kDa) and are believed to be the molecules responsible for the biological activities of AHCC (27, 28, 51). AHCC has been shown to have an enhancing effect on the immune system of humans (27) and rodents (10, 28, 47), including an increase of natural killer cell activity (25) and IL-12 production (49, 50).

Our laboratory has shown, in previous studies, that hindlimb unloading of mice decreases resistance to infection (4, 8, 17) and that the use of countermeasures reduces these effects in this model (5). Hindlimb-unloaded mice were far more susceptible than control mice to infection with *Klebsiella pneumoniae* (8) and *Pseudomonas aeruginosa* (4). Hindlimb-unloaded mice that received AHCC for 1 wk before and during the infection period overcame the effects of hindlimb unloading and showed improved survival, approaching levels seen in normally housed mice (5). The objective of this study was to clarify the mechanisms involved in AHCC-enhanced resistance to infection in the hindlimb-unloading model. We tested the hypothesis that oral administration of AHCC will enhance the function of the immune system of mice under hindlimb-unloading conditions, leading to increased resistance to infection.

MATERIALS AND METHODS

Animals. Specific pathogen-free female 9- to 11-wk-old Swiss/Webster mice, 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. Animals were housed in a quiet, isolated room with controlled temperature and light cycle, having access to food and water ad libitum. Experimental procedures commenced after 1-wk acclimation. All experimental manipulations were approved by the Morehouse School of Medicine/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 (4, 8). Briefly, hindlimb-unloaded mice

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Table 1. Effects of AHCC on body and spleen weight in mice under hindlimb-unloading conditions

Treatment Groups	Body Weight, g	Spleen Weight, mg	Spleen Weight Loss, %
NC	23.94±0.4	113.6±10.6	29.2±4.7
NC + HU	21.60±1.1	78.8±3.8*	
PBS	22.34±1.4	133.2±29.3	31.0±6.3
PBS + HU	21.74±1.0	84.8±6.8*	
AHCC	22.94±1.1	121.8±2.30	23.6±7.3
AHCC + HU	22.18±0.8	93.2±9.5*	

Values are means ± SE; $n = 5$ mice/group. NC, normally caged under no treatment; PBS, mice receiving phosphate-buffered saline by gavage; AHCC, mice-receiving active hexose correlated compound by gavage; HU, hindlimb unloading. * $P < 0.05$ vs. control.

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 unloading period. AHCC was administered by gavage to ensure that the mice always received the entire dose. Gavage was performed using a straight dosing needle (Ejay International, Glendora, CA), following the recommendations of the company for use in mice (22-gauge and 1.5 inches length). To ensure that the observed changes were not due to the gavage procedure, a group of mice received the excipient (PBS) used for the AHCC preparation. The dose was 1 g/kg, which is the reported maximum effective dose of the compound in humans. AHCC in powder was weighted and diluted in PBS; dose was adjusted to have the AHCC concentration in 300 μ l of volume.

Experimental groups. The following experimental groups were utilized in this study: 1) normally caged mice receiving by gavage either AHCC or PBS, and 2) hindlimb-unloaded mice receiving by gavage either AHCC or PBS. The normally caged group included a) mice not receiving any treatment, normal control; b) mice receiving the excipient (PBS); and c) mice receiving AHCC. The hindlimb-unloaded group included d) mice housed in normal cages, normally caged; e) mice receiving PBS and hindlimb unloaded; and f) mice receiving AHCC and hindlimb unloaded. Groups of five to six mice in each group were used for each study to allow for statistical analyses. Experiments were repeated at least twice using the same experimental conditions. A restrained control group, used in previous hindlimb-

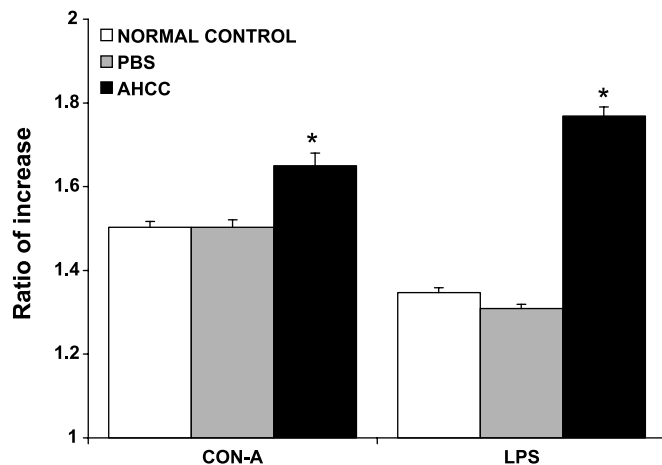


Fig. 1. Effects of oral administration of active hexose correlated compound (AHCC) on mitogen-induced spleen cell proliferation of normally caged mice. Con-A, concanavalin A; LPS, lipopolysaccharide. Data are means ± SE of the increase of proliferation of mitogen-stimulated cells compared with mitogen-nonstimulated control cells. * $P < 0.05$.

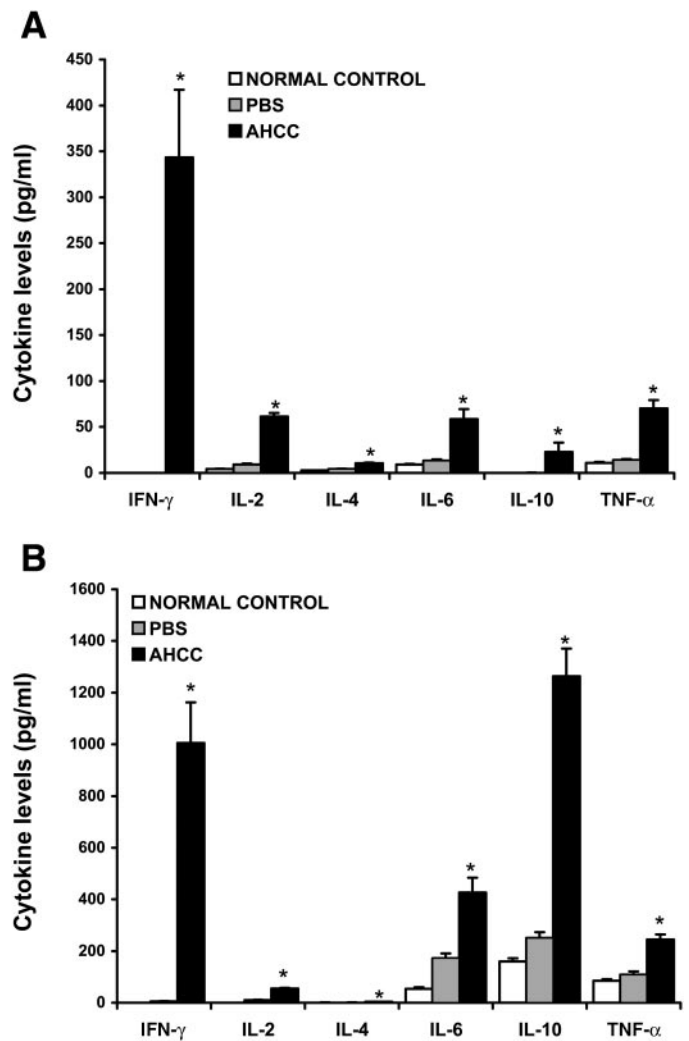


Fig. 2. Effects of oral administration of AHCC on cytokine levels produced by spleen cells isolated from normally caged mice. Cytokine production was measured in supernatants obtained from cultured spleen cells after stimulation with Con-A (A) and LPS (B). Data are means ± SE. * $P < 0.05$.

unloading studies, was not included because no differences between restrained and normally housed mice were observed in those studies (4, 8).

Sample collection. Mice were killed by cervical dislocation 10 days after the commencement of AHCC treatment in the two groups. Mice in the hindlimb-unloading group were hindlimb unloaded 8 days after AHCC treatment and 2 days before sample collection. A similar protocol was used in our previous studies showing the effects of AHCC on resistance to infection (5). Spleen and peritoneal cells were removed and processed according to the type of experiment. Spleen cells were cultured and incubated for 48 h for proliferation and cytokine measurements. Peritoneal cells were cultured for 24 h for cytokine measurements and at different time points for nitric oxide analysis. Body and spleen weight were recorded for each experimental group.

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

***K. pneumoniae* antigen preparation.** Bacteria from an isolated colony were grown in 250 ml of tryptic soy broth medium for 4 h at 37°C with gentle shaking. Bacterial suspension was washed two times

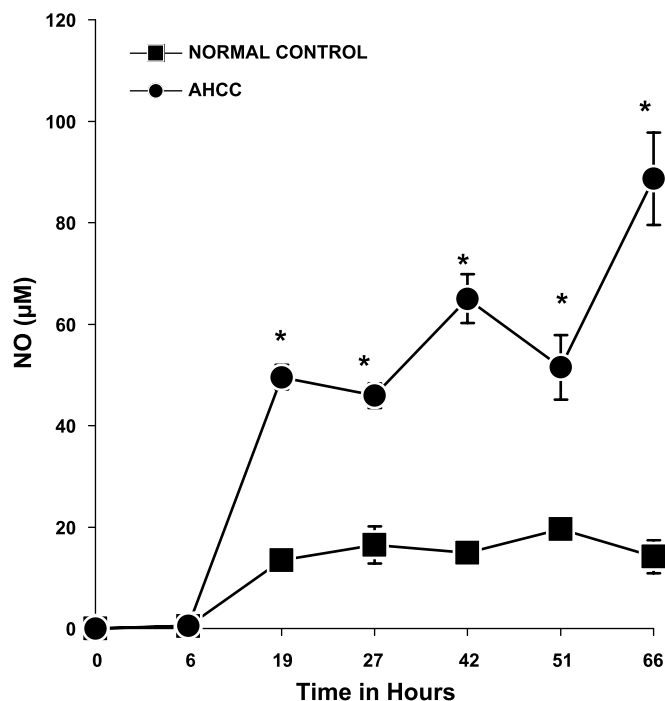


Fig. 3. Effects of oral administration of AHCC on nitric oxide (NO) released by peritoneal cells isolated from normally caged mice. Peritoneal cells were cultured and stimulated with LPS. Data are means \pm SE. * $P < 0.05$.

in PBS at 3,000 g for 10 min and resuspended in 10 ml of distilled water. Bacterial cells were 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 using a 0.2- μ m syringe filter (Pall, Ann Arbor, MI) and aliquoted at -80°C until use. One aliquot was removed, and protein concentration was measured by using a standard bicinchoninic acid assay (Pierce, Rockford, IL).

Spleen cell proliferation assay. The assay was performed as described previously, with some modifications. Briefly, spleens from mice in each experimental group were removed aseptically, pressed through a cell strainer (Becton Dickinson, Franklin Lakes, NJ), and placed in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Sigma), and 0.1% gentamicin (Sigma). Erythrocytes were removed from the cell suspension by treatment with lysis buffer (0.83% NH_4Cl in 0.1 mM Na_2EDTA and 1 M KHCO_3). Cell viability was determined by use of Trypan blue (Sigma), and cells were plated in triplicate at a density of 5×10^5 cells per well in a 96-well Falcon flat-bottom tissue culture plate (Becton Dickinson). Spleen cells were stimulated with 2.5 $\mu\text{g}/\text{ml}$ of concanavalin A (Con-A) and 5 $\mu\text{g}/\text{ml}$ of lipopolysaccharide (LPS) and cultured for 48 h in a water-jacketed incubator at 37°C and 5% CO_2 (Forma Scientific, Marietta, OH). Spleen cell proliferation was determined by using a colorimetric method following the manufacturer's instructions (Promega, Madison, WI). Optical density was recorded at 490 nm using a microplate reader (Spectramax 250 microplate spectrophotometer system, Molecular Devices, Sunnyvale, CA).

Peritoneal cell functional assays. These assays were performed as described previously, with some modifications (7). Briefly, mice that received injection of 2 ml ip of thioglycollate broth 5 days previous to sample collection were killed by cervical dislocation. Peritoneal cells harvested from mice under different treatments were placed in RPMI without phenol red (Sigma), supplemented with 5% FBS (Sigma), 1% penicillin/streptomycin (Sigma), and 0.1% gentamicin (Sigma),

counted, and plated (1 ml) in 24-well cell culture plates (Corning, New York, NY) at a density of 1×10^6 cells/well. Peritoneal cells were stimulated with LPS (10 $\mu\text{g}/\text{ml}$) and in some experiments with *K. pneumoniae* antigen (10 $\mu\text{g}/\text{ml}$). Plates were placed in a water-jacketed incubator at 37°C in 5% CO_2 (Forma Scientific). Supernatants were collected after 24-h incubation and stored at -80°C until use for cytokine production.

Nitrite determinations. For nitric oxide analysis, peritoneal cells harvested from mice were cultured and stimulated in similar fashion

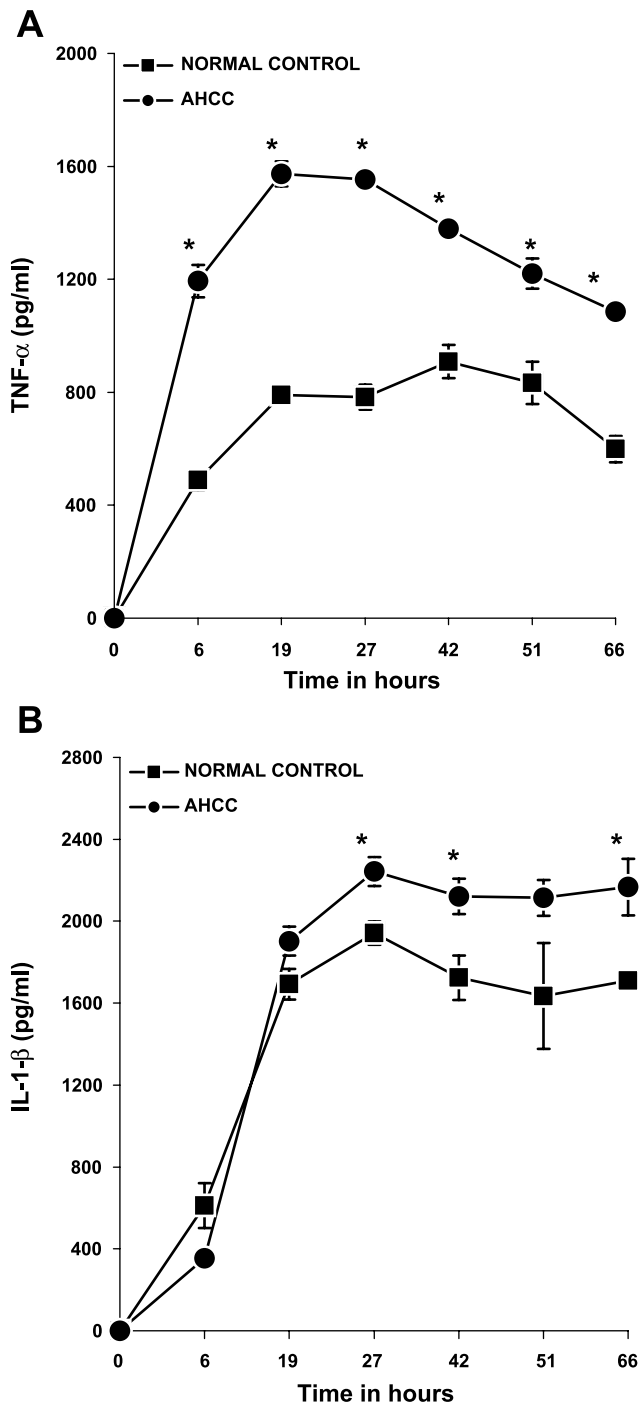


Fig. 4. Effects of oral administration of AHCC on cytokine levels produced by peritoneal cells isolated from normally caged mice. Cytokine production was measured in supernatants obtained from cultured peritoneal cells after stimulation with LPS. A: TNF- α . B: IL-1 β . Data are means \pm SE. * $P < 0.05$.

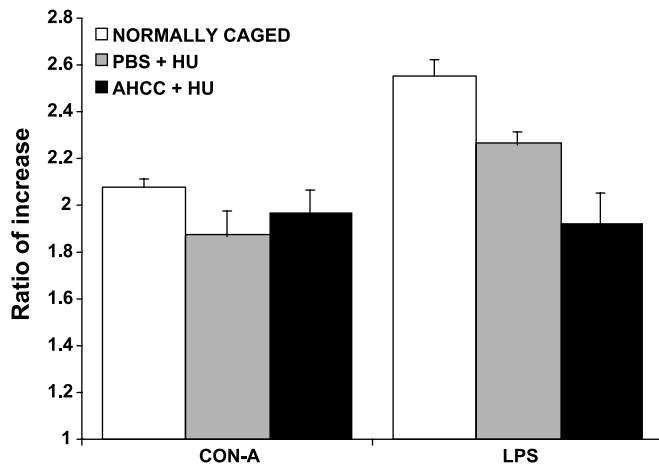


Fig. 5. Effects of oral administration of AHCC on mitogen-induced spleen cell proliferation of hindlimb-unloaded (HU) mice. Data are means \pm SE of the increase of proliferation of mitogen-stimulated cells compared with mitogen-nonstimulated control cells.

as above. Supernatants were collected at different time points after the commencement of culture and stimulation, one aliquot was used for immediate nitric oxide analysis, and the remainder was stored at -80°C until use for cytokine analysis. Nitrite concentrations were determined using the Greiss reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine hydrochloride in 5% phosphoric acid) with reference to a standard curve for sodium nitrate (18).

Cytokine measurement. Supernatant fluids from parallel cell cultures of spleen cell proliferation and from the peritoneal functional assays were used to determine cytokine production (6). Cellular debris was removed by spinning at 2,000 g for 5 min at 4°C . Concentrations of cytokines were determined by using the Douset ELISA system method according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Optical densities were measured at 450 nm with an ELISA plate reader (Spectramax 250 microplate spectrophotometer system).

Statistical analysis. Groups of five to six mice in each group were used for each study. At least two separate experiments for each determination were performed in this study. Data were analyzed by using Statview 5.0.1 with alpha set a priori at $P < 0.05$. Student's *t*-test was used to test statistical significance between any two groups. ANOVA followed by post hoc Fisher's test was used to test statistical differences between more than two groups and among groups at different time points.

RESULTS

Oral administration of AHCC does not restore the loss of spleen weight observed in mice under hindlimb-unloading conditions. There were no significant changes in body weight among treatments (Table 1). However, spleens isolated from

hindlimb-unloaded mice weighted significantly less than normally caged control mice. Although the percentage of spleen weight loss was reduced in mice receiving AHCC (23.6 ± 7.3) compared with mice receiving PBS (31 ± 6.3) and mice not receiving treatment (29.2 ± 4.7), a statistical difference was not reached.

AHCC enhances the function of the immune system in normally housed mice. Before studying the effects of AHCC in mice under hindlimb-unloading conditions, we investigated the effects of AHCC in normally housed mice. To assess the role of AHCC on spleen cell function, spleen cells were removed, cultured, and stimulated with mitogens. Spleen cell proliferation and cytokine production were measured and compared among groups. Mice receiving AHCC showed significant increase in proliferation in spleen cells stimulated with Con-A (1.65 ± 0.03) compared with control mice receiving PBS (1.5 ± 0.018) and normal control mice not receiving any treatment (1.5 ± 0.014). Similar results were found in spleen cells stimulated with LPS: the ratio of increase in proliferation was significantly higher in the AHCC group (1.77 ± 0.021) than the PBS (1.31 ± 0.01) and the normal control (1.35 ± 0.012) groups (Fig. 1).

Analysis of cytokine production showed a significant overall increase of cytokines with predominant T helper (Th) 1 cell response (IFN- γ and IL-2) in the AHCC group compared with control groups in cells stimulated with Con-A (Fig. 2A). A greater cytokine response with predominant Th2 (T helper cell 2) response (IL-4, IL-6, IL-10) was observed in the AHCC group in cells stimulated with LPS. However, IFN- γ levels also were significantly increased after LPS stimulation in this group (Fig. 2B).

The study of peritoneal cell function is critical in this work because we have used in previous studies the intraperitoneal route of inoculation to show the effects of hindlimb unloading on resistance to *K. pneumoniae* infection (5, 8). Peritoneal cells were removed, cultured, and stimulated with LPS. Nitric oxide and cytokine production were measured at different time points. Nitric oxide production was significantly increased starting after 19 h of LPS stimulation in the AHCC group compared with control group (Fig. 3). The increase in nitric oxide in the AHCC group was preceded by an early increase (6 h) of TNF- α (Fig. 4A) and a later increase (27 h) of IL-1 β (Fig. 4B).

AHCC does not affect spleen proliferation but enhances the Th1 response in mice under hindlimb-unloading conditions. Mice receiving either AHCC or PBS were subjected to hindlimb-unloading conditions to assess the role of this compound on cells of the immune system. There were no significant differences in proliferation among the groups, neither after Con-A nor after LPS stimulation (Fig. 5). Similar results (no

Table 2. Effects of AHCC on cytokine production measured in supernatants obtained from cultured spleen cells harvested from control and hindlimb-unloaded mice and stimulated with mitogens

Treatment Groups	LPS			Con-A		
	IL-6	IL-10	TNF- α	IFN- γ	IL-2	TNF- α
NC	39.76 \pm 3.27	269.82 \pm 36.4	25.17 \pm 1.46	17.5 \pm 2.5	159.5 \pm 2.5	68.83 \pm 3.1
PBS + HU	64.67 \pm 5.51	264.87 \pm 13.1	52.38 \pm 3.55	9.5 \pm 1.5	70.5 \pm 11.5	112.407 \pm 19.4
AHCC + HU	58.89 \pm 11.4	182.42 \pm 27.8	33.71 \pm 7.97	71.5 \pm 45.5*	299.5 \pm 104.5*	88.85 \pm 5.2

Values are means \pm SE in pg/ml; $n = 6$ mice per group. Values obtained from nonstimulated cells were subtracted from values from stimulated cells. Spleen cells were harvested from mice under different conditions. Cytokine levels in pg/ml were detected in supernatants obtained from cultured cells stimulated with lipopolysaccharide (LPS; 5 $\mu\text{g/ml}$) and concanavalin A (Con-A; 2.5 $\mu\text{g/ml}$). * $P < 0.05$ vs. control.

statistical differences) were found regarding cytokine levels after LPS stimulation (Table 2). However, AHCC increased significantly the Th1 (IFN- γ and IL-2) response in cells obtained from hindlimb-unloaded mice and stimulated with Con-A (Table 2).

AHCC restores the function of peritoneal cells suppressed by hindlimb-unloading conditions. Peritoneal cells obtained from hindlimb-unloaded mice receiving AHCC or PBS were cultured, and cytokine production was measured in supernatants collected 20 h after stimulation with LPS or *K. pneumoniae* antigen. Results were similar regarding the type of stimulant used; levels of IL-6, IL-1 β , and TNF- α were significantly increased in hindlimb-unloaded mice receiving AHCC compared with hindlimb-unloaded mice receiving PBS (Table 3). There were no statistical differences between normally caged mice and hindlimb-unloaded mice receiving AHCC, suggesting that AHCC restores to normal values the levels of cytokines that were suppressed under hindlimb-unloading conditions (Table 3).

AHCC increases nitric oxide production in peritoneal cells isolated from hindlimb-unloaded mice. Peritoneal cells isolated from hindlimb-unloaded mice under different treatments were cultured and stimulated with LPS. Nitric oxide and cytokine production were measured at several time points after stimulation. Nitric oxide levels were significantly increased after 19 h in the hindlimb-unloaded group receiving AHCC (Fig. 6). This nitric oxide increase was preceded by an early increase (6 h) of TNF- α (Fig. 7A) and IL-1 β levels (Fig. 8A). Surprisingly, peritoneal cells not stimulated with LPS in the AHCC group produced higher levels of TNF- α (Fig. 7B) and IL-1 β (Fig. 8B) compared with controls, which produced little or no cytokines.

DISCUSSION

Spaceflight conditions induce changes in the physiology of humans and animals (35). Development of countermeasures to prevent those changes has become a critical issue to ensure the safety of space travelers. It has been shown that hindlimb unloading, a ground-based rodent model of spaceflight conditions, alters the immune response (14, 24, 29, 30, 45) and compromises resistance to infection (4, 8, 17, 29, 30). We have also shown that AHCC can be used as a countermeasure to reduce the effects of hindlimb-unloading conditions (5). However, the mechanisms by which AHCC increases the rate of survival in this model remain to be established. Although several studies have shown the potential beneficial effects of AHCC on the immune system of humans (27) and rodents (10, 28, 47, 49, 50), very little is known about the effects of this compound on resistance to infection.

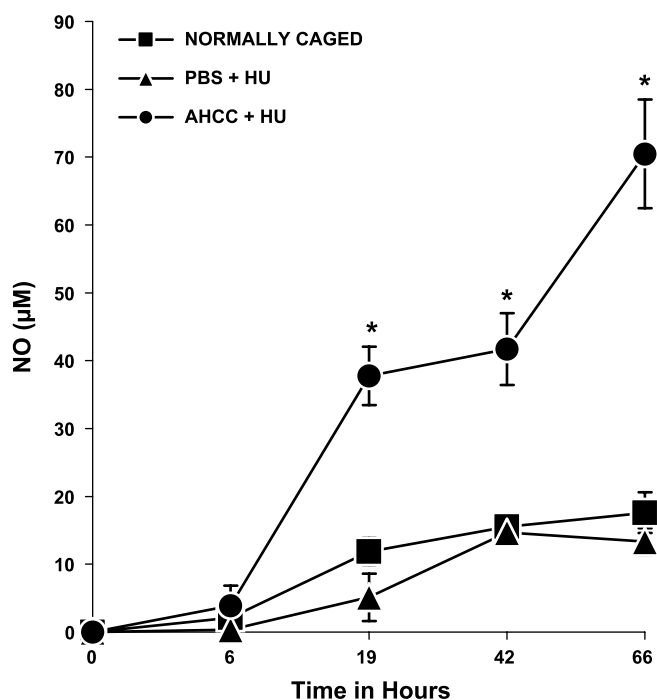


Fig. 6. Effects of oral administration of AHCC on NO released by peritoneal cells isolated from HU mice. Peritoneal cells were cultured and stimulated with LPS. Data are means \pm SE. * $P < 0.05$.

The objective of this study was to investigate the effects of AHCC on the function of the immune system. Our results supported the hypothesis that oral administration of AHCC would enhance the function of the immune system, which could result in increased resistance of the host to bacterial infection. Results of this study suggest that AHCC is a good enhancer of the immune system. However, it appears that infection combined with other external factors, such as hindlimb-unloading procedure, is a complex process that can lead to different outcomes of infection, depending on the overall status of the host. This is the first report on the effects of AHCC on immune cells affected by spaceflight-like conditions.

We reported previously that oral administration of AHCC had no significant effects on survival of normally housed mice and that AHCC was highly beneficial in mice under immunosuppressed conditions induced by the hindlimb-unloading model (5). We showed, in the present study, that AHCC was very effective at enhancing the immune system of mice under normal conditions. An overall increase of the immune function

Table 3. Effects of AHCC on cytokine production measured in supernatants obtained from cultured peritoneal cells harvested from control and hindlimb-unloaded mice and stimulated with either LPS or *K. pneumoniae* antigen

Treatment Groups	LPS			<i>K. pneumoniae</i> Antigen		
	IL-6	IL-1 β	TNF- α	IL-6	IL-1 β	TNF- α
NC	740.1 \pm 8.8	443.4 \pm 80.3	714.9 \pm 9.9	723.9 \pm 20.2	64.5 \pm 12.1	697.3 \pm 169.7
PBS + HU	714.3 \pm 12.6	369.3 \pm 98.0	225.5 \pm 37.4	715.7 \pm 14.2	106.6 \pm 22.9	225.5 \pm 15.7
AHCC + HU	779.5 \pm 9.4*	400.8 \pm 96.5	748.9 \pm 98.2*	776.3 \pm 9.0*	227.2 \pm 68.2*	583.5 \pm 70.2*

Values are means \pm SE in pg/ml; $n = 6$ mice/group. Values obtained from nonstimulated cells were subtracted from values from stimulated cells. Peritoneal cells were harvested from mice under different conditions. Cytokine levels in pg/ml were detected in supernatants obtained from cultured cells stimulated with LPS (5 μ g/ml) and *Klebsiella pneumoniae* antigen (5 μ g/ml). * $P < 0.05$ between AHCC + HU and its control PBS + HU.

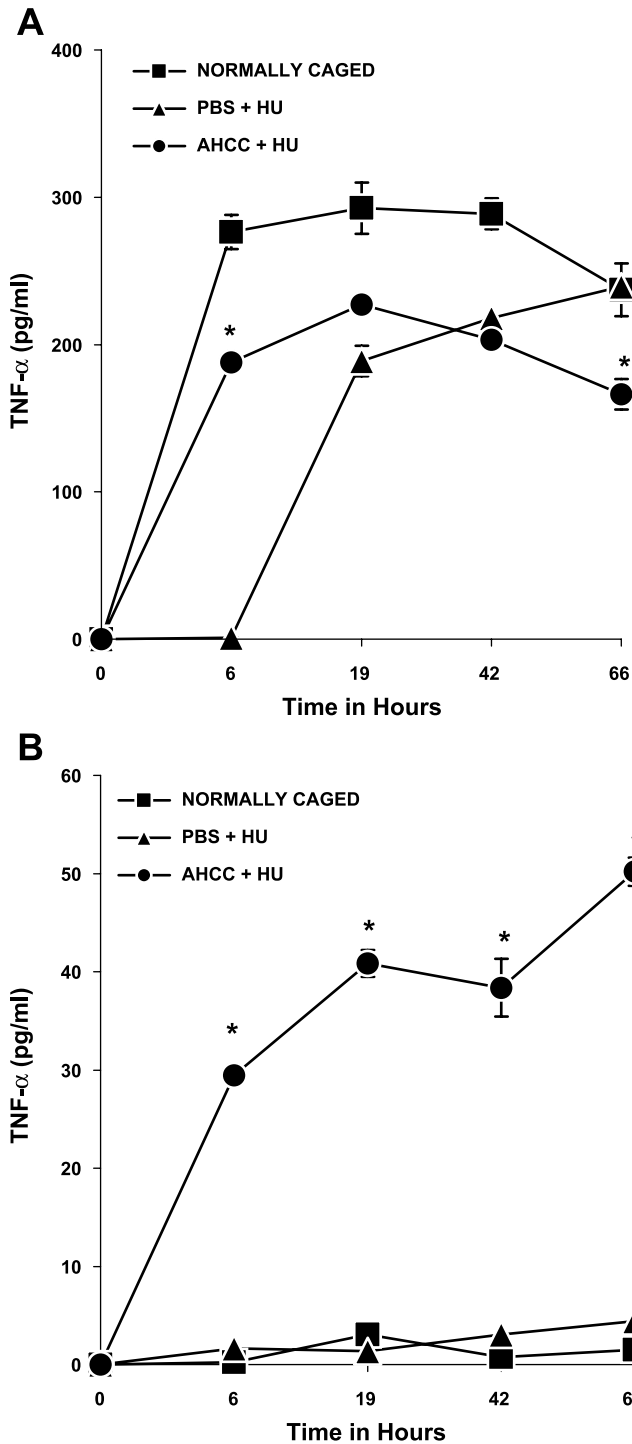


Fig. 7. Effects of oral administration of AHCC on TNF- α levels produced by peritoneal cells isolated from HU mice. Cytokine production was measured in supernatants obtained from cultured peritoneal cells with (A) and without (B) LPS stimulation. Data are means \pm SE. * $P < 0.05$.

above normal control values was seen in the AHCC group. AHCC also had an impact on the immune function of mice under hindlimb-unloading conditions. In fact, AHCC restored the function of the immune system, which was severely suppressed by hindlimb-unloading conditions, to values very close to values obtained from mice under normal conditions. There-

fore, whereas enhancement of the immune response in normal mice may have minimal beneficial effects on resistance to infection, restoration of the immune response to normal levels in immunosuppressed hindlimb-unloaded mice appears to be crucial for their survival.

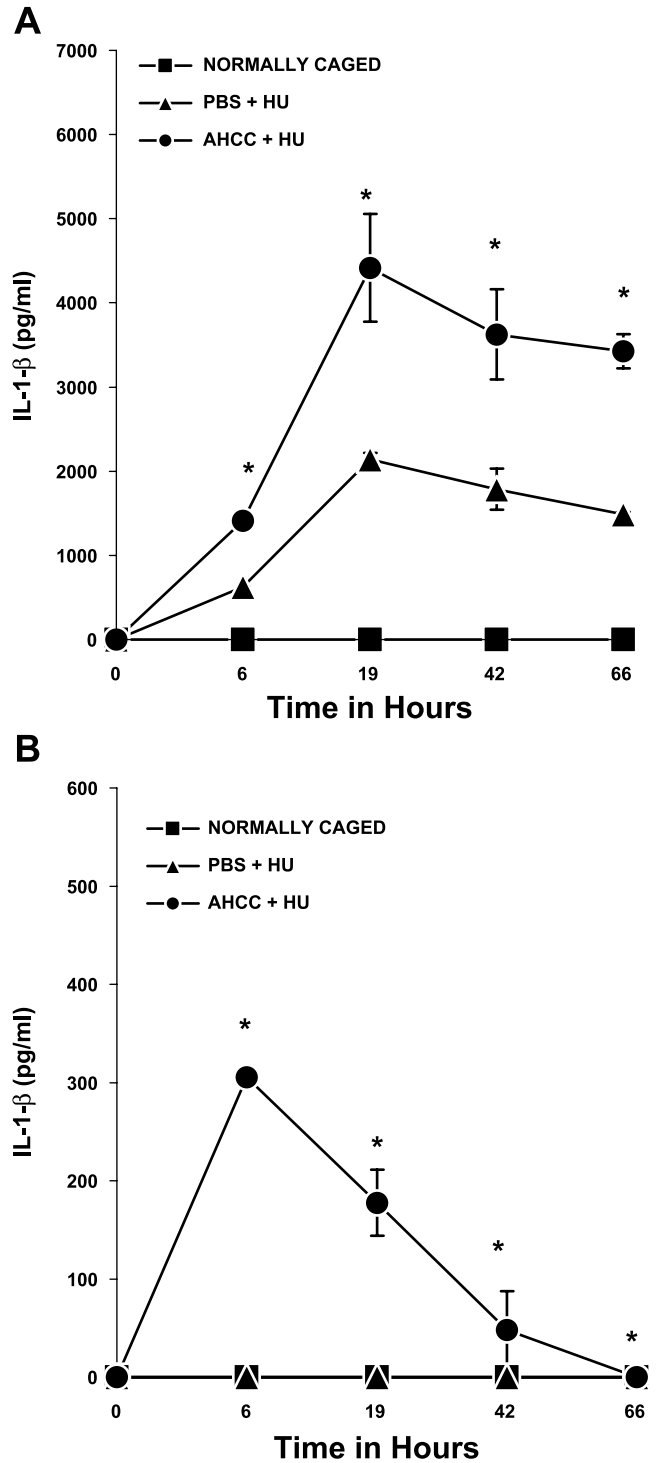


Fig. 8. Effects of oral administration of AHCC on IL-1 β levels produced by peritoneal cells isolated from HU mice. Cytokine production was measured in supernatants obtained from cultured peritoneal cells with (A) and without (B) LPS stimulation. Data are means \pm SE. * $P < 0.05$.

Our results are consistent with other studies showing that AHCC enhances the function of the immune system. It has been shown that AHCC increases natural killer cell activity (25), IL-12 (50), and nitric oxide production (28). Additional studies have shown that cytokine therapy reverses the immunosuppression seen in the hindlimb-unloading model (2, 3). Although other factors such as stress hormones and other molecules released during the hindlimb-unloading period may affect the outcome of infection, it seems clear that, in our model of intraperitoneal inoculation of bacteria, innate immunity plays a critical role in resistance to infection. The enhancement of peritoneal macrophages by AHCC may result in activation of several components of the innate immunity, including complement and acute phase proteins (32), inducing the synthesis and release of proinflammatory cytokines such as TNF, IL-1, IL-8, and IL-6. This activation of the immune system could contribute to the rapid clearance of bacteria from the system, resulting in reduction of mortality in mice treated with AHCC.

The molecular mechanisms involved in the enhancement of the immune system by AHCC under normal and hindlimb-unloading conditions remain unclear. However, it is probable that the high content of carbohydrates present in AHCC plays a role in the regulation of the immune system. In recent years, there has been a growing body of evidence that nonprotein antigens such as glycolipids can be recognized by T cells and that antigen presentation requires the binding to the CD1 molecule (37, 38). Also, experiments using model T-cell epitopes have demonstrated that carbohydrates can modulate T-cell responses in a variety of ways (13, 19). More in vitro studies are needed to clarify both the specific component(s) of AHCC that activates the immune system and the signaling pathways by which AHCC may be acting to upregulate the function of the immune system. In addition, several other factors must be considered in mice under hindlimb-unloading conditions that may have contributed to the immunological changes seen under hindlimb-unloading conditions. Factors involved may include those related to stress and hormonal changes, which may alter the immune system on one hand, and the effects of unloading and fluid shifts characteristics of this model, which may alter the physiology of the host on the other hand. Release of stress hormones such as corticosterone has been shown to inhibit immune responses and decrease resistance to infection (11, 12, 22). 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.

In conclusion, it seems clear that pretreatment followed by treatment with AHCC plays a protective role in resistance to infection. The overall role of AHCC may result in a decrease of tissue injury and reduction of mortality of hindlimb-unloaded mice infected with *K. pneumoniae*. It is also clear that AHCC is more effective when there are intrinsic defects in the immune system of the host, suggesting that AHCC may be useful to protect against microbial infections in immunosuppressed hosts. Further studies are required to fully define the potential and mechanism of AHCC as a countermeasure to minimize any detrimental effects in situations where the function of the immune system is compromised.

GRANTS

This study was funded in part by a grant from the Amino Up Chemical Company, Sapporo, Japan. The National Aeronautics and Space Administration (NASA) partially supported this work through NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute. Facilities utilized at Morehouse School of Medicine for these studies were supported by the National Institutes of Health under the Research Centers in Minority Institutions program award number 5G12 RR/AI03034.

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