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Research

Active hexose correlated compound activates immune function to decrease bacterial load in a murine model of intramuscular infection

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Abstract

Background: Infection is a serious, costly, and common complication of surgery and constitutes the principal cause of late death in patients undergoing surgery. The objective of this study was to clarify the mechanisms by which active hexose correlated compound (AHCC) increases survival in a murine model of intramuscular infection.

Methods: Food-deprived mice receiving either AHCC or excipient were infected with bacteria. Kinetics of bacterial load, white blood cell counts, cytokine levels, and antibody levels were compared between groups.

Results: AHCC-treated mice had reduced bacterial load at day 5 and cleared bacteria entirely at day 6. Levels of interleukin-12, tumor necrosis factor-α, and interleukin-6 peaked earlier in this group (day 3) compared with controls (day 5). Increased percentages of peripheral lymphocytes and monocytes and decreased numbers of polymorphonuclear cells were detected in the AHCC group.

Conclusions: AHCC appears to induce an early activation of the immune response, leading to an effective clearance of bacteria and rapid recovery. © 2008 Elsevier Inc. All rights reserved.

Keywords: Klebsiella pneumoniae; Mice; Cytokines; Humoral immunity; Peripheral leukocytes; Clearance

Surgical wounds and severe trauma in human beings and animals are very often associated with infection. Infection always has been a characteristic of human life and, in modern surgery, sepsis continues to be a significant problem for health care professionals worldwide [1]. Research aimed at finding new drugs and compounds has become a high priority. The use of modern aseptic procedures, prophylactic antibiotics, and immunostimulants including cytokines [2–5] has had limited success for the prevention and control of infection [6,7]. Nowadays, infection not only is the main cause of late death during trauma, but also because its incidence remains high, it represents a substantial burden for patients and health professionals in terms of morbidity, mortality, and economic cost [7,8].

Because of the limitations related to availability of human models, we use a mouse model of intramuscular infection [9]. This model simulates conditions that very often occur after trauma or surgery such as local microbial contamination and food deprivation [5,10]. Important components of this model are the nutritional and immunologic status of the host. Adequate nutrition and optimal function of the immune system are critical for resistance to infection. It is clear that the immune system plays a major role during infection and that its function is compromised seriously during severe trauma and surgical procedures [11–14]. Several studies have shown alterations in phagocytosis and antigen presentation in severely traumatized patients [12,14]. In this model, animals are deprived of food before and shortly after infection. Studies in animals have shown that acute starvation induces changes in immunologic parameters [15–18] including a reduction of circulating lymphocytes [15,18], decreased ability of spleen cells to respond to mitogens, and changes in normal T-helper/cytotoxic ratios [15].

We have been testing a natural compound that has shown a great potential to be used as a countermeasure to minimize detrimental effects in situations in which the function of the immune system is compromised by adverse conditions

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[9,19]. Active hexose correlated compound (AHCC) is an extract prepared from mycelia of the *Basidiomycete* mushroom (*Lentinula edodes*) [20]. Compounds isolated from mushrooms have been used successfully to revitalize hosts with impaired function of the immune response [20–24]. The positive effects of AHCC in the immune system of human beings and animals have been reported previously [25–30]. Several major advantages of AHCC include nontoxicity, good tolerance, no apparent side effects, and great potential for anticancer activity [20,26,29,31–35]. AHCC contains a mixture of polysaccharides, amino acids, and minerals. About 74% of AHCC is oligosaccharides and about 20% is of the α-1, 4 glucan type. The acetylated forms of the last group are low-molecular-weight oligosaccharides (~5 kd) that are believed to be responsible for the biological activities of AHCC [21,35,36].

We previously reported that oral administration of AHCC before and during the course of *Klebsiella pneumoniae* infection significantly improved the survival and prolonged the time of death of mice in the mouse model of intramuscular infection [9]. In an attempt to clarify the mechanisms involved in the protection of mice in this model, a time course of infection was established and several parameters were measured at various time points after infection. Although early activation of innate immunity and redistribution of peripheral cells appear to play a critical role in bacteria clearance, further investigation is necessary to fully define the mechanisms involved in the protection against bacterial infection induced by AHCC.

Materials and Methods

Bacteria

Klebsiella pneumoniae strain ATCC 43816 was obtained from American Type Culture Collection (Bethesda, MD). 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, each weighing 21–25 g) were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). Animals were housed in a quiet, isolated room with controlled temperature, light cycle, and having access to food and water ad libitum. Experimental procedures commenced after 1 week of acclimation. All experimental manipulations were approved by the Institutional Animal Care and Use Committee of the State University of New York University at Binghamton and were performed under the supervision of a veterinarian in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Administration of AHCC

Mice in the AHCC group received the compound for 1 week before and daily throughout the infection period. AHCC was administered by gavage to ensure that mice always received the entire dose. The dose was 1 g/kg, the reported maximum effective dose of the compound in animals [19]. Control mice received water, the excipient used for AHCC preparation.

Intramuscular infection model

Mice receiving AHCC and the control mice receiving the excipient were inoculated intramuscularly in the right thigh with 100 μL of phosphate-buffered saline (PBS) containing one lethal dose 20 (LD_{20}) of *K pneumoniae* (a dose at which 20% of animals will die). Mice were deprived of food but were allowed free access to water 24 hours before and 6 hours after infection. Under these circumstances, this model simulates some of the conditions that often occur after trauma or surgical procedures, including systemic contamination as a result of the wound [10].

Experimental groups

The following experimental groups were used for each course of infection: (1) infected mice treated with AHCC (AHCC group) and (2) infected mice treated with excipient (control group). Organ bacterial load, plasma levels of cytokines, differential white blood cell counts, and antibody levels were determined at different time points after infection.

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 to TSB and incubated at 37°C with shaking for aeration (Max Q Mini 4000 bench-top orbital shaker; Barnstead International, Dubuque, IA). Cultures were harvested when the absorbance of sample at 600 nm (A_{600}) reached .5 (DU530 spectrophotometer; Beckman Coulter, Fullerton, CA), which correlated previously with known bacterial counts. Bacteria were washed by pelleting at 1,900 × g, resuspended, and diluted to the appropriate dose in PBS, pH 7.4. Bacterial density was confirmed by plating 100 μL of appropriate 10-fold serial dilutions of bacteria cultures on tryptic soy agar (TSA) plates and the density was recorded as colony forming units per milliliter (CFU/mL). The lethal doses for 20% of mice infected with *K pneumoniae* were determined in previous studies by using the Reed-Muench estimation [9]. Proper concentrations were confirmed by plating 3 consecutive 10-fold dilutions of the suspension on TSA.

Bacterial organ load and differential white blood cell count determinations

Under deep anesthesia (isofluorane), lungs and livers were removed aseptically from AHCC-treated and control mice and placed in PBS. Tissues were weighed and homogenized completely by using a pellet pestle (Sigma, St. Louis, MO) to allow the release of bacteria. Whole blood was obtained by cardiac puncture with sodium citrate as an anticoagulant. One hundred microliters of the diluted blood and homogenized tissues were plated on TSA (three 1:10 serial dilutions) and incubated for 18 to 24 hours at 37°C. The CFU/mL measurement was determined by averaging the number of colonies counted on the 3 serially diluted TSA plates. For differential white blood cell counts, 5 μL of citrated blood smears were prepared on microscope slides, fixed, and stained using Hemacolor (EMD Chemical, Inc.,

Gibbstown, NJ). White blood cells were counted under 100× magnification, oil immersion microscopy. Plasma was obtained after centrifugation of citrated blood at 1,000 × g for 10 minutes and stored at –20°C until use.

Cytokine and chemokine analysis

Plasma levels of interleukin (IL)-12 p70, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-10, IL-6, and monocyte chemoattractant protein (MCP)-1 were measured simultaneously using the Cytometric Bead Array Multiplex Assay System (Mouse Inflammation kit; BD Biosciences, San Diego, CA) following the manufacturer's instructions. Briefly, 50 μL of cytokine capture beads slurry was mixed with 50 μL detection antibodies and incubated with 50 μL of either known concentrations of recombinant standards or plasma samples in a 96-well Multiscreen BV 1.2-μm filter plate (Millipore, Burlington, MA) for 2 hours at room temperature in the dark. Beads were washed with 200 μL wash buffer and vacuum aspirated using the Multiscreen Vacuum Manifold (Millipore Corp., Burlington, MA, USA). Beads were resuspended in 120 μL wash buffer and agitated with an MTS 2/4 Digital Microtiter Shaker (IKA Works; VWR, West Chester, PA) before acquisition. Analytes were detected and measured by flow cytometry using the FACSArray Bioanalyzer with the BD FACSArray Software Experiment Wizard FACSArray Bioanlyzer [(BD Biosciences, San Diego, CA)] and analyzed with BD [Cytometric Bead Array (CBA)] Software version 1.4 (BD Biosciences, San Diego, CA).

K pneumoniae *antigen preparation*

An isolated colony of *K pneumoniae* was grown in 250 mL of TSB medium for 4 hours at 37°C with shaking for aeration. Bacterial cells were washed twice in PBS at 1,900 × g for 10 minutes and resuspended in 10 mL of distilled water. The bacterial suspension was sonicated with 10 repeated 30-second 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 minutes at 4°C. The supernatant containing the antigen was filtered (.22 μm; Sigma), aliquoted, and stored at −80°C until use. An aliquot was removed for protein determination using a standard Pierce bicinchoninic acid assay (Pierce, Rockford, IL).

Enzyme-linked immunosorbent assay for detection of immunoglobulin G and immunoglobulin M antibodies to K pneumoniae

Specific immunoglobulin (Ig)G and IgM anti–*K pneumoniae* antibodies levels were detected in plasma by using an enzyme-linked immunosorbent assay (ELISA) as previously described with some modifications [9]. Briefly, 96-well Nunc-Immuno MaxiSorp Surface microtiter plates (BioWorld Laboratory Essentials, Dublin, OH) were coated with 100 μL of a solution containing 5 μg/mL of *K pneumoniae* antigen in coating buffer (.05 mol/L carbonate/bicarbonate, pH 9.6; Sigma). Plates were kept overnight at 2°C to 8°C. After washing 3× with washing buffer (PBS, pH 7.4, .05% Tween 20), nonspecific sites were blocked with 275 μL of blocking buffer (1% bovine serum albumin, 5% sucrose in PBS, pH 7.4) for 1 hour at room temperature and washed as described earlier. Plasma samples were diluted in 1% bovine serum albumin in PBS (reagent diluent) at 1:200 for IgG and 1:100 for IgM detection and 100 μL of this dilution were added and incubated at 37°C for 2 hours. Secondary antibodies conjugated to horseradish peroxidase were diluted in reagent diluent; 100 μL of a 1:20,000 dilution of goat anti-mouse IgM (Sigma) and 100 μL of a 1:40,000 dilution of rabbit anti-mouse IgG (Sigma) were plated and incubated at 37°C for 2 hours; 100 μL of substrate, tetramethylbenzidine kit (R&D Systems, Minneapolis, MN) were added to washed wells and developed at room temperature for 20 minutes. Reaction was stopped with 50 μL of 2 N sulfuric acid (H_2SO_4) and optical density was determined at 450 nm using an Elx808 Ultra Microplate Reader (Biotek Instruments, Inc. Wanoosky, VT).

Statistical analysis

Groups of 4 to 8 mice in each group for each time point were used in each experiment. At least 2 separate experiments for each determination were performed in this study. Data were analyzed by using Statview 5.0.1 (SAS Institute Inc., Cary, NC) with α set a priori at a P value of less than .05. The Student t test was used to test statistical significance between any 2 groups. Analysis of variance followed by a post hoc Fisher test was used to test statistical differences between more than 2 groups and among groups at different time points.

Results

Oral administration of AHCC promotes the clearance of bacteria from blood and organs in food-deprived and K pneumoniae*–infected mice*

We have shown in previous studies that AHCC increases the survival of mice in a rodent model of intramuscular infection [9]. To assess the role of AHCC on the progress of infection in this model, AHCC-treated and control mice were infected with sublethal doses of *K pneumoniae*, and the presence of bacteria was determined in blood and organs in successive days after infection. Fig. 1 shows the kinetics of bacterial organ load of food-deprived and infected mice receiving either AHCC or the excipient water. Mice receiving AHCC started to remove bacteria at day 5, and at day 6 no traces of bacteria could be found circulating in blood (Fig. 1A), or present in the lungs (Fig. 1B) or livers (Fig. 1C). In contrast, control mice receiving water significantly increased the number of bacteria detected in all 3 tissues at day 5 ($P < .05$) and continued to increase at day 6 ($P < .05$). Although AHCC-treated mice recovered completely and overcame infection, control mice looked extremely sick and some of them died at these last time points tested (days 5 and 6).

AHCC induces a strong early immune response mediated by IL-12, TNF-α, IL-6 cytokines, and MCP-1 chemokine in the surgical infection rodent model

Proinflammatory cytokines and chemokines are known to be involved in the clearance and control of a variety of pathogens including *K pneumoniae* [37–40]. To assess the role of these molecules on clearance of bacteria in this

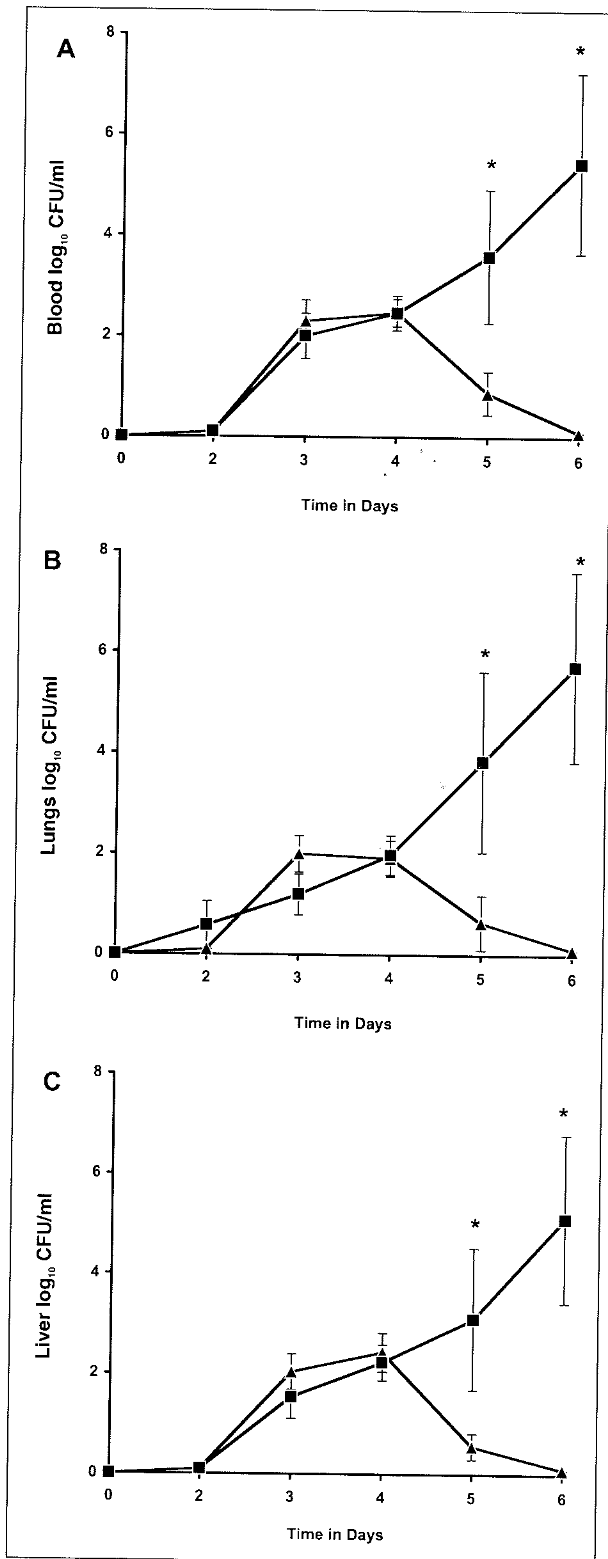

Fig. 1. Effect of oral administration of AHCC on clearance of bacteria of food-deprived mice infected with *K pneumoniae* in the intramuscular model of infection. Blood and organs (lungs and livers) were removed aseptically and bacterial load was determined in these tissues at different time points after infection (n = 4–8 mice per treatment/per time point). Data are expressed as the means ± SE of $\log_{10}$ CFU/mL of bacteria at days 0, 2, 3, 4, 5, 6 (*$P < .05$). (A) Blood, (B) lungs, and (C) liver. ▲, AHCC; ■, control.

model, levels of cytokine production were measured in plasma samples at different time points after infection (Fig. 2). Twenty-four hours after infection (day 2), levels of all proinflammatory molecules were relatively low in both groups except for MCP-1 in the AHCC group, which had about a 2.5-fold increase compared with controls (Fig. 2A). Major statistical differences were seen at day 3, levels of IL-12, TNF-α, and IL-6 were increased significantly in the AHCC group ($P < .05$) compared with controls (Fig. 2B) at this time point. Levels of cytokines decreased significantly in the AHCC group at day 4 except for IL-6 (Fig. 2C), and returned to normal levels at day 5 (Fig. 2D). In contrast, levels of cytokines in control mice were relatively low until day 5 in which levels of TNF-α, IFN-γ, MCP-1, and IL-6 had a significant increase (Fig. 2D).

AHCC modulates the whole blood lymphocyte, polymorphonuclear, and monocyte cell counts in this model

Immune cells play a critical role in host defense against bacterial and other infections. To investigate the role of AHCC on the distribution of circulating white blood cells, the percentages of these types of cells were determined in whole blood isolated from AHCC and control mice. At day 2, a significant decrease of the percentages of polymorphonuclear (PMNs) with a significant increase in the percentages of monocytes was observed in the AHCC group (Fig. 3A). At day 3, a significant increase in the percentage of total white blood cells ($P < .05$) and lymphocytes ($P < .05$) was found in the AHCC group (Fig. 3B). However, the percentage of circulating PMNs consistently was lower in the AHCC group throughout the course of infection. Similar trends to those observed at day 3, but without statistical significance, were observed at day 4 (Fig. 3C) and day 5 (Fig. 3D).

*AHCC has no effect on specific anti–*K pneumoniae *antibody production in this murine model of intramuscular infection*

To assess the role of AHCC on antibody production, specific IgG and IgM antibodies against *K pneumoniae* were tested by ELISA in plasma samples collected from AHCC-treated and control animals (Table 1). Low levels of anti–*K pneumoniae* IgG were detected until day 5 in both groups; these levels increased substantially at day 6. Although results showed an early increase of these antibodies in the AHCC group at days 2 and 3 followed by a decrease in subsequent days compared with controls, no statistical significance was found between the 2 groups at any time point. In contrast to IgG-specific antibodies, IgM antibodies started to increase at day 5 and substantially greater levels were reached at day 6 in both groups. Levels of this antibody were consistently lower in the AHCC group during the course of infection, but once again no statistical significance was found between these 2 groups.

Comments

One of the most important aspects in the care of surgical patients continues to be the prevention and diligent control of microbial contamination. Among postoperative compli-

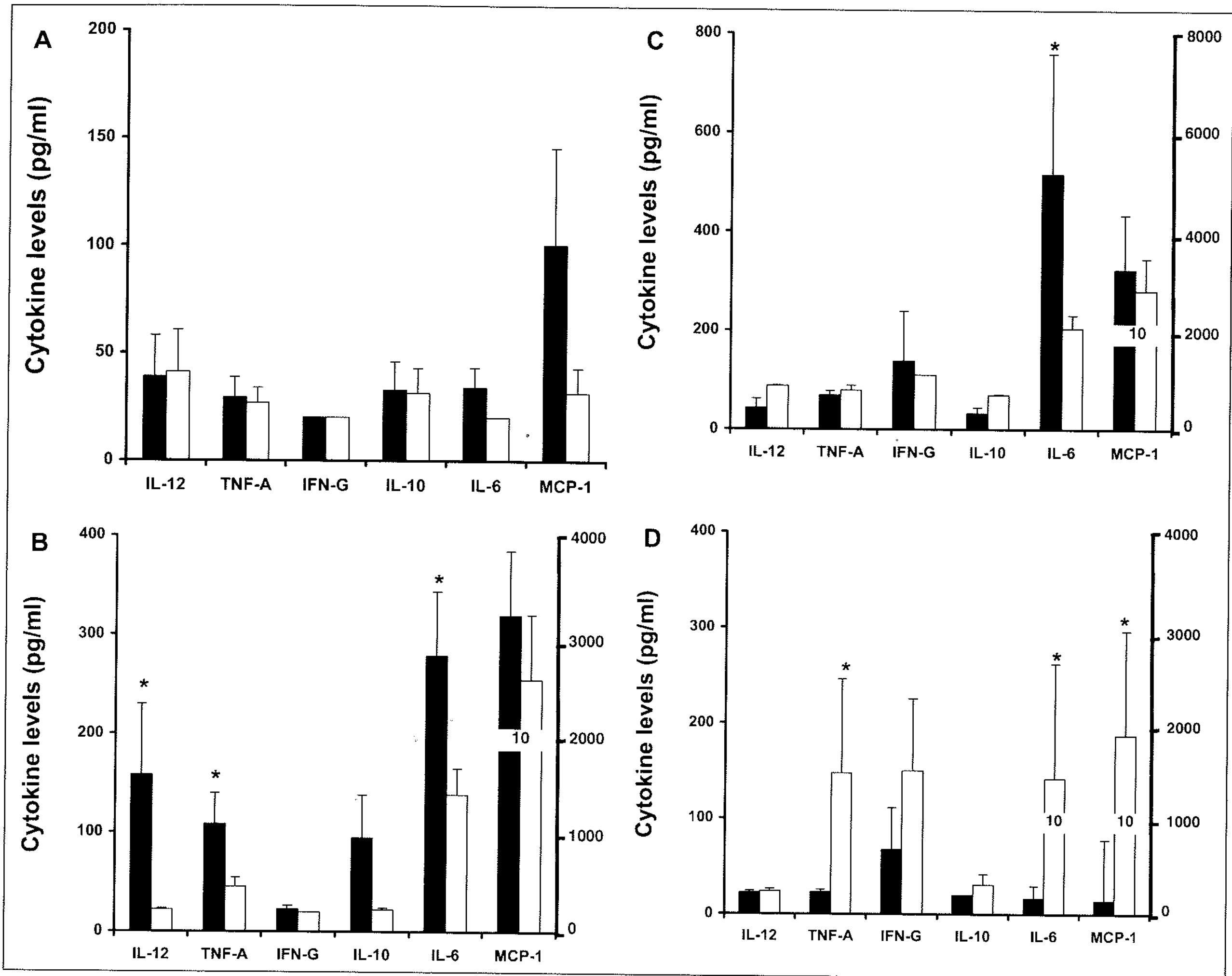

Fig. 2. Effect of oral administration of AHCC on the kinetics of proinflammatory cytokines-chemokines production of food-deprived mice infected with *K pneumoniae* in the intramuscular model of infection (n = 4–8 mice per treatment/per time point). Values are means ± SE of the concentrations of proinflammatory cytokines expressed in pg/mL (*$P < .05$). Proinflammatory cytokines-chemokines were measured in plasma at days (A) 2, (B) 3, (C) 4, and (D) 5 after infection. Levels of MCP-1 in B, C, and D are 10 times less than measured concentrations so that levels of this chemokine could fit in the graphics (an additional y-axis has been added to the right side of the figures). Basal levels of these cytokines obtained from mice before infection were subtracted from values after infection. ■, AHCC; □, control.

cations, infection constitutes the major limiting factor for recovery of patients after surgery or severe trauma [41]. Malnutrition is an important risk factor and has been associated with a higher incidence of postoperative infections [18,42,43]. Abstinence from food and drink before and after surgery is a common protocol to reduce the complications associated with anesthesia such as the inhalation of stomach contents into the lungs during and after the procedure. In our mouse model of intramuscular infection, food deprivation is a critical factor because of its impact on the immune system. Several studies have shown that acute starvation induces a transient impairment of the immune system with full recovery after the refeeding period [15–18]. This transient compromise of the immune system could be a critical factor for the control and elimination of pathogens. We previously showed that food-deprived mice were extremely susceptible to infection and that this susceptibility was reversed by AHCC [9]. The objective of the present study was to explore the probable mechanisms by which AHCC acts on the host to improve the resistance to *K pneumoniae* infection. To accomplish this objective, we evaluated the cellular and humoral response of the host and its correlation with clearance of bacteria during the course of infection.

Results from our study suggest that AHCC plays an important role in the clearance of bacteria by inducing an early and balanced activation of innate immunity. The first proinflammatory chemokine induced by AHCC at day 2 was MCP-1. This chemokine has been reported to have chemoattractant activity for monocytes, T cells, mast cells, and basophiles [44]. Day 3 appears to be critical in this model, mice receiving AHCC produced significantly higher levels of critical proinflammatory cytokines such as IL-12, TNF-α, and IL-6. Host defense against bacterial infection requires the generation of a vigorous inflammatory response. This process depends on an intricate balance of the production of mediators, cytokines, and chemokines. Production of these molecules has to be sufficient to control the replication and dissemination of bacterial pathogens [45],

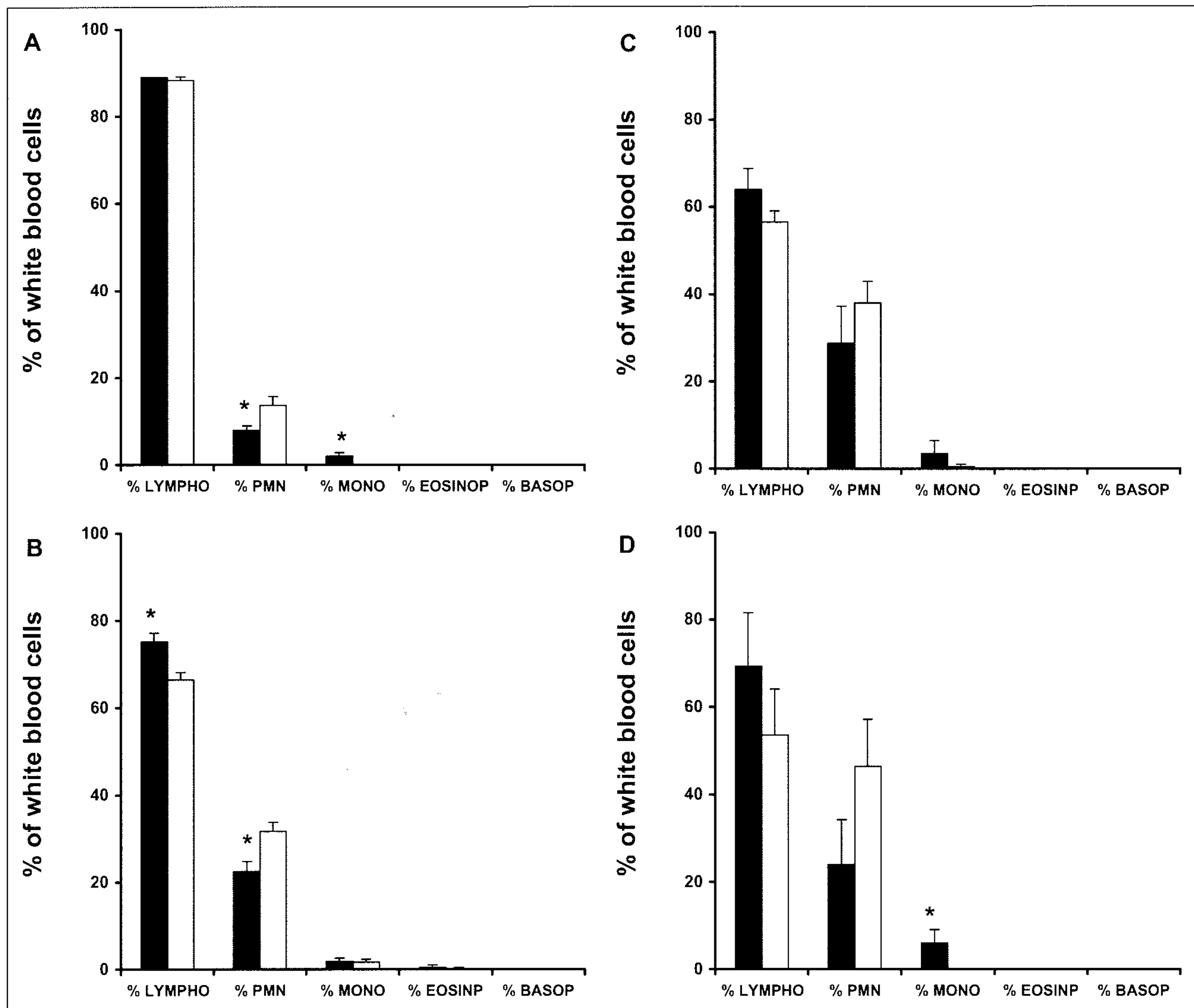

Fig. 3. Effects of oral administration of AHCC on the concentration of white blood cells circulating in blood of food-deprived mice infected with *K pneumoniae* in the intramuscular model of infection (n = 4–8 mice per treatment/per time point). Data are expressed as the means ± SE of the percentages of white blood cells counted in blood (*$P < .05$). Data were obtained at days (A) 2, (B) 3, (C) 4, and (D) 5 after infection. Data obtained before infection were as follows: % LYMPHO, percentage of lymphocytes (83.2 ± 2.6); % PMN, percentage of PMNs (13.75 ± 1.38); % MONO, percentage of monocytes (2.5 ± .87); % BASOP, percentage of basophiles (.5 ± .8); and % EOSINOP, percentage of eosinophiles (0). ■, AHCC; □, control.

but not excessive at the point to induce the negative characteristics associated with sepsis [44]. Levels of production of cytokines returned to normal at day 5, coinciding with the total clearance of bacteria and full recovery of mice receiving AHCC. In contrast, control mice receiving the excipient produced very high levels of cytokines at day 5, coinciding

Table 1
Kinetics of anti-*K pneumoniae* antibody levels measured in plasma samples obtained from food-deprived mice receiving either AHCC or excipient at different time points after infection in the intramuscular model of surgical soft-tissue infection

Treatment	Day 2	Day 3	Day 4	Day 5	Day 6
IgG levels (OD) × 10^{-3}					
AHCC	271 ± 128	332 ± 127	104 ± 25	242 ± 95	1,391 ± 245
Control	229 ± 120	274 ± 60	290 ± 208	323 ± 55	1,906 ± 501
IgM levels (OD) × 10^{-3}					
AHCC	22 ± 5	301 ± 41	214 ± 40	523 ± 286	1,805 ± 220
Control	43 ± 7	335 ± 35	241 ± 32	648 ± 142	1,872 ± 189

Values are means ± SE given as optical density (OD); n = 4–8 for each treatment for each time point. Plasma samples were tested for specific IgG and IgM antibodies using an ELISA. Background levels of antibodies detected in noninfected mice were subtracted from levels of infected mice.

with high numbers of bacteria detected in organs and extreme sickness observed in those mice. These results suggest that these high levels of proinflammatory cytokines observed in the control group were the result of massive dissemination of bacteria resulting in sepsis. The sepsis syndrome is an acute state characterized by multiorgan dysfunction, coagulopathy, shock, and eventually death [44]. Research over the past 20 years has helped to reinforce the importance of inflammatory response in clearing *K pneumoniae* infection. Any interference with a rapid response mediated by cytokines such as IL-12, TNF-α, and IL-6 results in severe disease and dissemination of infection [46]. In our model, it appears that AHCC overcame the deleterious effects on the immune system caused not only by acute starvation [15–18] but also by the pathogen itself [46]. One of the major mechanisms for *K pneumoniae* to survive and disseminate into the host is to suppress the immune system and its capsule appears to play an important role in this mechanism [46]. Increased numbers of peripheral lymphocytes and macrophages with a decrease of PMNs was observed in the AHCC group at day 2 and became more evident at day 3. A characteristic complication of *K pneumoniae* infection is its ability to disseminate to the peripheral blood, resulting in a systemic infection in addition to localized pulmonary infection [47]. It also is important to mention the critical role that the recruitment and activation of neutrophils plays in the host defense against acute bacterial infection [45]. Our results suggest that AHCC helps to avoid bacterial dissemination by increasing immune cells such as lymphocytes and monocytes that are important in the clearing of circulating bacteria and also helps to redistribute immune cells that are very important at the local organ level such as PMNs, especially neutrophils. AHCC appears to have no effect on humoral immunity in this particular model because no significant differences were detected in antibody levels at any time point after infection. These results are consistent with similar studies on the effects of AHCC on the immune system using different models [9,19]. This could be because humoral immunity is activated late during the course of infection and early activation of innate immunity is responsible for the clearance of bacteria and resolution of infection.

The results of this study showed that AHCC was very effective at improving the outcome of food-deprived mice by providing an early activation of the immune system. It also was clear that food-deprived mice receiving the excipient were not able to overcome the effects of food deprivation because those mice were not able to mount an adequate immune response. It appears that the conditions of the host at the time of infection are crucial to the final outcome of infection. Timing of food deprivation appears to be critical to resistance to infection as well. It has been shown that resistance to fungal infection was diminished profoundly when the period of food deprivation ranged from 48 hours before to 24 hours after infection. Food deprivation initiated immediately or at 24 or 48 hours after infection resulted in moderate or no change at all in resistance to infection [48]. Similarly, no changes in mortality occurred when food deprivation was initiated 72 hours before infection but finished at the time of infection [48]. These findings are consistent with previous studies in which AHCC restored the immune system of mice affected by exposure to adverse conditions [19,49]. Based on previous and present results, it appears that AHCC is very effective when the immune system is compromised. AHCC did not significantly improve the immune function or survival in normal mice infected with bacteria [19,49].

Our results are consistent with previous studies showing that AHCC has beneficial effects on the function of the immune system [21,25–36,50,51] and in diseases such as cancer [21,25,29,32,52] and diabetes [33], and infections [19,50,51,54]. It has been shown that this compound enhances several aspects of the immune response including natural killer cell activity [unpublished data] and cytokine production [29,31]. At the same time, AHCC has shown positive effects in cancer patients by prolonging survival and improving the prognosis and quality of life of these patients [21,52]. In animal models, it has been reported that AHCC increases the number of tumor Ag-specific CD8(+) T cells, the frequency of tumor Ag-specific IFN-γ–producing CD8(+) T cells, and the number of natural killer and gamma-delta T cells [32]. Finally, AHCC was shown to increase resistance to infection in different rodent models in which the immune system is compromised [19,50,51,53].

The mechanisms by which AHCC increases survival in food-deprived hosts remain unclear. Our findings suggest that improvement in resistance to infection is the result of enhancement of the innate immune response rather than a direct cytotoxic effect of AHCC on bacteria. It is worth exploring other mechanisms involving the neuroendocrine system that appear to be linked with the effects of food restriction [41,54–56]. It has been shown that food restriction increases the production of corticosterone levels [56] and affects the production of catecholamines [55]. The effects of food deprivation on corticosterone and catecholamines could be enhanced by stress related–trauma and the imminence of surgical procedures. These could be key factors for suppression of the immune system and resistance to infection. Another possibility to consider in this model is related with the direct effect of catecholamines on bacterial growth. In vitro studies have shown that catecholamines, especially noradrenaline, enhance the growth of a variety of bacterial pathogens including *K pneumoniae* [57–59]. It is possible that AHCC modulates not only the function of the immune system but also the secretion of these hormones, but proof of this possibility will require additional experimentation in the future.

Overall, pretreatment followed by continued treatment with AHCC protects mice in this model from death and overcomes the effects of acute food deprivation and infection. AHCC may be effective when there are intrinsic defects in the immune system of the host by providing a balanced enhancement of the innate immune response. It is important to emphasize that this compound has not been tested for treatment. We are currently using it as a prophylactic measure only; therefore, the use of AHCC to treat bacterial infections remains to be investigated.

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