

Oral Administration of Active Hexose Correlated Compound Enhances Host Resistance to West Nile Encephalitis in Mice^{1,2}

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Abstract

West Nile virus (WNV) poses a serious threat to public health, especially to the elderly and the immuno-compromised. Neither vaccines nor treatments are available for humans. Active hexose correlated compound (AHCC) is an extract of *Lentinula edodes* of the *Basidiomycete* family of fungi rich in α -glucans. In this study, we evaluated the effect of AHCC on host susceptibility in the murine model of WNV infection. Mice orally administered with AHCC (600 mg/kg) every other day for 1 wk before and at d 1 and 3 postinfection were assessed using viremia levels, survival rate, and protective immunity. AHCC administration in young (6- to 8-wk-old) mice attenuated viremia and mortality following lethal WNV infection. WNV-specific IgM and IgG production and $\gamma\delta$ T cell expansion were also enhanced in these mice. Aged (21- to 22-mo-old) mice were more susceptible to WNV infection than young mice, partially due to the dysfunction of $\gamma\delta$ T cell subsets. AHCC administration in aged mice enhanced the protective $V\gamma 1^+$ T cell response as well as WNV-specific IgG but not IgM antibodies production. AHCC administration in aged mice attenuated viremia levels but led to no difference in mortality rate. Overall, our data suggests that AHCC enhances protective host immune responses against WNV infection in young and aged mice. Dietary supplementation with AHCC may be potentially immunotherapeutic for WNV-susceptible populations. *J. Nutr.* 139: 598–602, 2009.

Introduction

West Nile virus (WNV),⁹ a single-stranded, mosquito-borne RNA virus-induced neurological disease has become a public health concern in North America since its arrival in 1999 (1,2). Human infection symptoms include fever, headache, myalgia, meningitis, and encephalitis (2). Severe neurological disease (meningitis or encephalitis) that required long-term rehabilitation and death has been observed in over 30% of the confirmed WNV cases and occurred with an increased frequency in the

elderly and immuno-compromised patients (3,4). Treatment is currently nonspecific and supportive (2). The murine model has been an effective in vivo experimental model to investigate viral pathogenesis and the host immunity in humans. Following the initial subcutaneous or intraperitoneal injection in mice, WNV induces a systemic infection and eventually invades the central nervous system (CNS). The severity and symptoms observed in mice mimic those in severe cases in humans infected by mosquito bites (5,6). Studies from animal models suggest that type 1 interferons (IFN), $\gamma\delta$ T cells, and early humoral responses have been shown to be important in limiting viremia and virus dissemination into the CNS (6–10). Moreover, the proinflammatory cytokine response induced upon WNV infection could modulate the blood brain barrier permeability, which in turn may enable viral entry into the brain and induce lethal encephalitis (11,12). Therefore, enhancing protective immune responses and attenuating proinflammatory responses are the potential targets in immunotherapy of WNV encephalitis.

Active hexose correlated compound (AHCC), an extract prepared from mycelia of the *Basidiomycete* mushroom (*Lentinula edodes*), contains oligosaccharides, amino acids, lipids, and minerals (13). About 74% of AHCC are composed of oligosaccharides, which are enriched in low molecular weight acetylated

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⁹ Abbreviations used: AHCC, active hexose correlated compound; CNS, central nervous system; IFN, interferon; LD₁₀₀, lethal dose 100%; PE, phycoerythrin; PFU, plaque-forming unit; Q-PCR, quantitative PCR; WNV, West Nile virus; WNVE, West Nile virus envelope.

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α -1,4-glucans. The biological effects of AHCC have been attributed to its glucan fraction (14,15). AHCC is well tolerated and is largely free of adverse effects. It has been shown that AHCC has a positive effect on the immune system of humans (14) and rodents (15–17). Oral administration of AHCC has been shown to enhance NK activity and $\gamma\delta$ T cell expansion in human and rodents with malignancies (15,18). In the trinitrobenzenesulfonic acid model of colitis in rats, AHCC acts as a prebiotic and is antiinflammatory (19). Moreover, AHCC treatment enhances host resistance to *Klebsiella pneumoniae* and influenza virus infection (20,21). In this study, we evaluated the effect of AHCC treatment on host immune responses of young and aged mice infected with WNV.

Materials and Methods

Mice. Young (6- to 8-wk-old) and aged (21- to 22-mo-old) C57BL/6 mice were purchased from Jackson Laboratories and the National Institute of Aging, respectively. Groups were age- and sex-matched for each experiment and were housed under identical conditions. All animal experiments were approved by the Animal Care and Use Committee at Colorado State University.

Infection of mice. A total of 1000 plaque-forming units (PFU) corresponds to lethal dose 100% (LD_{100}) in young mice and 100 PFU represents LD_{100} in aged mice for WNV isolates CT 2741 or NY99, as described in our previous work (9,22). To better evaluate the AHCC effect on host susceptibility, young or aged mice were inoculated intraperitoneally with 800 PFU or 80 PFU of WNV isolate NY99, a dose close to their respective LD_{100} . Infected mice were monitored twice daily for morbidity until d 28 postinfection. When mice showed signs of morbidity (obvious severe illness, unable to right self when tipped on side or back, obvious extreme weight loss), they were immediately collected and killed by CO_2 .

Oral feeding with AHCC. AHCC (Amino Up) was dissolved in distilled water. Mice were orally administered with AHCC (600 mg/kg) by gavage every other day for 1 wk before infection and at d 1 and 3 postinfection in a 200- μ L volume. Control mice received 200 μ L distilled water. Similar doses of AHCC were used previously based on body weight and did not produce toxic effects in mice (18–20).

Quantitative PCR for viral load and cytokine production. RNA was extracted from blood using an RNeasy extraction kit (Qiagen). cDNA was then synthesized using the ProSTAR First-strand RT-PCR kit (Stratagene). The sequences of the primer-probe sets for the WNV envelope (WNVE) and IFN α and PCR reaction conditions were described previously (12,23). Probes contained a 5' reporter, 6-carboxy-fluorescein, and a 3' quencher, TAMRA (Applied Biosystems). The assay was performed on an iCycler (Bio-Rad). To normalize the samples, the same amount of cDNA was used in a quantitative PCR (Q-PCR) for β -actin. The ratio of the amount of amplified gene compared with the amount of β -actin cDNA represented the relative levels in each sample.

ELISA. We determined WNV-specific IgM and IgG at d 4 postinfection by ELISA as described previously (24). Microtiter plates were coated with recombinant WNVE protein (24) overnight at 4°C at 100 ng/well. Sera were added at 1/30 or 1/100 dilution in PBS with 2% bovine serum albumin and incubated for 1 h at room temperature. This was followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) at a dilution of 1/1000 in PBS-Tween for 1 h. Color was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) and intensity determined at an absorbance of 405 nm using a spectrophotometer.

Flow cytometry. Splenocytes were stained with fluorescein isothiocyanate-conjugated hamster anti-mouse TCR δ (BD Biosciences). To identify $\gamma\delta$ T cell subpopulations, this was followed by incubation with biotinylated

monoclonal antibody 2.11 for V γ 1 or monoclonal antibody UC3 for V γ 4, and finally staining with phycoerythrin (PE)-streptavidin (e-Bioscience) (22). Cells were fixed in 1% paraformaldehyde in PBS and examined using a Coulter XL instrument (Beckman Coulter). Data were analyzed using FCS express 2 (De Novo Software).

Cytokine assays. We measured IFN γ production of splenic $\gamma\delta$ T cells by intracellular cytokine assay as previously described (9,22). Briefly, splenocytes were stimulated with 50 μ g/L phorbol myristate acetate (Sigma-Aldrich) and 500 μ g/L ionomycin (Sigma-Aldrich) for 5 h at 37°C. Golgi-plug (BD Biosciences) was added during the final 3.5 h. Cells were harvested, stained with antibody for TCR $\gamma\delta$, fixed in 2% paraformaldehyde, and permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN γ or PE-conjugated rat IgG $_1$ (BD Biosciences). Cells were analyzed using a Coulter XL instrument. Sera inflammatory cytokines were assessed using mouse inflammation kit (BD Biosciences) by a FACSArray analyzer (BD Biosciences).

Statistical analysis. Survival curve comparisons were performed using Prism software (GraphPad Software) statistical analysis, which uses the log rank test (equivalent to the Mantel-Haenszel test). Values for viral burden, cytokine production, antibody titer, and T cell number experiments were presented as means \pm SEM. *P*-values of these experiments within each age group were calculated with an unpaired Student's *t* test or Mann-Whitney test. Significance was accepted at *P* < 0.05.

Results

Susceptibility of young mice to WNV. AHCC-administered young mice (54% survival) were more resistant to lethal WNV infection than controls (21% survival) within a 4-wk interval (Fig. 1; *P* < 0.05). We further measured viral load by Q-PCR. Around the peak of infection in the blood (d 4), viremia in AHCC-treated mice decreased to 19% of control levels (Fig. 1; *P* < 0.05). These data suggest that AHCC treatment attenuates virus replication in the periphery during WNV infection.

Humoral and innate immune responses in young mice. B cell-mediated humoral immune responses are critical for host defenses against disseminated infection by WNV (6,25). We next examined WNV-specific IgM and IgG levels in the sera following WNV infection (Table 1). At d 4 postinfection, there were higher levels of WNV-specific IgM (*P* < 0.05) and IgG (*P* < 0.05) in AHCC-treated mice than in controls, suggesting an increase on humoral responses with AHCC treatment during WNV infection.

Type 1 IFN is critical in controlling dissemination of WNV (7,8). Q-PCR results revealed that at d 1 postinfection, IFN α production in AHCC-treated mice ($0.97 \pm 0.18 \times 10^{-3}$; *P* > 0.05) did not differ from control mice ($0.68 \pm 0.23 \times 10^{-3}$). Further, the production of proinflammatory cytokines, including tumor necrosis factor- α , interleukin-6, and interleukin-12, in serum of the 2 groups did not differ at the peak of infection in the blood (d 4; data not shown).

$\gamma\delta$ T cell expansion in young mice following WNV challenge. We previously showed that $\gamma\delta$ T cells, a nonclassical T cell subset, respond rapidly following WNV infection, limiting viremia and invasion of the CNS and protecting the host from lethal encephalitis (9). AHCC treatment enhances $\gamma\delta$ T cell number (18). Here, the expansion of splenic $\gamma\delta$ T cells at d 3 postinfection in AHCC-treated mice increased 59% as measured by percentage (Table 1; *P* < 0.05) and 83% by cell number (Table 1; *P* < 0.05). The percentage of IFN γ -producing $\gamma\delta$ T cells in AHCC-treated

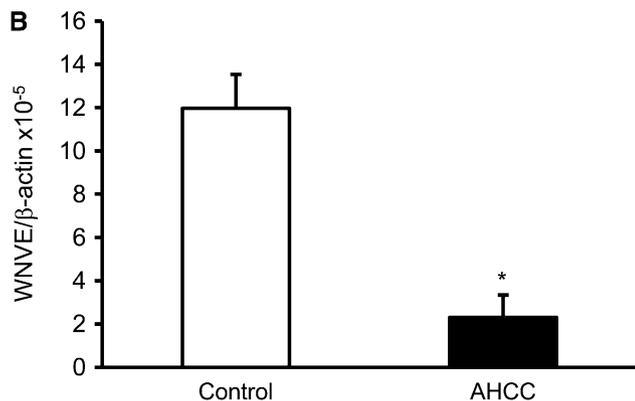
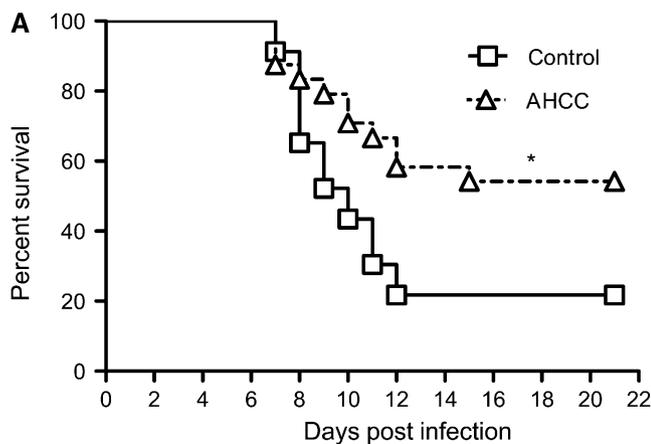


FIGURE 1 AHCC enhances host resistance to WNV infection in young mice. (A) Percent survival; $n = 24$ (AHCC treated) or 23 (control). (B) Viral load. The y-axis depicts the ratio of the amplified WNVE cDNA to β -actin cDNA of each group. Values are means \pm SEM, $n = 5$. Data shown were representative of 2 independent experiments. *Different from control, $P < 0.05$.

mice (7.59 ± 0.01 ; $P > 0.05$) did not differ from that of controls (7.57 ± 0.59). However, the total number of $\gamma\delta^+ \text{IFN-}\gamma^+$ cells in these mice was higher due to the differences in T cell expansion.

AHCC administration in aged mice following WNV challenge. A major risk factor for fatality of WNV encephalitis in humans is aging (3,4,26). Aged mice are much more susceptible to WNV infection than young mice following WNV challenge (22). Here, we observed an enhanced splenic $\gamma\delta$ T cell expansion at d 3 postinfection in AHCC-treated aged mice than controls as measured by the percentage (26% higher; $P < 0.05$) and total cell number (83% higher; $P < 0.05$) (Table 2). Upon WNV infection, the $V\gamma 1^+$ T cell subset was important for protecting the host, whereas $V\gamma 4^+$ T cells were involved in pathogenesis.

TABLE 1 AHCC treatment in protective immunity of young mice following WNV infection¹

Group	IgG		$\gamma\delta$ T cells	
	OD	%	%	$n \times 10^6$
Control	0.20 ± 0.04	0.11 ± 0.02	3.4 ± 0.29	2.30 ± 0.23
AHCC	$0.32 \pm 0.04^*$	$0.21 \pm 0.04^*$	$5.4 \pm 0.35^*$	$4.20 \pm 0.27^*$

¹ Values are means \pm SEM, $n = 14$ (IgG or IgM) or 4 ($\gamma\delta$ T cells). Data shown are representative of 2 independent experiments. *Different from control, $P < 0.05$.

Higher susceptibility to WNV infection in aged mice was partially due to the dysfunction of $\gamma\delta$ T cell subsets (22). In this study, we further found that AHCC enhanced $V\gamma 1^+$ T cell expansion ($P < 0.05$) but did not affect $V\gamma 4^+$ T cell response ($P > 0.05$) at d 3 postinfection (Table 2). Moreover, the WNV-specific IgG ($P < 0.05$) but not the IgM response (0.70 ± 0.12 vs. 0.57 ± 0.14 , AHCC-treated vs. control; $P > 0.05$) increased in these mice (Fig. 2). Finally, viremia at d 4 postinfection in AHCC-treated aged mice was reduced to 24% of control levels (Fig. 2; $P < 0.05$). Through 4 wk postinfection, 54.5% of AHCC-treated aged mice survived WNV infection ($P = 0.16$) and 30% of controls survived (Fig. 2; $P > 0.05$).

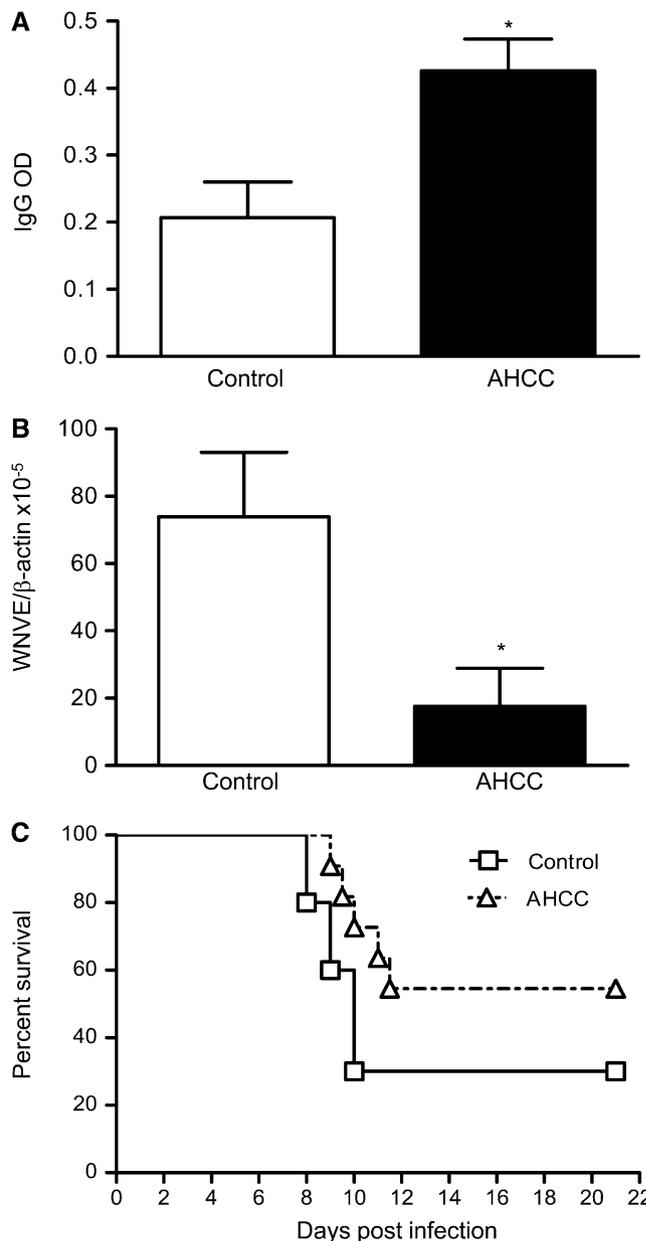


FIGURE 2 WNV infection in controls and AHCC-treated aged mice. (A) WNV-specific IgG antibodies in sera. Values are means \pm SEM, $n = 8$. (B) Viremia levels. Values are means \pm SEM, $n = 9$ (AHCC-treated) or 10 (control). (C) Percent survival. $n = 10$ (control) or 11 (AHCC-treated). *Different from control, $P < 0.05$.

TABLE 2 Percent and number of $\gamma\delta$ T cells in aged mice following WNV infection¹

Group	$\gamma\delta$ T cells		V γ 1 subsets		V γ 4 subsets	
	%	$n \times 10^6$	%	$n \times 10^4$	%	$n \times 10^4$
Control	2.03 \pm 0.15	1.2 \pm 0.18	1.24 \pm 0.04	72.7 \pm 2.35	0.70 \pm 0.15	41.1 \pm 13.1
AHCC	2.55 \pm 0.12*	2.2 \pm 0.22*	1.78 \pm 0.12*	155 \pm 7.04*	0.69 \pm 0.14	60.0 \pm 12.0

¹ Values are means \pm SEM, $n = 4$. Data shown are representative of 2 independent experiments. *Different from control, $P < 0.05$.

Discussion

Although viral pathogenesis is not clearly understood yet, it was suggested that WNV infects the brain in part via hematogenous spread, as an increased viral burden in serum correlates with earlier viral entry into the brain (25). Systemic WNV replication-induced proinflammatory responses could modulate blood brain barrier permeability, which may enable viral entry into the CNS (11,12). Hence, it is critical to control viremia levels during early stages of WNV infection. Previous studies have suggested important roles for type 1 IFN, $\gamma\delta$ T cells, and early humoral responses in limiting viremia and virus dissemination into the CNS (6–10). In this study, we have demonstrated that oral administration of AHCC in WNV-susceptible young mice enhanced early IgG and IgM responses and the magnitude of $\gamma\delta$ T cell expansion. Nevertheless, IFN α levels and proinflammatory responses were not affected. AHCC administration in these mice led to attenuated viremia and an increase in survival following WNV challenge. Overall, these results suggest that oral administration of AHCC enhances protective immunity and reduces lethality from WNV challenge in young susceptible mice.

In humans, aging is one of the well-documented risk factors for WNV encephalitis (3,4,26). We have previously demonstrated that aged mice are more susceptible to WNV infection, partially due to the dysfunction of $\gamma\delta$ T cell subsets in these mice (22). Here, our results have shown that AHCC administration increased the protective V γ 1⁺ T cell responses in aged mice. AHCC treatment also partially enhanced early humoral responses (IgG, not IgM) following WNV challenge. Although AHCC administration in aged mice still attenuated viremia levels, the survival rate in these mice did not improve significantly. These data suggest that oral administration of AHCC partially enhances host protective immunity against WNV in aged mice.

The mechanisms through which AHCC modulates protective immune responses in young and aged mice during WNV infection is currently under investigation. Earlier reports suggest that α -glucans isolated from plants or fungi could activate the immune system through toll-like receptor signaling (27,28). It is likely that AHCC, which contains a high content of α -1, 4 glucans, acts through these innate immune receptors and is either directly or indirectly involved in the activation of dendritic cells and $\gamma\delta$ T cells. The decline in immunity, including innate and adaptive immunity, occurs with advancing age (29,30). Therefore, we speculate that the impaired innate immune signaling with aging might contribute to the reduced magnitude of enhancement of protective immunity against WNV after AHCC administration in aged mice.

WNV-induced neurological disease has become a public concern in recent years. Yet, immunotherapy and vaccine are not available for human use. AHCC, an extract prepared from the nonedible portion of the shiitake mushroom, has been used as a dietary supplement in Asia and more recently in the US and Europe. In this study, 600 mg/kg of AHCC was administered to

mice every other day. According to guidance published by the Center for Drug Evaluation and Research at the U.S. FDA (31), an oral dose of 1 g/(kg⁻¹ · d) in a 20-g mouse is comparable to a human equivalent dose of \sim 5 g/d in a 60-kg human. Therefore, the dose in our study is equivalent to \sim 3 g/2 d in a 60-kg human. Similar doses have been tested in human studies. For example, one recent phase I trial has shown that 9 g/d of AHCC for 14 d had minimal adverse effects and was well tolerated by 85% of the subjects (32). Another study has suggested that 3 g/d of AHCC intake for 4 wk had a positive effect on specific innate immunity (33). Overall, our study clearly demonstrates that AHCC supplementation can improve the protective immune responses during WNV infection in the susceptible hosts.

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