Short-term supplementation with active hexose correlated compound improves the antibody response to influenza B vaccine

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Administration of bioactive nutritional supplements near or at the time of immunization has been a recent approach to stimulate human immune response to vaccination. Active hexose correlated compound (AHCC), a mushroom extract, has been shown to protect mice against lethal primary influenza infection. Moreover, when AHCC was administered pre-vaccination in mice, they showed improved protection from lethal avian flu infection when compared to mice vaccinated alone. In this study, we hypothesized that AHCC will also improve the immune responses of healthy individuals to influenza vaccine. A randomized controlled study was performed with 30 healthy adults to evaluate the effects of AHCC supplementation on the immune response to the 2009-2010 seasonal influenza vaccine. Blood was drawn pre-vaccination and 3 wk post-vaccination. Immediately post-vaccination, the AHCC group began supplementation with AHCC (3 g/d). Flow cytometric analysis of lymphocyte subpopulations revealed that AHCC supplementation increased NKT cells (P < .1), and CD8 T cells (P < .05) post-vaccination compared to controls. Analysis of antibody production 3 weeks post-vaccination revealed that AHCC supplementation significantly improved protective antibody titers to influenza B, while the improvement was not significant in the control group. Overall, our study showed that AHCC supplementation improved some lymphocyte percentages and influenza B antibody titers over the control. Future studies are required to determine the kinetics of AHCC supplementation to improve the overall response to influenza vaccination.

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1. Introduction

Influenza infection is a major public health threat worldwide resulting in 36000 deaths and over 200000 hospitalizations each year [1]. While influenza vaccination effectively protects 70% to 90% of young individuals from influenza disease, it is only about 45% effective in immunocompromised populations, such as in cancer patients and the elderly [2]. There is a growing interest in identifying alternative strategies that may improve the immune response to influenza vaccination to reduce morbidity and mortality from infection and its secondary complications. One approach could be to administer bioactive nutritional supplements along with immunization to maintain or stimulate the immune response.

Active hexose correlated compound (AHCC) is a dietary supplement prepared from Basidiomycetes mushrooms, largely comprised of α-1,4-glucans [3]. Animal studies have demonstrated that AHCC increases survival during acute infections...
such as influenza virus [4–6], avian flu [5], West Nile virus [7], and opportunistic infections [8–11]. Our laboratory has shown that AHCC supplementation, even in small doses, increases survival of young mice against influenza infection [4,6]. These studies provide clear evidence that AHCC has the capacity to improve or maintain immune function to primary virus infections, including influenza, in animal models.

Importantly, studies in human populations have demonstrated that AHCC can improve immune parameters in immunocompromised populations such as the elderly [12] and cancer patients, with minimal adverse consequences [13]. Supplementation with AHCC increases dendritic cell (DC) number and function [14], natural killer (NK) cell cytotoxicity [15], and CD4 and CD8 T cell function [12]. However, no studies have examined if short-term supplementation with AHCC may improve the immune response after influenza vaccination in healthy individuals. Such studies are essential given the ineffectiveness of current influenza vaccination strategies in vulnerable human populations.

In the current study, we examined whether AHCC supplementation could improve the efficacy of seasonal influenza vaccination in a pilot study of healthy individuals. Our hypothesis was that short-term supplementation with AHCC would enhance the immune response to influenza vaccination. To test our hypothesis we analyzed lymphocyte phenotypes, cytokines, and protective antibody titers against the influenza vaccine. Our data indicated that AHCC supplementation results in sustained levels of B cells, NK cells, CD4 T cells, and T cells; while showing improved NKT cells ($P < .1$) and CD8 T cells ($P < .05$). AHCC supplementation also demonstrated a significant increase in antibody titers against influenza B. These results suggest that this pilot study showed some improvements in the markers of vaccine efficacy with AHCC supplementation.

### 2. Methods and materials

#### 2.1. Subjects

All protocols were approved by the Institutional Review Board of Michigan State University. The distribution of study subjects by age and gender is illustrated in Table 1. A total of 30 healthy individuals initially enrolled in the study from the medical office of Dr. Lawrence Kempf in Manhattan, NY, USA. A total of 29 subjects completed the study, indicating a 97% retention rate. Before enrollment, all participants gave written informed consent. Subjects also provided information about their general health status at enrollment and at the end of the study. Subjects were excluded from the study if they had diseases associated with immune disorders or if they took medications which are known to alter immune function.

All subjects were vaccinated in October and November of 2009 with the recommended commercially available subvirus trivalent influenza vaccine Flushield (Wyeth Laboratories, PA, USA). On day 0, baseline blood samples were collected from subjects prior to receipt of influenza vaccination. On day 21, post-vaccination blood samples were collected. The blood samples were collected prior to the first confirmed case of influenza in the New York area.

#### 2.2. Supplementation with AHCC

This study was a randomized controlled trial in which subjects were randomly separated into two groups. The AHCC group was supplemented with 3 g/d of AHCC for 3 weeks beginning on day 0, after blood samples and vaccination were completed. The control group was vaccinated; however, they did not receive the AHCC supplement or a placebo in this pilot study.

#### 2.3. Sample preparation

Pre and post-vaccination plasma and serum samples were drawn from all subjects between 06:00 and 08:00 h after an overnight fast (12 h from previous meal). Samples were shipped overnight at 4°C for analysis at Michigan State University (East Lansing, MI, USA). At both time points, blood samples were drawn from all individuals. Serum and plasma were processed and stored at −80°C until further analysis for antibody titers and cytokines. Peripheral blood mononuclear cells (PBMC) were isolated from plasma with histopaque 1770 (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol [16].

#### 2.4. Flow cytometry

Immune phenotyping of PBMCs was performed using previously published protocols [17], $1 \times 10^6$ PBMCs were stained with fluorochrome conjugated antibodies to identify lymphocyte populations. The antibodies used are listed here (Pharmingen, San Diego, CA, USA): CD8 (FITC), CD56 (PE), CD4 (PE), CD3 (PerCP-Cy5.5), CD16 (PerCP-Cy5.5), CD45RA (PerCP-Cy5.5), NKp46 (APC), and CD19 (APC). Sample data was acquired on the Accuri C6 (BD Accuri Cytometers, MI, USA) and analyzed using FlowJo software (Tree Star, OR, USA). Lymphocytes were gated using FSC vs SSC and cell populations were identified as followed: [CD3+] cells = T cells, [CD19+] cells = B cells, [CD56+/CD3+] cells = NK cells, [CD56+/CD3+] cells = NKT cells.

#### 2.5. Antibody titers

Serum samples were sent to Enzo Clinical Diagnostic Laboratories (Farmingdale, NY, USA) for analysis of antibody production against the influenza A and B strains in the 2009-2010 vaccine. Titers were determined using the standard Hemagglutinin Inhibition Assay (HI test) and values were reported using geometric mean of serum anti-HA antibody titers [16]. Antibody titers ≥40 post-vaccination were considered
protective (seroprotection) and a 4-fold rise in specific antibody titers post-vaccination was considered a seropositive response (seroconversion) to the current vaccine.

2.6. Cytokine analysis

Custom Bio-Plex Pro Assay (Biorad, Hercules, CA, USA) was utilized to assess the following cytokines: IL-2, IL-4, IL-6, IL-10 and IFN-γ [18]. Briefly, undiluted frozen plasma samples were thawed on ice and assessed according to the manufacturer’s instructions and via Luminex 100 technology (Luminex, Austin, TX, USA).

2.7. Statistical analyses

Statistical analysis was performed using Graph Pad Prism 4 (Graph Pad Software, San Diego, CA, USA). Unpaired t-tests were performed to identify statistical significance of fold change pre- to post-vaccination between groups. A Z-test for proportions with a 95% confidence interval was used to determine significance between proportions. Repeated measures analysis of variance with Tukey’s post hoc test were performed on the antibody titer data, represented as geometric means ± SEM. Statistical significance was set at $P < .05$, trends were set at $P < .1$.

3. Results

3.1. Demographic characteristics of participants

A total of 30 healthy individuals were recruited for this study and were randomly assigned into either the AHCC supplemented group or the control group, which did not receive a placebo. Table 1 shows the demographics of the population. There were 14 individuals in the AHCC group (mean age 60.8 years) and 15 individuals in the control group (mean age 57.8 years). There was no statistical difference between the ages of the two groups. There was only one drop-out in the study from AHCC group, indicating 97% compliance for the overall study. The ratio of males to females was similar in the control group (7:8); however, the ratio of males to females was skewed in the AHCC group (9:5). There were no differences in mean age based upon gender.

Fig. 1 – Lymphocyte populations fold change over three wk post-influenza vaccination. White bars indicate control group (n = 15), black bars indicate AHCC supplemented group (n = 14). Data is presented as means ± SEM. Statistical analysis: paired t tests. *$P < .05$; #$P < .1$. 

![Graph showing lymphocyte populations fold change over three wk post-influenza vaccination.](image-url)
3.2. Effect of AHCC on lymphocyte populations 3 wk after influenza vaccination

We assessed by flow cytometry whether or not short-term supplementation with AHCC altered the phenotype of lymphocyte populations in response to vaccination. Fig. 1 shows the pre- to post-vaccination fold change of B cells, CD3 T cells, CD8 T cells, CD4 T cells, NKT cells, and NK cells. When compared to the control, the AHCC group showed a trend for increased NK-T cell fold change (P < .1) and a significant increase in CD8+ cytotoxic T cells fold change (P < .05). However, there were no significant differences in the fold changes of: B cells, T cells, CD4 T cells, or NK cells.

3.3. Effect of AHCC on serum anti-influenza A and B antibody levels

To evaluate the effect of AHCC on vaccine efficiency, we measured the anti-influenza A and B antibody titers in the serum 3 wk post-vaccination. The antibody titers to influenza A and B increased pre- to post-vaccination for both the AHCC and control groups (Fig. 2). The increase in anti-influenza A antibody titers were not statistically significant. The increase in anti-influenza B titers were statistically significant in the AHCC group, this increase was not statistically significant in the control group.

We then analyzed the percentage of individuals who achieved titers ≥40 and had a 4-fold rise post-vaccination to each of the strains of influenza. Table 2 indicates that an equal percentage of individuals in the AHCC and control groups achieved titers ≥40 to influenza A post-vaccination. However, more individuals showed a 4-fold rise to influenza A post-vaccination in the control group. A higher percentage of individuals in the AHCC group achieved titers ≥40 to influenza B post-vaccination, as well as 4-fold rise (Table 2). None of these findings were significant.

3.4. Effect of AHCC on plasma cytokine levels after influenza vaccination

To have a better understanding of the immunomodulatory effects of AHCC post-influenza vaccination, we also performed a bioplex pro assay for the following cytokines: IL-2, IL-4, IL-6, IL-10 and IFN-γ. The cytokines were chosen to differentiate the effects of AHCC on Th1/Th2 responses (IL-2, IFN-γ/IL-4), to indicate inflammation (IL-6), and to determine the function of regulatory T cells (IL-10) (Table 3). Data were obtained for IL-4, IL-6, IL-10 and IFN-γ, while IL-2 was not detected. We did not observe statistically significant differences due to the low number of detectable samples. IL-6 and IL-10 were the only cytokines in which the majority of the samples had a detectable value. However, there were no significant differences in inflammation or T-reg activity with vaccination or with AHCC supplementation. Interestingly, IFN-γ was elevated in the control group compared to AHCC; this increase was not significant.

4. Discussion

This pilot study evaluated the effect of short-term supplementation with AHCC on the immune response to influenza vaccination. We support our hypothesis; short term supplementation with AHCC did enhance the immune response to influenza vaccination. Both the AHCC and control group showed an increase in antibody titers to influenza B post-vaccination. This improvement was only significant in the AHCC group. In addition, more participants from the AHCC group had improvements in their influenza B antibody titers, in terms of seroprotection (titers ≥40) and seroconversion (4-fold rise in titers). Flow cytometric analysis of the phenotypic changes on lymphocyte subpopulations revealed that AHCC supplementation increases CD8 T cells (P < .05), and NKT cells (P < .1) post-vaccination compared to controls. This could indicate that short-term AHCC supplementation may be a therapeutic strategy to improve the cell-mediated immune response after influenza vaccination. Analysis of cytokine profiles did not reveal any significant inflammatory effect of AHCC. Collectively, these data indicate no adverse effects of AHCC supplementation, and trends in the data suggest that future studies increasing the numbers of participants and the duration of supplementation may yield more positive results.
In a murine model of West Nile viral infection, AHCC supplementation resulted in elevated antibody titers [7]. A trivalent split vaccine, like the one used in this trial, is expected to promote high antibody responses [17]. In our study, we saw a significant improvement of antibody titers to influenza B with AHCC supplementation, while there were no significant changes in the control group. Also, in regard to influenza A, more participants supplemented with AHCC achieved seroprotection and seroconversion, when compared to the control. In regard to influenza A, there was no difference between the AHCC and control groups seroprotection rates. However, more individuals in the control group experienced seroconversion when compared to the AHCC group. Overall, about 60% of the participants achieved seroprotection to influenza A, while only about 15% to 20% of participants achieved seroprotection to influenza B, depending on the group. The lower than expected vaccine response could be due to our study group age, almost half of the participants were >60 years. When looking specifically at these aged participants, we noticed they had very small changes in their antibody titers pre- to post-vaccination and almost none of them achieved seroprotection. This confirms previous published studies describing low immune responses to flu vaccine among the elderly and other immunocomprised individuals [2,19-21]. Future age and immune status controlled studies are required to examine the effect of AHCC in enhancing immune responses to vaccination.

Many animal studies support the idea that AHCC enhances survival from several primary infections [4-11], a protective effect attributed to AHCC’s actions on DCs, CD8 T cells and NK cells. The bioactive compound of AHCC is acetylated α-glucans but the exact mechanism of action is not known. β-glucans are another component of mushroom extracts which have received special interest in vaccination, because they are recognized by innate immune cell receptors and activate immune responses [22,23]. Thus, it is possible that AHCC also works through activating innate immunity. In our study, although we saw a trending increase in NKT cells, we did not see any change in NK cell percentages pre- and post-vaccination. There are several reports indicating that AHCC supplementation enhances NK cell cytotoxicity [4,6,15,20,24] and increases the number of DCs in humans, especially myeloid DCs [14]. To further understand the role of AHCC on the innate immune system, future studies should determine the effect of AHCC on NK cell and DC numbers, subsets, activation, and function at earlier time points post-vaccination.

The increased fold changes of T cells pre to post vaccination indicate that any immunomodulatory role of AHCC is probably associated with the activation of the Th1 pathway. In the absence of viral insult, supplementation of healthy adults with AHCC for 4 wk does not alter the percentages of T cells [6,14]. It does however increase the number of myeloid DCs, [14] which present antigens to promote activation and proliferation of antigen specific T cells. In this study, we did not examine DCs, but the increased percentage of CD8 T cells could indicate increased activation and proliferation. Additional studies support that AHCC supplementation enhances antigen specific activation and proliferation of T cells [20] and production of IFN-γ and TNF-α after incubation with mitogens or monoclonal antibodies [12].

While this pilot study does show promising results for future study, we did have some limitations. The control group did not have a placebo and was not given any type of treatment. In a future study we recommend using a placebo, so the participants can be blinded as to what group they are in. In order to determine innate immune changes it may also be beneficial to look at lymphocyte populations prior to the 3-wk time period set in this study, perhaps at 1 wk post-vaccination. There was also a delay in analysis of samples, as they had to ship overnight on ice, which is not ideal. Additionally, our flow cytometric gating strategy may have included NKT cells in our T cell gate; this is because we did not gate T cells as CD56-. Complete blood count was not measured; therefore absolute numbers of lymphocytes could not be calculated. Finally, pre-existing antibodies may have impeded a significant increase in titers and confounded the effect of AHCC in the immune response to influenza.

In this study, AHCC supplementation with influenza vaccination showed an increase in antibody titers over the control, and some improved lymphocyte populations. However, overall there is little evidence that orally ingested compounds can show measurable effects in the in vivo immune response in humans. AHCC has been shown to protect mice from primary infections, but a protective effect in humans from influenza infection is very difficult to prove. Studies aiming to show a preventive role of AHCC against common colds and influenza infections would require very large numbers of participants, and would need to record the occurrence and severity of infections. Future studies are

| Table 2 – Antibody titer levels from plasma of pre- and post-vaccinated healthy adults 1 |
|----------------------------------------|------------------|
| Percentage of participants with:      |                  |
|                                       | Titer >40 pre | Titer >40 post | 4-fold rise |
| Influenza A                           |                |
| Control                               | 28.6% 4(14) 2  | 57.1% 8(14)    | 14.3% 2(14) |
| AHCC                                  | 28.6% 4(14)    | 57.1% 8(14)    | 0.0% 0(14)  |
| Influenza B                           |                |
| Control                               | 7.1% 1(14)     | 14.3% 2(14)    | 14.3% 2(14) |
| AHCC                                  | 7.1% 1(14)     | 21.4% 3(14)    | 21.4% 3(14) |

1 Statistical analysis: Z-test for proportions with a 95% confidence interval. No significant differences were found. 2 Data presented as: number of participants (total participants in group).

| Table 3 – Plasma cytokine levels of healthy vaccinated adults in control and AHCC groups 1 |
|----------------------------------------|------------------|
| Control (pg/mL) n = 15                 | AHCC (pg/mL) n = 14 |
|                                       | Pre | Post | Pre | Post |
| IL-2 ND 2                              | ND  | ND   | ND  | ND   |
| IFN-γ 550 ± 224.7 2                    | 595 ± 271.0     | 260.2 ± 39.3  | 191.7 ± 56.1 |
| IL-4 3.6 ± 1.8                         | 6.1 ± 1.8      | 2.2 ± 0.0     | 2.2 ± 0.0     |
| IL-6 15.6 ± 4.3                        | 18.7 ± 6.5     | 28.2 ± 5.2    | 15.6 ± 3.9   |
| IL-10 7.3 ± 2.3                        | 7.7 ± 1.1      | 17.8 ± 8.8    | 7.7 ± 1.4   |

1 No significant differences were found. 2 ND stands for not detectable. 3 Data presented as means SEM.
critical to determine the potential immune-enhancing potential of AHCC, especially with influenza vaccination in immunocompromised or aging individuals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nutres.2012.11.001.

References