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Effects of active hexose correlated compound on frequency of $CD4^+$ and $CD8^+$ T cells producing interferon-γ and/or tumor necrosis factor–α in healthy adults

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ABSTRACT

Active hexose correlated compound (AHCC) is a natural compound with the potential to be used as an immunoenhancer in cases in which the immune system is compromised. The purpose of this study was to evaluate the effects of this compound on the immune function of healthy adults aged 50 years or more. The production of interferon (IFN)–γ and tumor necrosis factor (TNF)–α by $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry in peripheral blood obtained from subjects at different time points after AHCC intake. The frequency of $CD4^+$ and $CD8^+$ T cells producing IFN-γ alone, TNF-α alone, or both increased during AHCC intake compared with baseline values. Furthermore, the frequency of such cells remained high even 30 days after discontinuing AHCC. Overall, these findings suggest that AHCC enhances $CD4^+$ and $CD8^+$ T cell immune responses in healthy elderly persons taking at least 30 days to obtain such effect, which remained up to 30 days after discontinuing treatment with this compound.

1. Introduction

Increased numbers of substances have been used to revitalize the immune response of hosts with impaired function of the immune system [1,2]. Many immune enhancers are natural compounds that have proved to be nontoxic for humans and with great potential for anticancer activity [3–6]. Active hexose correlated compound (AHCC), an extract prepared from mycelia of the *basidiomycete* mushroom *Lentinula edodes*, has received special attention in the past few years by its reported benefits [1,7]. AHCC is commercially used as a nutritional supplement and contains a mixture of polysaccharides, amino acids, and minerals. In addition, AHCC is orally bioavailable, well tolerated by human beings, and free of adverse effects [1,8]. Chemical analysis has shown that about 74% of AHCC is oligosaccharides containing about 20% of the α-1, 4-glucan type. The partially acetylated forms of α-1,4-glucan have low molecular weight (5 kDa), and are believed to be the molecules responsible for the biologic activities of AHCC [2,9,10].

AHCC has been shown to have an enhancing effect on immune function of humans [2,11] and rodents [9,12–14] including an increase of natural killer (NK) cell activity [15], interleukin-12 production [16,17] and resistance to bacterial infection [7,18]. Of interest, it has been found that the effects of AHCC are more evident in hosts with the impaired immune function [18]. In fact, we have demonstrated that AHCC can enhance interferon (IFN)–γ production by $CD8^+$ T cells, and the numbers of NK and $\gamma\delta$ T cells in a murine tumor model [19]. However, it is largely unknown whether AHCC could enhance IFN-γ and tumor necrosis factor (TNF) –α production by $CD4^+$and $CD8^+$T cells in human, in particular, elderly adults with increased risk of infection and malignancy. Thus, we addressed this question by measuring the frequency of IFN-γ–producing and/or TNF-α–producing $CD4^+$ and $CD8^+$ T cells in adults aged 50 years or more before, during, and after AHCC intake. The results of our study showed increased production of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells during and after AHCC intake compared with baselines, suggesting the potential role for AHCC in improving host defense against infections and malignancy in humans by enhancing T cell immune function.

2. Subjects and methods

2.1. Study design

This was an open-label trial in which subjects older than 50 years were treated with AHCC for 60 days. Peripheral blood was collected at baseline, at 30 and 60 days during AHCC treatment, and 30 days after treatment had been discontinued. Subjects were interviewed and explained the aims of the study at the screening visit. After signing the consent form, the inclusion and exclusion criteria for the subjects were confirmed. Subjects had a screening laboratory evaluation (complete blood count, liver function tests,

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blood urea nitrogen, creatinine, glucose, electrolytes), a brief physical examination, 12-lead electrocardiogram, and vital signs, and 30 subjects were finally enrolled in the trial.

2.2. Subjects

Inclusion criteria were age of 50 years or more, normal mental status, no supplements except multivitamins in the last 60 days, no immunization in the last 4 months. Exclusion criteria were use of corticosteroids, methotrexate, interferon, or other immunotherapy, use of any other supplements except multivitamins within 60 days before enrollment, rheumatoid arthritis, systemic lupus erythematosis, multiple sclerosis, or other immunologic illness, hepatitis A, B, or C, organ transplant, history of cancer chemotherapy within the last 12 months, lymphoma, cirrhosis, nephritis, uncompensated heart failure, or any significant active or uncontrolled illness. Subjects were assigned a number from a master sheet containing 30 identifier numbers. The number was added to the three initials of the subject to compose the study ID.

2.3. Flow cytometry

Heparinized peripheral blood was aliquoted in 15 ml conical polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) at 1 ml per tube as previously described [20]. Peripheral blood was stimulated for 6 hours with phytohemaglutinin (PHA 20 μg/ml; Sigma-Aldrich, St. Louis, MO) or phosphate-buffered saline (PBS) in the presence of the co-stimulatory anti-CD28 and anti-CD49d monoclonal antibodies (1 μg/ml, BD Pharmingen, San Diego, CA). Culture tubes were incubated in a humidified 37°C, 5% CO_2 incubator with the last 4 hours of incubation in the presence of Brefeldin A (BFA) (10 μg/ml, Sigma-Aldrich), a secretion inhibitor, to enhance the accumulation of cytokines in the cytoplasm. At 6 hours, 100 μl of 20 mmol/l EDTA for a final concentration of 2 mmol/l EDTA were added. Red blood cells in blood samples were then lysed, and remaining cells were fixed with FACS Lysing Solution (Becton Dickinson, San Jose, CA) and stored at −80°C until further staining. After thawing cells were re-suspended in BD FACS Permeabilization Solution (Becton Dickinson) and stained with an-

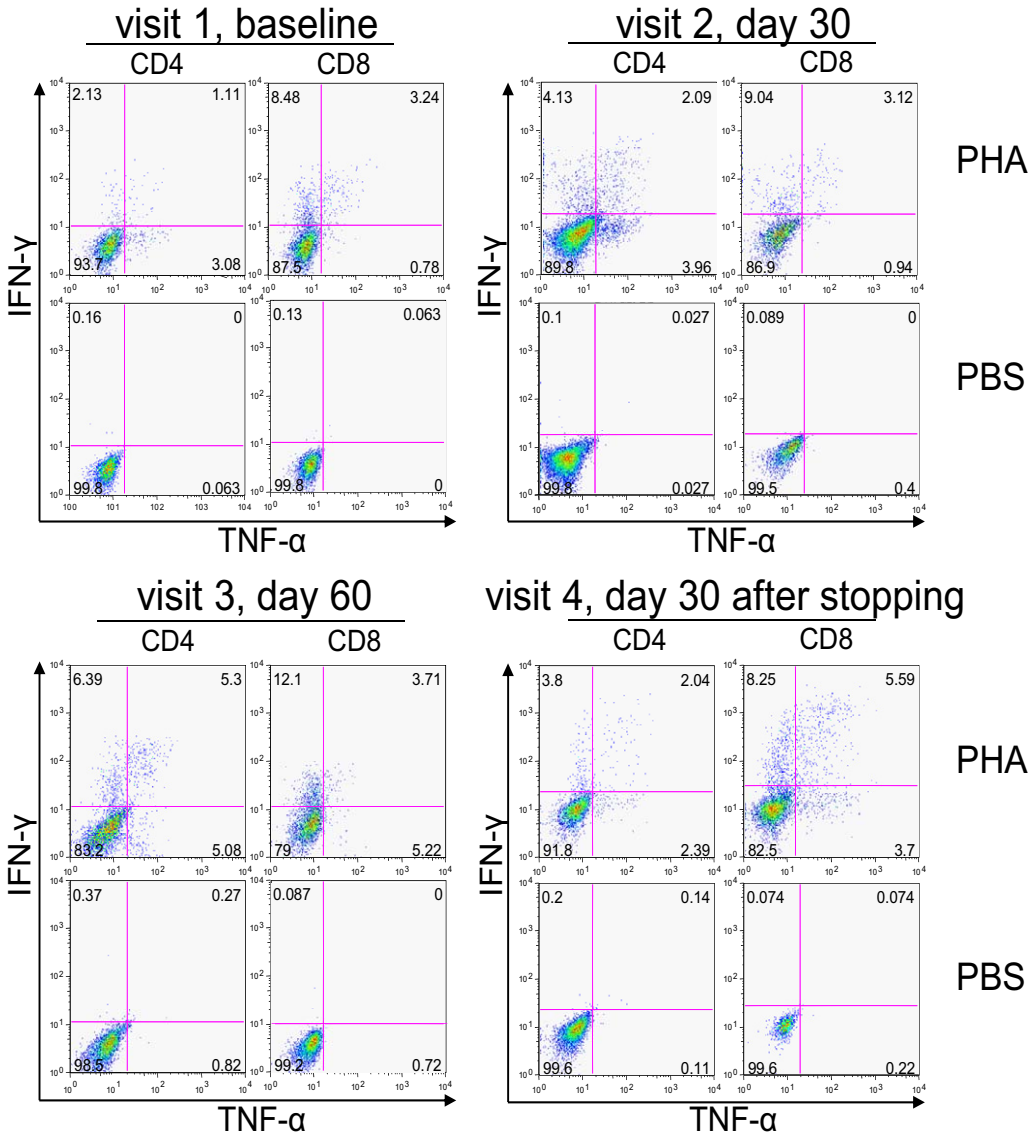

Fig. 1. Analysis of $CD4^+$ and $CD8^+$ T cells producing cytokines in response to phytohemaglutinin using flow cytometry. Peripheral blood was obtained from individuals before and after taking active hexose correlated compound (AHCC) and stimulated with phytohemaglutinin (PHA) or phosphate buffered saline (PBS) for 6 hours in the presence of brefeldin A (BFA, Golgi plug) during the last 4 hours of stimulation. Stimulated cells were fixed, permeabilized, and stained with antibodies to IFN-γ and TNF-α as well as to CD4 or CD8. Stained cells were analyzed on a FACSCalibur. Visits 1, 2, 3, and 4 indicate before, 30, 60 days on AHCC and 30 days after discontinuing AHCC, respectively.

tibodies to CD8-APC, CD4-cychorome, and IFN-γ- or TNF-α-FITC. Data were acquired on a FACSCAlibur system and analyzed using FlowJo software (Tree Star, Ashland, OR). The frequency of $CD4^+$ or $CD8^+$ T cells producing cytokines in the negative control stimulated with PBS was subtracted from the frequency of the same cell population in a sample stimulated with PHA. These values obtained after subtracting the negative control were used for the statistical analyses.

2.4. AHCC administration

AHCC was provided as 500-mg capsules. Total daily dose was 3 g (3 capsules twice daily). The subjects completed a drug compliance card indicating the ingestion of each dose. Subjects who had taken less than 80% of the prescribed dose (missing more than 12 individual doses in 30 days) would be dropped from the primary analysis.

2.5. Study documentation

Subjects were identified in the Case Report Form by the subject's initials and the subject study number. The clinical study protocol and consent form were reviewed and approved by the Institutional Review Board, in compliance with the requirements of 21 CFR 56. Clinical study complied also with US Good Clinical Practice, Title 21 of the US Code of Federal Regulations, the International Conference on Harmonization, the Declaration of Helsinki, and with all applicable laws and regulations of the locale and country where the study was conducted.

2.6. Informed consent and authorization

The objectives of the research, the nature, possible hazards and adverse reactions of the product were explained by a member of the research team during the screening interview both, verbally as well as written on the informed consent form. Subjects were given ample opportunity to inquire about the details of the study, and to read and understand the consent form before signing it. Where applicable, the initial informed consent document was accompanied by a signed and dated Health Insurance Portabilit and Accountability Act (HIPAA) authorization document as required by the institution and/or research facility. All information regarding this study, including but not limited to this protocol were kept strictly confidential.

2.7. Statistical analysis

Based on previous experience, we estimated that 30 treated subjects would provide more than 80% power at the two-tailed alpha level of 0.05 to detect any significant differences in immunologic activity between baseline and end of treatment.The mean frequency of cytokine-producing $CD4^+$ or $CD8^+$ T cells was compared by the Mann–Whitney *U* test.

3. Results and discussion

The production of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells was measured in healthy elderly persons at baseline, at 30 and 60 days of AHCC intake (visits 1, 2, and 3, respectively), and at 30 days after the last dose of AHCC (visit 4). Peripheral blood cells were obtained from the subjects and stimulated with PHA or PBS. The frequency of $CD4^+$ and $CD8^+$ T cell subsets, including cells producing IFN-γ alone, TNF-α alone and both IFN-γ and TNF-α was determined using flow cytometry. As shown in Figs. 1 and 2, the frequency of the $CD4^+$ T cell subsets producing the cytokines was higher at visits 2 and 3 than at visit 1. In addition, the frequency of $CD4^+$ T cells producing IFN-γ alone and TNF-α alone was higher at visit 2 than at visit 1. At 30 days after AHCC was discontinued (visit 4), the frequency of $CD4^+$ T cells producing IFN-γ alone, TNF-α alone, and a combination of IFN-γ and TNF-α decreased uniformly, suggesting the role for AHCC in enhancing $CD4^+$ T cell responses

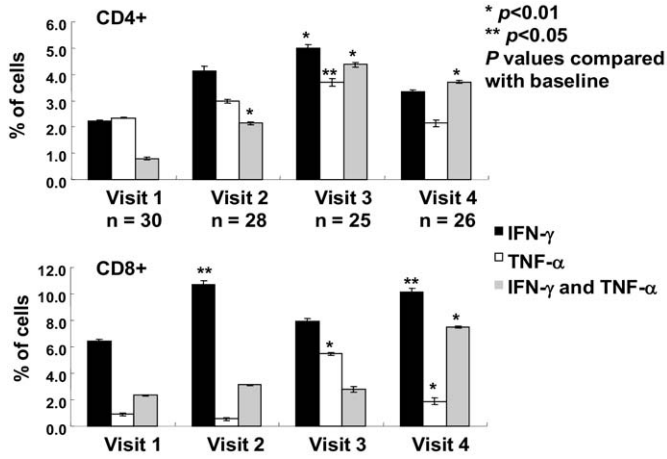

Fig. 2. Changes in the frequency of $CD4^+$ and $CD8^+$ T cells producing cytokines before and after active hexose correlated compound (AHCC). As described in Fig. 1, the frequency of $CD4^+$ and $CD8^+$ T cells producing IFN-γ, TNF-α or both cytokines was determined before and after AHCC intake. Visits 1, 2, 3, and 4 indicate before AHCC, 30 and 60 days on AHCC, and 30 days after discontinuing AHCC, respectively.

during the intake of the compound. Of interest, the frequency of $CD4^+$ T cells producing the combination of IFN-γ and TNF-α at visit 4 was still higher compared with the frequency of the same cells at the baseline (visit 1). This implies that the effect of AHCC on $CD4^+$ T cells could last for several weeks even after this compound is discontinued.

In analyzing $CD8^+$ T cell responses, the frequency of $CD8^+$ T cells producing IFN-γ alone increased at visits 2 and 3 as well as at 30 days after discontinuing AHCC (visit 4) compared with the baseline although these differences were not statistically significant except at visit 4. The frequency of $CD8^+$ T cells producing TNF-α alone was higher at visits 3 and 4 than at visit 1. The frequency of $CD8^+$ T cells producing a combination of IFN-γ and TNF-α was not different during the first 3 visits. However, it was significantly higher at visit 4 compared with the baseline. Of interest, the frequency of $CD8^+$ T cells producing IFN-γ alone, TNF-α alone and both cytokines at 30 days after discontinuing AHCC (visit 4) was higher than that of the baseline. This finding suggests that the effect of AHCC on $CD8^+$ T cells could remain even after discontinuing the compound.

Although several studies have shown the potential beneficial effects of AHCC on the immune system of human beings [2,11] and rodents [9,12–14], very little is known about the mechanisms involved in the enhancement of the immune system by AHCC. Previous studies in rodents have suggested that AHCC appears to benefit the most hosts with impaired function of the immune system [14,18]. The objective of this study was to investigate the effects of AHCC on the function of the immune system in healthy adults aged 50 or more who may have increased risk of infections and malignancy. Our results supported the hypothesis that oral administration of AHCC would enhance the function of the immune system in human healthy adults. Our results are also consistent with other studies showing the role of AHCC on the function of the immune system. It has been shown that AHCC increases NK cell activity [15], IL-12 [16,17], and regulates nitric oxide production [21].

In conclusion, our results suggest that AHCC can enhance $CD4^+$ and $CD8^+$ T cell immune responses in healthy persons via increasing production of cytokines IFN-γ and TNF-α from T cells. Effects were seen after 30 days, and remained up to 30 days after discontinuing the compound. AHCC may improve immune response against pathogens through this mechanism. In summary, our results suggest that AHCC can enhance $CD4^+$ and $CD8^+$ T cell immune responses in healthy people via increasing production of the cytokines IFN-γ and TNF-α from T cells. Further studies are required to

fully define the potential and mechanism of AHCC as a countermeasure to minimize any detrimental effects in situations in which the function of the immune system is compromised.

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