Dietary Supplementation with White Button Mushroom Enhances Natural Killer Cell Activity in C57BL/6 Mice

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Abstract

Mushrooms are reported to possess antitumor, antiviral, and antibacterial properties. These effects of mushrooms are suggested to be due to their ability to modify immune cell function. However, a majority of these studies evaluated the effect of administering extracts of exotic mushrooms through parental routes, whereas little is known about the immunological effect of a dietary intake of white button mushrooms, which represent 90% of mushrooms consumed in the U.S. In this study, we fed C57BL/6 mice a diet containing 0, 2, or 10% (wt/wt) white button mushroom powder for 10 wk and examined indices of innate and cell-mediated immunity. Mushroom supplementation enhanced natural killer (NK) cell activity, and IFNγ and tumor necrosis factor-α (TNFα) production, but only tended to increase IL-2 (P = 0.09) and did not affect IL-10 production by splenocytes. There were significant correlations between NK activity and production of IFNγ (r = 0.615, P < 0.001) and TNFα (r = 0.423, P = 0.032) in splenocytes. Mushroom supplementation did not affect macrophage production of IL-6, TNFα, prostaglandin E2, nitric oxide, and H2O2, nor did it alter the percentage of total T cells, helper T cells (CD4+), cytotoxic or suppressive T cells (CD8+), regulatory T cells (CD4+ /CD25+), total B cells, macrophages, and NK cells in spleens. These results suggest that increased intake of white button mushrooms may promote innate immunity against tumors and viruses through the enhancement of a key component, NK activity. This effect might be mediated through increased IFNγ and TNFα production.

Introduction

Immune function, particularly innate and T cell–mediated immune response, is critical to preventing and controlling microbial infection (1–4) and neoplasia (5,6). There are, however, limited strategies available to efficiently modulate the immune response. Nutritional interventions that involve optimizing the intake of essential nutrients and utilizing promising functional foods have become an increasingly favored approach to the modulation of immune cell function. Mushrooms have long been suggested to possess immunomodulatory properties (7–11).

The substances present in mushrooms with immunomodulatory and antitumor activity are mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides, and polysaccharide proteins (10,12–14). Mushrooms also contain a spectrum of nutrients at varied levels, such as B vitamins, vitamin D, and minerals (potassium, copper, magnesium, selenium, and zinc). In recent years, the consumption of mushrooms, either as whole mushrooms or extracted supplements, has increased. Although historically, mushroom consumption has been associated with the maintenance of health and warding off disease, scientific data supporting their health benefits are limited. Epidemiological data regarding the health benefits of mushroom consumption is essentially nonexistent.

Studies relating the mushroom’s medicinal use started 4 decades ago, and some clinical and experimental studies thus far have suggested that mushrooms and their polysaccharide components inhibit tumor growth. This effect of mushrooms may be due to their ability to modulate immune system function rather than having a direct effect on cancer cells (8,9,11). In the majority of early studies, purified mushroom polysaccharides were administered through a parental route or added to cell cultures. More recent studies, however, reported that oral supplementation with a variety of different mushroom species are effective in modulating certain immune functions (7,9,15,16).

Mushroom products have been evaluated for their pharmaceutical potential, and studies thus far have predominantly focused on tumor-bearing animals or cancer patients. Whether...
an increase in the dietary consumption of mushrooms or mushroom supplements can also have an impact on healthy animals or subjects has not been established. This is despite the fact that mushrooms and their subcomponents are widely consumed by the general population as part of a normal diet. A majority of studies on mushrooms have been conducted outside of the U.S and almost all have used exotic mushrooms. In contrast, few studies have evaluated the potential health benefits of white button mushrooms (Agaricus bisporus), a strain of mushroom that constitutes 90% of mushrooms consumed in the U.S. Furthermore, little is known about their potential immunological effects. Because the mushrooms of different strains have a varied composition of nutrients and other functional ingredients, the results obtained from studies of these strains might not be applicable to white button mushrooms. Our objective in this study, therefore, was to determine the effect of white button mushrooms on innate and T cell–mediated immune functions.

Materials and Methods

Diets. Fresh white button mushrooms were provided by Franklin Farms through the Mushroom Council. Mushroom stems were first cut off at level of fruit body (crown) and each mushroom was cut into quarters. The cut pieces of mushroom were freeze-dried for 5 d and then ground to powder in a grinder (Norton). Dry matter was 7.5% of fresh weight. Mushroom powder was added at 2 or 10% (wt:wt) to AIN-93M diet (17), purchased from Research Diets, and thoroughly mixed. A control mix was added to the control diet at 10% (wt:wt) and to 2% mushroom diet at 8% (wt:wt) to equalize the levels of total energy and macronutrients in the experimental diets. The control mix was calculated to best match the mushroom powder in energy, total carbohydrates, dietary fiber, and protein and was prepared by mixing 25% casein, 37.5% corn starch, and 37.5% cellulose. We chose the 2 and 10% levels of supplementation to represent the high intake of fresh mushrooms achievable through diet (2%) and supplementation (10%).

Animals. Male C57BL/6NIA mice (4 mo of age) were purchased from the National Institute on Aging colonies at Harlan Sprague Dawley. After a 2-wk acclimation period, all mice were weight-matched and placed into 1 of 3 experimental groups. Mice were housed individually and maintained in an environmentally controlled atmosphere (temperature 23°C, relative humidity 45%) with a 12:12-h light:dark cycle. Mice were given free access to water and group pair fed their respective diet. To do this, mice were initially given a weighed portion of food daily. If any mouse did not eat the entire portion of food, the weighed portion for all mice was decreased to the amount that the mouse ate the previous day. If all the mice consumed all of the diet, then the quantity of diet given was increased until an individual mouse did not consume all the food. By group feeding, we decreased the variability among the mice both within each diet group and among the diet groups. All mice were observed daily for clinical signs of disease, and body weight was recorded at wk 0 (start), 1, 2, 4, 6, 8, and 10 (end). At the end of study, mice were killed by CO2 asphyxiation and exanguination. All conditions and handling of the animals were approved by the Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals.

Splenocyte isolation. Spleens were aseptically removed and placed in sterile RPMI 1640 (Biowhittaker) medium-supplemented with 25 mM L-HEPES (Invitrogen Gibco), 2 mM L-glutamine (Gibco), 100 kU/L penicillin and 100 mg/L streptomycin (Gibco). Medium, supplemented as described, was referred to as complete RPMI. Single cell suspensions were prepared as previously described (18). All the experiments were conducted under a condition of 37°C, atmosphere of 5% CO2, and a 95% humidity, unless indicated otherwise.

Cellular composition of splenocytes. The percentages of major component cell types in spleen were determined using fluorescent-activated cell sorting (FACS) analysis. Splenocytes (1 × 106 cells/sample) were stained with following anti-mouse antibodies: FITC-conjugated anti-CD3 (T cells), PE-conjugated anti-CD19 (B cells), FITC-conjugated anti-CD4 (T helper (Th) cells), PE-conjugated anti-CD8 (T suppressor or cytotoxic cells), APC-conjugated anti-CD25 (IL-2 receptor α chain), FITC-conjugated anti-natural killer-1.1 (NK-1.1 cells), and APC-conjugated anti-mouse F4/80 (macrophages (Mφ)). F4/80 antibody was from Caltag and all the other antibodies were from BD PharMingen. Stained cells were analyzed on a FACSCalibur (BD Biosciences) and the results were analyzed using the Summit software, version 4.0 (DakoCytomation).

Natural killer cell activity assay. NK cell activity was assessed using radioisotope 51Cr release assay. YAC-1 cells, a murine lymphoma cell line purchased from ATCC, were incubated with 51Cr (Perkin Elmer Life and Analytical Sciences) (25 μCi/1 × 106 cells) for 90 min at 37°C. Labeled YAC-1 cells were added to appropriately diluted splenocytes in a round-bottomed 96-well plate (Nunc) for final effector:target cell ratios of 10:1, 25:1, 50:1, and 100:1. The cocultured cells were incubated in triplicate for 4 h at 37°C. Spontaneous release was measured as the amount of 51Cr released by target cells alone, and maximum release was measured by the amount of 51Cr released after the addition of 5% Triton X solution. Supernatant was collected after brief centrifugation (250 × g for 1 min) and counted in a Cobra II gamma counter (Packard Instruments) for radioactivity as counts per min (cpm). NK cell activity was expressed as a specific lysis percentage: (sample cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) × 100.

Mitogenic response of splenocytes. Splenocytes (1 × 105 cells/well), in 96-well, flat-bottom plates (Becton Dickinson Labware), were cultured in the presence or absence of the Th1 cell mitogen concanavalin A (Con A; Sigma) at 0.5, 1.5, or 3 mg/L, phytohemagglutinin P (PHA; Difco Laboratories) at 2, 5, or 20 mg/L, or LPS (Sigma) at 0.1, 1, or 10 mg/L for 72 h. Cultures were pulsed with 0.5 μg [3H]-thymidine (Perkin Elmer) during the final 4 h of incubation. The cells were harvested onto glass fiber filter mats (Wallac) by a Tomtec harvester (Wallac) and cell proliferation was quantitated as the amount of [3H]-thymidine incorporated into DNA as determined by liquid-scintillation counting in a 1205 Betaplate counter (Wallac). The counter had an efficiency of >90% for [3H]. Results are expressed as Bq.

Cytokine and prostaglandin E2 production by splenocytes. Splenocytes (4 × 106 cells/well) in 24-well culture plates (Becton Dickinson Labware) were cultured in the presence of Con A (1.5 mg/L) or LPS (1 mg/L) for 24 h for inflammatory IL-6, tumor necrosis factor-α (TNFα), IL-12p70, and prostaglandin E2 (PGE2) production or in the presence of Con A (1.5 mg/L) or PHA (5 mg/L) for 48 h for IFNγ, IL-2, and IL-10 production. Cell-free supernatants were collected at the end of incubation and stored at −70°C for later analysis. All of the cytokines were measured using ELISA. The reagents for IL-6, TNFα, IFNγ, IL-2, IL-10, and IL-12p70 assays were from BD Pharmingen. PGE2 was measured using radioimmunoassay as previously described (19).

Cytokine. PGE2, NO, and H2O2 production by Mφ. Peritoneal exudate cells were obtained by peritoneal lavage and enriched for Mφ using the method of Kumagai et al. (20). Peritoneal Mφ prepared in this manner were at least 90% pure, as assessed by the expression of Mac-1 and F4/80 cell surface antibody. Mφ (~5 × 105 cells/well) were incubated in 24-well culture plates (Becton Dickinson Labware) in the presence of LPS (1 mg/L) for 24 h. Cell-free supernatants were analyzed for cytokine, PGE2, and NO production. Cells were lysed in 1 mol/L NaOH for total cell protein analysis using the bicinchoninic acid protein assay kit (Pierce). IL-6, TNFα, IL-12, and PGE2 were analyzed as described above and NO was measured using

6 Abbreviations used: Con A, concanavalin A; cpm, counts per minute; FACS, fluorescent-activated cell sorting; Mφ, macrophages; NK, natural killer; PGE2, prostaglandin E2; PHA, phytohemagglutinin; Th, helper T cells; TNFα, tumor necrosis factor-α.

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Griess assay as previously described (21). All the concentrations were normalized with total cell protein.

For measurement of H2O2 production, Mø (−5 × 105 cells/well) were incubated in 96-well culture plates (Becton Dickinson Labware) in the presence of phorbol 12-myristate 13-acetate (Sigma) at 10 µg/mL or zymosan (Sigma) at 1 g/L for 1 h. Supernatants were collected and analyzed for H2O2 production using Amplex Red hydrogen peroxide/ peroxidase assay kit (Molecular Probes). Total cell protein was measured as described above and used to normalize H2O2 concentrations.

Statistical analysis. All results were expressed as means ± SEM. Statistical analysis was conducted using Systat 10 statistical software. Significant differences were determined using ANOVA for overall effect of mushroom consumption and was followed by Fisher’s least significance difference (LSD) post-hoc test for individual comparisons. Pearson correlation was used to determine associations between NK activity and production of IFNγ or TNFα. Significance was set at P < 0.05.

Results

General condition of mice. Mice in all diet groups remained healthy throughout the experiment. Daily food intakes of group pair-fed mice varied from 3 to 3.6 g/d. Body weight did not differ among the diet groups at the start of the study and increased from 1.6 to 1.7 g in the 3 dietary groups after the 10-wk feeding period with no difference in body weight gain among the diet groups (data not shown).

Subpopulations of splenocytes. The percentage of total B cells (CD19+), total T cells (CD3+), Th cells (CD4+), suppressive/cytotoxic T cells (CD8+), regulatory T cells (CD4+/CD25+), NK cells (NK 1.1+), and Mø (F4/80+) did not differ among the 3 diet groups (data not shown).

NK cell activity. For all the effector:target cell ratios tested, NK cell activity was significantly enhanced by mushroom supplementation in a dose-dependent manner (Fig. 1). Similar results (data not shown) were obtained when NK activity was expressed as killing activity per given number of NK cells and calculated based on the total splenocytes used in this assay and the percentage of NK cells in the splenocytes obtained from the aforementioned FACS assay.

Discussion

This study demonstrates that dietary supplementation with white button mushrooms enhances NK cell activity and IFNγ and TNFα production. The results suggest that the consumption of white button mushrooms, a strain constituting 90% of mushrooms consumed in the U.S., can modulate the body’s innate immunity. To our knowledge, for the first time, these results demonstrate that dietary supplementation with white button mushrooms significantly enhances the ability of spleen NK cells to lyse the target tumor cells in a dose-dependent manner. This increased NK capacity mainly represents a more vigorous cytolytic activity of NK cells toward target cells and is not due to an increase in the number of total NK cells because the percentage of NK cells in the spleen was not altered by mushroom supplementation. Other strains of edible mushrooms have also been shown to increase NK activity; however, these studies were mostly conducted in tumor-bearing or virus-infected animals (15,22–24).

NK cells are a group of specialized lymphocytes characterized by their ability to spontaneously kill pathogen-infected cells and tumor cells and are thus a key component of the innate immune system (25). This function of NK cells is mediated and regulated by the immunoregulatory cytokines produced by NK cells themselves as well as other cells such as T cells and Mø. IFNγ is the most important cytokine in mediating NK activity. IFNγ is thought to play an essential role in NK cell-mediated suppression of viral and bacterial infection (26–28) and the inhibition of tumor initiation and metastasis (26,29). To a lesser degree, TNFα may also contribute to the antiviral and antitumor effects of NK cells (26,28). In this study, the enhancement of NK

Th 1 and Th 2 cytokine production. Con-A stimulated secretion of Th 1 cytokine IFNγ was significantly greater in the group fed the 10% mushroom diet (25.9 ± 4.2 kU/L) than in the control group (16.5 ± 1.9 kU/L) and was intermediate in the 2% mushroom diet group (22.0 ± 2.9 kU/L). TNFα production was significantly higher in the group fed the 10% mushroom diet (60 ± 18 ng/L) than in those fed the control diet (30 ± 5 ng/L). The 2% mushroom-fed mice tended to have higher (P = 0.09) IL-2 production compared with the control mice (data not shown). IL-10 (Th 2 cytokine), IL-12p70, IL-6, and PGE2 (lipid inflammatory mediator and T cell suppressor) production did not differ among diet groups (data not shown).
activity by mushroom supplementation was accompanied by an increase in splenocyte production of IFN-γ. Furthermore, there were significant positive correlations between NK activity and IFN-γ production. We also found a higher TNFα production, but only in mice fed 10% mushroom and when compared with controls. Accordingly, a weak but significant correlation was found between NK activity and TNFα production. These results are consistent with a previous study in which i.p. injection of D-Fraction extracted from maitake mushroom increased IFNγ and TNFα production by whole spleen cells in carcinoma-bearing C3H/HeN mice (24). Because IFNγ and TNFα are produced by different cell types found in spleen (T cells, NK cells, and Mφ), further studies using intracellular cytokine staining and flow cytometry are needed to determine the cellular origin of the mushroom-induced increase in these cytokines. Our results using peritoneal macrophages, however, indicate that the effect of mushroom is not mediated through Mφ.

Helper T cells are designated as Th 1 and Th 2 subpopulations based on the patterns of their cytokine secretion. Th 1 cells, characterized by IL-2, IFNγ, and TNFα production, are mainly involved in cell-mediated immunity to intracellular infections. Th 2 cells, characterized by IL-4, IL-5, and IL-10 production, are mainly involved in humoral immunity against extracellular infections. Mushroom polysaccharides have been shown to induce polarization of Th response but with a varied manner. Although some of these mushroom-derived compounds induce Th 1 response, others favor Th 2 response. It has been suggested that the effect of mushrooms on Th cell polarization is dependent upon the type of the mushroom-derived polysaccharides and animal strain used (7). In this study, mushroom supplementation increased IFNγ and TNFα production and tended to increase in IL-2 production, but did not affect IL-10 production. These results indicate that white button mushrooms may induce a shift toward Th 1 response. This is further supported by our in vitro experiment in which extract of white button mushroom promoted maturation and the antigen-presenting function of bone marrow-derived dendritic cells (Z. Ren, Z. Guo, S. N. Meydani, and D. Wu, unpublished results), which have been shown to induce Th 1 response (30,31). Whereas enhanced Th 1 response is beneficial for antimicrobial and antitumor defense, it may be undesirable in certain autoimmune and inflammatory disorders where overactive Th 1 response plays a role. Thus, further studies using appropriate animal models are needed to determine the clinical significance of the observed immunomodulatory effects of white button mushrooms.

The polysaccharides, particularly β-1,3-glucans, are believed to be the active components in mushrooms and are therefore most frequently used in the studies to determine the effect of mushrooms. It is still not well understood how polysaccharides affect systemic immune function after oral ingestion given the fact that they are dietary fibers. However, several studies have, indeed, observed a tumor-inhibitory effect after oral administration of certain glucans (16,24,32–34). We previously reported that feeding mice a diet containing extract of the mycelia of Coriolus versicolor increased delayed-type hypersensitivity skin response, an in vivo measure of cell-mediated immunity, in old mice (18). Together with the findings of our dietary supplementation study, it is reasonable to suggest that, in contrast to common thought, immunomodulatory effects of mushrooms may not entirely depend on the distribution of these polysaccharides into the target tissues. Alternatively, because the cells (dendritic cells, Mφ, lymphocytes) in gut-associated lymphoid tissues have direct access to gut content and continuously recirculate in the blood and lymphatic systems, they can be activated by mushroom polysaccharides in the gut and move to blood and other systemic immune organs. It is also possible that when whole mushroom extracts are used, some nonpolysaccharide molecules present in mushrooms, such as lipids,
vitamins, flavonoids, minerals, and trace elements, with the immunomodulating property might contribute to the observed effects. However, the 2 and 10% mushroom powder added to the diets in this study do not substantially increase the level of nutrients that are known to have immunostimulatory effects above those present in the basal AIN-93M diet. For example, based on the food intake of mice and nutrient composition of raw white button mushrooms (35), we calculated that the 2 and 10% mushroom powder in diets provided only 2.4 and 12% of more zinc and 7 and 35% more selenium than those found in the basal diet, respectively. Therefore, it seems unlikely that the nonpolysaccharide nutrients present in mushrooms play a major role in inducing the enhancement of NK activity following mushroom consumption.

In conclusion, the results of this study demonstrate that dietary supplementation with white button mushrooms enhances NK cell activity. This effect of mushrooms may be mediated through increased production of IFNγ and TNFα. These results suggest that consumption of white button mushrooms may increase innate immunity to tumors and viral infections. Future studies are needed to determine the clinical significance of these findings, particularly in those with impaired immune functions, such as elderly, and in those with cancer.

**Literature Cited**