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A phase I/II trial of a polysaccharide extract from *Grifola frondosa* (Maitake mushroom) in breast cancer patients: immunological effects

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Abstract

Background—Cancer patients commonly use dietary supplements to “boost immune function”. A polysaccharide extract from *Grifola frondosa* (Maitake extract) showed immunomodulatory effects in preclinical studies and therefore the potential for clinical use. Whether oral administration in human produces measurable immunologic effects, however, is unknown.

Methods—In a phase I/II dose escalation trial, 34 postmenopausal breast cancer patients, free of disease after initial treatment, were enrolled sequentially in five cohorts. Maitake liquid extract was taken orally at 0.1, 0.5, 1.5, 3, or 5 mg/kg twice daily for 3 weeks. Peripheral blood was collected at days −7, 0 (prior to the first dosing), 7, 14, and 21 for ex vivo analyses. The primary endpoints were safety and tolerability.

Results—No dose-limiting toxicity was encountered. Two patients withdrew prior to completion of the study due to grade I possibly related side effects: nausea and joint swelling in one patient; rash and pruritus in the second. There was a statistically significant association between Maitake and immunologic function ($p < 0.0005$). Increasing doses of Maitake increased some immunologic parameters and depressed others; the dose–response curves for many endpoints were non-monotonic with intermediate doses having either immune enhancing or immune suppressant effects compared with both high and low doses.

Conclusions—Oral administration of a polysaccharide extract from Maitake mushroom is associated with both immunologically stimulatory and inhibitory measurable effects in peripheral blood. Cancer patients should be made aware of the fact that botanical agents produce more complex effects than assumed, and may depress as well as enhance immune function.

Keywords

Clinical trial; Breast cancer; Immunology; Maitake; Mushroom; Botanical; Herbal; Dietary supplement; Optimal dose; Complementary and alternative medicine

Introduction

Grifola frondosa (common names: Sheep’s head, Ram’s head, Hen of the woods, Maitake) is an edible polypore mushroom used in East Asian traditional medicine. Polysaccharide extracted from the fruit body of *G. frondosa* (Maitake extract) has demonstrated immunomodulatory effects in previous research. Maitake extract enhanced NK cell response (Kodama et al. 2002a, 2003); (Suzuki et al. 1989), macrophage and cytotoxic T cell activity (Takeyama et al. 1987) and antibody response (Suzuki et al. 1985), a study that also showed activation of alternative complement pathways (Suzuki et al. 1985). In addition, Maitake extract may modulate antigen presentation. The transfer of dendritic cells from tumor-bearing mice previously treated with Maitake extract protected the recipient mice against tumor implantation (Harada et al. 2003). Maitake extract increases interferon-gamma (IFN- γ) production in the mouse splenocytes. Further, the production of IL-12 was upregulated in RAW 264.7 macrophages in response to Maitake extract (Kodama et al. 2002b). Maitake also reversed immunosuppression resulting from chemotherapeutic agents (Kodama et al. 2005) and enhanced both growth and differentiation of mouse bone marrow cells subjected to doxorubicin chemotherapy, suggesting that Maitake extract reduces the suppression of hematopoiesis by certain chemotherapeutic agents (Lin et al. 2004).

The main component of Maitake extract is a glucan/protein (ratio 80:20–99:1) complex with a molecular weight about one million Dalton. The configuration of the glucan includes both 1, 6 main chain with 1, 3 side branches and 1, 6 main chain with 1, 6 side branches (Mayell 2001). Beta-glucans are known to interact with members of pattern recognition receptors, including complement receptor type 3 (CR3) and Dectin-1 (Taylor et al. 2007; Herre et al. 2004; Willment et al. 2005; Brown et al. 2002; Kennedy et al. 2007). Cytotoxic activation of

yeast beta-glucan-primed NK cells by opsonized tumors was accompanied by a tumor-localized secretion of interferon-alpha (IFN- α), IFN- γ , tumor necrosis factor-alpha (TNF- α), and interleukin-6 (IL-6) (Ross et al. 1999). In mouse studies, co-administration of barley or oat beta-glucans and tumor-specific antibody suppressed the growth of subcutaneous xenograft tumors (Cheung et al. 2002) and reduced the number of lung metastases (Penna et al. 1996), suggesting a role for beta-glucan in stimulating host innate immunity.

Many cancer patients use Maitake extract as a dietary supplement in efforts to enhance immune function. Although preclinical studies suggest that this medicinal mushroom extract may have anti-tumor activity, clinical evidence from rigorously designed prospective trials with therapeutic endpoints have not been conducted. Before such trials can be designed, the most appropriate oral dose of Maitake must be determined. There are no prior reports of a systematic dose escalation study of this agent. In this study, we used dose-limiting toxicities and immunological parameters as endpoints. Because the dose-response for this agent as for other botanicals may be non-monotonic—that is, an intermediate dose may have larger effects than low or high doses—we decided to have five dosage cohorts and then calculate a dose-response curve, a design that we previously described (Vickers 2006).

Materials and methods

Reagents

The study agent (Maitake extract) is an extract from the fruit body of the Maitake medicinal mushroom (*Grifola frondosa*). It was provided by Yuikiguni Maitake Corp. through the Tradeworks Group, Vermont. Maitake mushrooms are grown in a factory with strict controls of temperature, humidity, illumination, ventilation, and other environmental variables. This minimizes batch-to-batch variation as well as impurities. The raw Maitake was extracted with hot water and alcohol as described by Nanba et al. (1987). Gaia Herbs, Inc. formulated and packaged the final product in a facility that meets the “current good manufacturing practice (cGMP)” standard.

The extract was formulated in liquid form and packaged in a 60 ml light resistant amber glass bottle and stored in a refrigerator at 4°C under dark conditions until use. The lot of Maitake extract used in this study was sent to NAMS (Northwood, OH) for endotoxin contamination tests using the *Limulus* amoebocyte lysate assay. The results indicated that there was no detectable endotoxin activity (maximum level, 0.012 endotoxin units/mg).

Antibodies

The fluorescence conjugated antibodies for surface staining in this study were purchased from CALTAG/Invitrogen (Carlsbad, CA). Fluorescence direct conjugated anti-human IFN- γ , IL-2, IL-10, IL-12 and TNF- α antibodies were obtained from BD biosciences (San Diego, CA).

Patient population

Participants were enrolled at the Memorial Sloan-Kettering Cancer Center (MSKCC) Breast Center between 3/2004 and 1/2007. Non-MSKCC patients were evaluated by an MSKCC physician before enrollment in the study. Subjects were postmenopausal women (natural or treatment-induced menopause) at least age 18 with resected stage I, II, or III breast cancer currently free of disease. Subjects were excluded if they had a concurrent second primary malignancy, had completed radiation or chemotherapy within the past 3 months or hormonal therapy within the past 2 months, used herbal or nutritional supplements (other than vitamins or minerals) within the preceding 6 weeks, had a history of allergy to mushrooms,

used mushroom or other beta-glucan-containing supplements, had a history of autoimmune disease, or were currently using steroids or other immunosuppressants.

Phase I/II study design

Two baseline blood samples were taken from breast cancer patients 1 week before the first dose and on day 0, prior to ingesting the first dose of the Maitake extract. After ingestion of various doses of the Maitake extract (0.1, 0.5, 1.5, 3.0, and 5.0 mg/kg twice daily), blood samples were collected on days 7, 14, and 21. Immunophenotyping was carried out on freshly drawn peripheral blood, and surface phenotype was assessed by standard techniques such as fluorescence activated cell sorting (FACS) analysis.

Safety monitoring

Safety monitoring was performed continuously, and study participants were interviewed and examined by a physician at the beginning and at the completion of the study. A comprehensive "Review of Systems" was obtained during the encounters, and study subjects were instructed to report any new symptoms during the study period to the research assistants or the investigators. Adverse events were graded using National Cancer Institute, Common Terminology Criteria for Adverse Events, Version 3.0.

FACS analysis

All samples were fixed with 1.5% formaldehyde in PBS and analyzed by FACS. Monoclonal antibody combinations used in this study were: (1) CD14/CD45 to define lymphocytes and monocytes; (2) CD3/CD19 to define T- and B-lymphocytes; (3) CD3/CD8 and CD3/CD4 to define T cell subsets; (4) CD3/CD16+ CD56 and CD3/CD161/CD56 to detect NK cell subsets; (5) CD4/CD25 to define activated T cells; (6) CD45/CD34/CD33 to detect stem cells; and (7) appropriate isotype controls to detect leukocytes reactive against mouse antigens and nonspecific fluorescence.

Cytokine production analysis

Intracellular cytokine production was assessed using peripheral blood that had been incubated with and without activators, phorbol myristate acetate (PMA) combined with ionomycin, or lipopolysaccharide (LPS), in the presence of Brefeldin A. After incubation with or without activators, cells were stained for surface antigens, then permeabilized and stained for intracellular cytokines. Intracellular cytokine production was assessed using monoclonal antibodies specific for IL-2, IL-10, IL-12, IFN- γ and TNF- α . Intracellular controls and unstimulated cultures were used to compare the response in cells identified by surface staining. PMA and ionomycin were used to stimulate T cell cytokine response, and LPS was used to activate monocyte response.

Neutrophil/monocyte respiratory oxygen burst

Blood samples were assayed to determine monocyte and neutrophil oxidative burst in response to *N*-formyl-MetLeu-Phe (fMLP), PMA, and *Escherichia coli* (*E. coli*) by flow cytometry. Whole blood samples were activated by opsonized fMLP, PMA, and *E. coli*. Oxidative burst was detected as reduction of the dye, dihydrorhodamine.

Statistical analyses

Dose-response curves were calculated for each immune endpoint using a mixed-effect model with assessment time and baseline values as co-variates. Quadratic regression was applied to determine the optimal biological dose at which immune response was maximized. If the quadratic term was statistically significant at $p < 0.005$, it was retained in the model, otherwise only a linear term was used. A statistically significant association between dose

and immune response was defined as a $p < 0.005$ for the linear term or for the joint test of the linear and quadratic term as appropriate. Given that a large number of markers were examined, we would expect that some might be statistically significant by chance. For a global test of a dose–response relationship between Maitake and the immune endpoints, we used a permutation method. Exploratory analyses examined potential interactions between dose and time. All analyses were conducted using Stata 9.2 (Stata Corp., College Station, TX).

Results

A total of 34 eligible study subjects were enrolled from 3/2004 to 1/2007. Subjects were enrolled into one of the five dose cohorts (0.2, 1, 3, 6, or 10 mg/kg per day) sequentially. If a subject dropped out before providing evaluable efficacy endpoint, another subject was enrolled in the same cohort, so that each cohort included six evaluable subjects. The characteristics of enrolled subjects are shown in Table 1.

No serious adverse event was observed during the study period in any study subject. No dose-limiting toxicity was encountered. Two patients withdrew prior to the completion of the study due to side effects, one (cohort 1 mg/kg per day) withdrew after complaining of grade I nausea and joint swelling; the other (cohort 10 mg/kg per day) withdrew due to grade I allergic reaction (rash and pruritus) after two doses of Maitake extract. The allergic reaction was subsided without intervention. Three other subjects were consented, but did not receive Maitake extract due to change of mind or logistical problems. Subject flowchart is shown in Fig. 1.

There was a statistically significant relationship ($p < 0.005$) between dose and response for 25 of the 146 measured parameters. By a permutation test, the probability of a result at least this extreme under the null hypothesis was $p < 0.0005$. We saw no evidence of interactions between dose and time for any endpoint.

The dose–response curves for the 20 immunological relevant parameters for which there was a statistically significant dose–response effect are shown in Figs. 2, 3, 4, and 5. Different types of dose–response curves are displayed: a relatively smooth increase or decrease in the immune parameter with increasing dose of Maitake (Fig. 2); greatest effect at an intermediate dose, with a smaller effect at the highest dose (Fig. 3); and an initial small decrease followed by increasing immune effect with rising dose (Figs. 4, 5). For some parameters, changes were modest—such as less than 10% difference from baseline—even though the differences were statistically significant.

The largest functional changes (those more than 50% higher than baseline) were seen in granulocyte response to PMA stimulation, IL-10 production from CD14+ cell stimulated by PMA, IL-10 production from CD3+ cell stimulated by PMA, IL-2 production from CD56+ CD3+ cell unstimulated, and TNF- α production from CD3+ cells stimulated by LPS. All of these parameters had the largest increase at an intermediate dose of Maitake extract at around 5–7 mg/kg per day (Fig. 3). The largest phenotypical changes were seen with CD3+ CD56+ NK T cell and CD4+ CD25+ T cell (50% higher than base-line), both at a high dose of Maitake extract (10 mg/kg per day) (Figs. 4, 5). While most parameters showed increase in value from the baseline, IFN- γ production CD45RA+ CD4+ cell stimulated by PMA was decreased by about 20% at a high dose of Maitake (10 mg/kg per day) (Fig. 2).

Discussion

Polysaccharide extracts from a variety of mushrooms have demonstrated immunomodulatory effects in many in vitro studies. However, clinical evaluation of

immunological effects at different oral dosage levels had not been conducted. In this dose escalation trial, we show that (1) oral administration of Maitake mushroom extract is associated with significant changes in certain immunologic parameters in the peripheral blood, (2) Maitake extract appears to have a stimulatory effect on some parameters and a suppressive effect on others, and (3) the “optimal dose” of Maitake extract, the dose associated with the largest immunological effect, varies for different immunological parameters.

There appears to be no “maximum dose”, only “optimal dose” depending on the immunologic endpoint. For most functional parameters, such as granulocyte oxidative burst response to PMA, IL-10 production from CD14+ cells stimulated by PMA, IL-10 production from CD3+ cells stimulated by PMA, IL-2 production from unstimulated CD56+ CD3+ cells, TNF- α production from CD3+ cells stimulated by LPS, monocytes oxidative burst response stimulated by fMLP, and TNF- α production from unstimulated CD3+ cells, the “optimal dose” was at around 5–7 mg/kg per day of Maitake extract (Fig. 3). For augmented granulocytes response to fMLP, augmented IFN- γ production from unstimulated CD45RO+ CD4+ memory T helper cells and suppressed IFN- γ production from CD45RA+ CD4+ cells stimulated by PMA, the “optimal dose” is at the highest tested dose—10 mg/kg per day (Fig. 2). The “optimal dose” for phenotypical markers is also at the high dose of 10 mg/kg per day (Figs. 4, 5).

Our data support the notion that the maximum tolerable dose of botanical agents, especially those that function as immunomodulators, may not produce optimal clinical effects. This could be because most botanical agents contain more than a single constituent. Its individual components may act antagonistically on a diversity of target cells or molecules with varied potency. Another notable finding is that this agent appears to be associated with the production of both stimulatory (IL-2) and suppressive (IL-10) cytokines. This is contrary to the usual public perception that medicinal mushroom extracts boost immune function.

The clinical significance of the immunologic changes we observed is unknown. IL-10 is thought to be an anti-inflammatory cytokine involved in limiting collateral damage during infection, autoimmune processes and allergy (O’Garra et al. 2004; Couper et al. 2008; Hawrylowicz 2005; (Wu et al. 2007)). If Maitake extract increases the production of this cytokine, it suggests that Maitake extract may reduce inflammation in the setting of infection, autoimmune processes or allergy. On the other hand, the production of IFN- γ and IL-2, both immunostimulatory cytokines, also increased. The clinical effect of the balance of these cytokines is unknown. In general, the immunologic changes we observed were moderate, all below 100% of the baseline. The clinical significance of these moderate changes remains to be evaluated. In our trial, no apparent clinical changes were observed, but our study was not designed to look for clinical efficacy endpoints.

In summary, we found in this phase I/II trial of breast cancer survivors that oral administration of Maitake medicinal mushroom extract over a 3-week period is well tolerated. No dose-limiting toxicity was experienced up to 10 mg/kg per day. The intermediate dose (5–7 mg/kg per day) was associated with the most prominent functional changes, such as increased production of IL-2, IL-10, TNF- α and IFN- γ by subsets of T cells.

The public health implication of these findings is significant, as our data show that the immunologic effects of orally administered Maitake extract are complex and dependent on cell type and specific cytokine. It is therefore more accurate to view this medicinal mushroom extract as an immunomodulator, rather than as an immune enhancer. Cancer patients should be made aware of the fact that botanical agents produce more complex

effects than assumed, and may depress as well as enhance immune function. Moreover, the clinical effect in cancer prevention or treatment remains uncertain.

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Fig. 1.
Study participants flow chart (description and number of participants)

Fig. 2. Maitake dose (g/kg per day) versus immune factor change from baseline (%). Numbered 1 to 5: 1 geometric mean of granulocytes response to fMLP, 2 granulocytes response to fMLP, 3 IFN- γ production from CD45RO+ CD4+ cells unstimulated, 4 granulocytes response stimulated by PMA, 5 IFN- γ production from CD45RA+ CD4+ cells stimulated by PMA

Fig. 3. Maitake dose (g/kg per day) versus immune factor change from baseline (%). Numbered 1 to 7: 1 granulocytes response to PMA, 2 IL-10 production from CD14+ cells stimulated by PMA, 3 IL-10 production from CD3+ cells stimulated by PMA, 4 IL-2 production from CD56+ CD3+ cells unstimulated, 5 TNF- α production from CD3+ cells stimulated by LPS, 6 monocytes response stimulated by fMLP, 7 TNF- α production from unstimulated CD3+ cells

Fig. 4. Maitake dose (g/kg per day) versus immune factor change from baseline (%). Numbered 1 to 4: 1 CD3+ CD56+ NK T cells, 2 CD3+ T cells, 3 CD3+ CD161+ NK T cells, 4 CD3- CD19+ B cells

Fig. 5. Maitake dose (g/kg per day) versus immune factor change from baseline (%). Numbered 1 to 4: 1 CD4+ CD25+ T cells, 2 CD3+ CD25+ T cells, 3 CD45RA+ CD4+ cells, 4 CD45RO+ CD8+ cells

Table 1

Subject characteristics

Age	38–77 (median = 55)
Breast cancer stage	I (12)
	II (16)
	III (6)
Race	White non-Hispanic (25)
	White Hispanic (2)
	Black non-Hispanic (4)
	Asian/Pacific Islanders (2)
	Unknown (1)