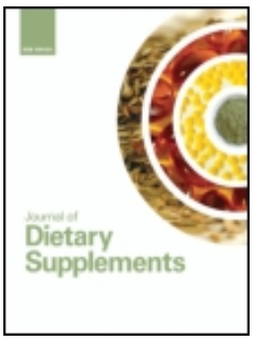

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Impact of Ingestion of Rice Bran and Shitake Mushroom Extract on Lymphocyte Function and Cytokine Production in Healthy Rats

Scott Giese, BS
George Richard Sabell, BS,
Mary Coussons-Read, PhD

ABSTRACT. This article provides a controlled evaluation of the ability of dietary supplementation with a commercially available rice bran extract modified with shitake mushroom extract (MGN-3) to support the immune function by assessing the ability of immunocytes to proliferate and produce cytokines in response to a mitogenic challenge. Twenty-four male Lewis rats were fed a control diet (Maypo sweetened oatmeal) or Maypo containing the recommended daily dose of MGN-3 for 2 weeks. This treatment modestly enhanced mitogen enhanced proliferation of splenocytes and interferon-gamma (IFN-g) production, and significantly increased proliferation of splenocytes to the superantigen toxic shock syndrome toxin-1 (TSST-1) as well as natural killer (NK) cell activity and production of interleukin-2 (IL-2) by stimulated lymphocytes. These data support the contention that ingestion of MGN-3 can support immune cell function. These data add to a growing body of data showing that ingestion of MGN-3 improves the ability of immune cells to proliferate the lyse tumor cells, suggesting that it may have utility as a dietary aid to support the immune system.

Scott Giese, George Richard SaBell, and Mary Coussons-Read are affiliated with the University of Colorado at Denver and Health Sciences Center, Department of Psychology, Department of Psychology, CB 144; POB 173364, Denver, CO, USA.

Address correspondence to: Mary Coussons-Read, PhD, University of Colorado at Denver and Health Sciences Center, Department of Psychology, CB 173; POB 173364, Denver, CO 80217–3364 (E-mail: Mary.Coussons-Read@cudenver.edu)

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KEYWORDS. Rice bran; shitake mushroom extract; rat; immune

INTRODUCTION

Numerous survey studies have shown that use of alternative therapies for enhancing the ability of the immune system to respond to illness and for the treatment of cancer and other illnesses is a common practice in the US (Cassileth, Lusk, Strouse, & Bodenheimer, 1984; Eisenberg et al., 1993; Eliason, Myszkowski, Marbella, & Rasmann, 1996; Lerner & Kennedy, 1992; Oneschuk, Fennell, & Hanson, 1998). These therapies range from the practice of meditation and massage techniques to the daily ingestion of dietary supplements. However, there has been little scientific research to validate the claims made by the promoters of these therapies, and thus, it is unknown whether the claims of improved health made by the consumers of these products are due to an actual physiological phenomenon or a placebo effect. (Begbie, Kerestes, & Bell, 1996; Lerner & Kennedy, 1992; McGinnis, 1991). Despite this lack of sound scientific evidence, the use of nutritional supplements to support health and immunity has risen to the forefront of the alternative medicine industry. With the huge variety of vitamins, herbal compounds, minerals, animal tissue, plant extracts, and various other therapies available, the supplement industry has become a multibillion-dollar industry (Eliason et al., 1996; Lerner & Kennedy, 1992; Montbriand, 1994; Oneschuck, Fennell, Hanson, & Bruera, 1998). This underscores the importance of isolating and differentiating between those compounds that may have innate immunosupportive ability and those that have no traceable physiological effects but are merely placebos. Moreover, assessing the effectiveness and physiological impact of these treatments in promoting health is critical, not only to ensure that the claims made by the purveyors of these products are bona fide, but also if there are any negative side-effects associated with the ingestion and use of these products.

This article provides a controlled evaluation of the effectiveness of one popular nutritional supplement in supporting cellular immune function in laboratory rats. The product tested here is a commercially available rice bran extract modified with shitake mushroom extracts and marketed under the name of MGN-3, which its manufacturer (Lane Labs, Inc., NJ) claims, significantly enhances the natural killer cell function. Natural killer (NK) cells are cytotoxic lymphocytes, which are major components of the innate immune system, and are instrumental in the rejection of tumors and virally-infected cells. Although a limited number of studies have assessed the effectiveness of this product in increasing immune cell function by

adding components of the product to cultured cells, (Ghoneum, 1998a; Ghoneum & Abedi, 2004; Ghoneum & Gollapudi, 2003; Ghoneum, & Jewett, 2000), no published studies have explicitly addressed the effects of ingestion of this compound on immune cell function, despite the fact that the product is marketed as a dietary supplement to be taken orally. The active ingredient of MGN-3 is an arabinoxylane from rice bran that has been enzymatically treated and modified with extract from shitake mushrooms (Hyphomycetes mycelia). This process appears to alter the arabinoxylane in a manner which renders it more readily available to interact with immune cells, and separate studies have shown that both the MGN-3 compound and mushroom extracts alone can support some aspects of immune cell function. For example, it has been previously reported that MGN-3 can enhance the ability of natural killer (NK) cells from cancer patients to kill tumor cells in a cell culture (Ghoneum, 1998a; Ghoneum, 1998b), and that it can help to reduce the rate of infection of cultured cells with HIV in cultured human lymphocytes (Ghoneum, 1998a). Although these and other studies have assessed aspects of immune cell function following the ingestion of MGN-3 in humans (Ghoneum, 1998b; Ghoneum & Jewett, 2000), controlled laboratory studies addressing the effectiveness of this compound are somewhat limited. For example, the above studies assessed immunomodulatory activity in cells from human peripheral blood, and because the studies were conducted in humans, the researchers were unable to fully control other variables, such as diet, drug use, exercise, and other subject characteristics, which might have affected immune cell function. In addition, these studies addressed the effectiveness of MGN-3 in modulating function in infected cells or in cells taken from patients who were using the preparation to treat a diagnosed disease (i.e., cancer). Many consumers who purchase and consume such products do so to enhance immune function to protect against cancer and infection, and are not overtly ill when they begin using the product. Moreover, these studies did not assess cytokine production, lymphocyte proliferation, and NK cell activity in the same subjects, but rather measured these different groups of outcomes in different people; assessment of these parameters in the same patients would be preferable because it could provide a more comprehensive assessment of the functional consequences of ingestion of MGN-3 on immune cell function.

More recently, Ghoneum and colleagues have undertaken studies of the effects of MGN-3 in mice and on cell lines, which have provided additional information about the effects of this compound. Injection of MGN-3 enhanced NK cell activity in aged mice, and addition of MGN-3 to culture increased the production of cytokines by culture human and

murine cell lines (Ghoneum & Abedi, 2004; Ghoneum, & Jewett, 2000). Although these studies further suggest that MGN-3 may support aspects of immune function, no published studies have performed controlled animal-based experiments to assess if ingestion of the compound, as directed by the manufacturer, has any impact on the activity of lymphocytes, nor have the studies addressed the duration of treatment necessary for observation of any immune benefit. To address these questions, the present study looked at the ability of short-term dietary supplementation with this product to enhance NK cell activity, lymphocyte proliferation to challenge, and thecytokine production of inflammatory cytokines in rats. The purpose of this approach was to measure the impact of ingestion of the suggested dose of MGN-3 on aspects of immune cell function in rats, in orderto provide an assessment of whether dietary supplementation with MGN-3 affects specific aspects of immune cell function in a manner which could enhance disease resistance.

MATERIALS AND METHODS

Subjects

Twenty-four healthy male Lewis rats, 150–175 g in weight, were purchased from Harlan-Sprague Dawley (Indianapolis, IN). Upon arrival, the animals were individually housed in polycarbonate cages with stainless-steel covers in a climate-controlled colony room, where a light-dark (12 hr) cycle was maintained through artificial illumination. The animals were permitted to acclimate for 2 weeks before the beginning of the experiment. Rats had 24-hr unlimited access to standard rat chow and water.

Experimental Treatment

The subjects were randomly divided into two groups of twelve each . Group 1 was the control group and received 15 g of Maypo brand oatmeal (Homestat Farm, PA), mixed according to the manufacturer's directions, in addition to their regularly available rodent diet daily for 14 days. Group 2 received 15 g of prepared Maypo supplemented with 0.25 g of MGN-3 daily in addition to their regularly available rodent diet. MGN-3 is an arabinoxylane from rice bran, a polysaccharide that contains $\beta 1$, and 4-xylopyronase hemicellulose, which has been enzymatically treated with an extract from hypomycetes mycelia (Purchased from Lane Labs, NJ). This process increases the ability of the compounds to interact and act on immune cells. One gram, three times daily (total of 3 grams per day) is the

manufacturer's recommended daily dosage of MGN-3 for adult humans, and the dosage used here was scaled to the rat's body size (0.25 grams per day). This dose was based only on body size, and did not account for other aspects of physiology such as liver function. Food and water intake were monitored and both groups were weighed daily. Without exception, all rats consumed all of the Maypo each day, regardless of control condition or MGN-3 condition, and thus, the dosage of MGN-3 was the same across all animals tested.

Tissue Collection

After the 14-day treatment period, all rats were sacrificed by cervical dislocation with a clamp and the spleens were removed. No anesthesia was used in this procedure due to the possible effects of anesthesia on the activity of the immune cells. The spleens were finely ground in complete RMPI-1640 (RMPI-1640 + 10% fetal bovine serum + 200 mM gentamycin) (ICN Technologies Costa Mesa, CA) and washed twice before being resuspended in complete RMPI (Roswell Park Memorial Institute)-1640. The cells were then quantified using Trypan blue exclusion and the suspensions were adjusted to 5×10^6 cells/ml in complete RPMI-1640.

Lymphocyte Proliferation Assays

The mitogens Concanavalin-A (Con-A) and Pyhtohaemagluttinin (PHA) were used to assess T-lymphocyte responsiveness in cultures of cells taken from the spleens of rats, fed either the control diet, or the diet supplemented with MGN-3 (see above for tissue collection procedure). In addition, proliferation of lymphocytes to the superantigen Toxic Shock Syndrome Toxin 1 (TSST-1) was measured (Sigma Chemical Co St. Louis, MO). Briefly, Con-A, PHA, and TSST-1 were prepared in concentrations of 0, 0.1, 1.0, and 10.0 μg/ml in supplemented RPMI-1640 to provide a background, suboptimal, and optimal concentrations of each substance. One hundred microliters of these preparations were added in triplicate to the wells of 96-well, flat-bottom, microtiter plates (Costar). Then 100 μl of the adjusted spleen cell suspensions were added to each well yielding final mitogen and superantigen concentrations of 0. 0.05, 0.5, and 5.0 μg/ml. The plates were incubated at 37°C in a humidified incubator, with 5% CO_2. The cultures were pulsed with 1 μCi[3] H-thymidine (ICN, Costa Mesa, CA) (specific activity = 6.7 Ci mmol) in 50 μl of supplemented RPMI-1640 during the last 6 hr of a 48-hr incubation. The cultures were

harvested onto glass fiber filter paper using a 12-well Skatron Combi harvester. The incorporation of ^{3}H-thymidine into dividing cells (a measure of the rapidity of cell division and proliferation) was determined with a liquid scintillation counter (Beckman Model LS 1701) and expressed as the mean of the triplicate counts/minute (CPMs).

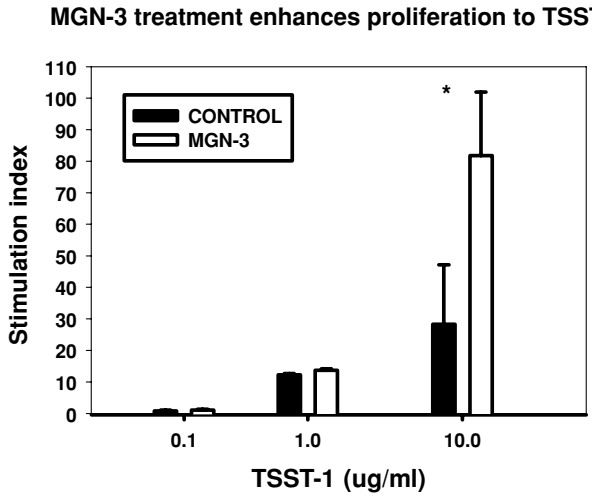
Natural Killer Cell Activity

Natural killer cell activity in the splenic lymphocyte population was assessed by using a standard cytotoxicity assay, details of which have been previously published (i.e., Coussons-Read, Dykstra, & Lysle, 1992; Coussons-Read, Dykstra, & Lysle, 1994). A murine T-cell lymphoma, YAC-1 (ATCC), was used as the target cell and was labeled with sodium chromate-51 (ICN, Costa Mesa, CA) for 70 min (0.2 mCi/7.5 $\times$ 10^{6}, 1 mice = 37 mBq). The labeled targets were plated on a 96-well microtiter plate with spleen cell suspensions, in triplicate, to yield effector cell (NK cells)/target cell (tumor cell) ratios of 100:1, 50:1, 25:1, and 12.5:1. The plates were incubated for 5 hr and then centrifuged. Control wells containing only labeled targets determined spontaneous release (leaking of radioactivity from cells) and cells containing labeled targets lysed with 10% trichloracetic acid were used to determine maximum possible release of radioactivity (the maximum amount that could come out if all the cells were lysed). Supernatants were harvested and the amount of ^{51}Cr released in the supernatant was determined with a liquid scintillation counter (Beckman). Lytic units were calculated using a computer program based on the equations of Pross and Maroun, (Pross & Maroun, 1984); Lytic units (LU) were defined as the number of NK cells used which causes 20% lysis of 5 $\times$ 10^{3} target cells (tumor cells).

Cytokine Production

One milliliter of the adjusted spleen cell suspensions prepared as above were cultured with 50 ug/ml Con-A in 12-well flat-bottom tissue culture plates (Falcon). The plates were incubated at 37°C in a humidified incubator, with 5% CO_2. At the end of a 48-hr incubation, the supernatants and cells were harvested and rapidly frozen and thawed twice to lyse the cultured cells to liberate intracellular cytokines. The lysed cultures were centrifuged at 1,000 g for 15 min, and the supernatants harvested and frozen at 70°C until cytokine assessment. Levels of the cytokines interleukin 2 (IL-2) and interferon gamma (IFNg) were assessed in culture

FIGURE 1. Mean (± SE) levels of stimulation (CPM/background) by TSST-1in spleen cell suspensions from control (solid bars) and MGN-3 (open bars) treated rats ($n = 12$). Asterisks indicate significant differences between groups ($p < .05$).

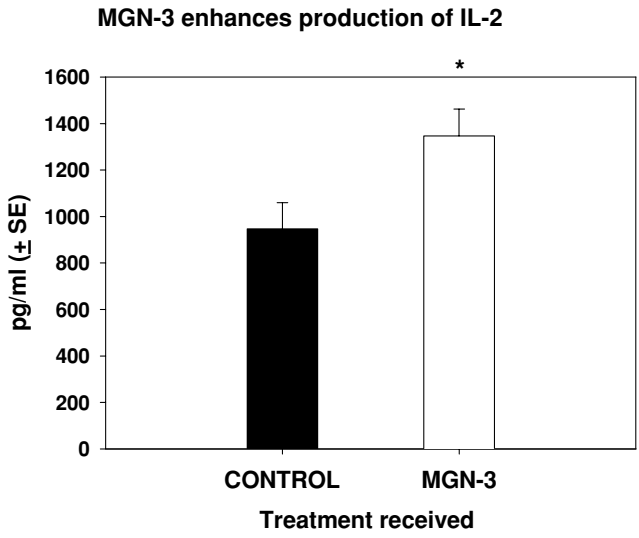


supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biosource Europe). Samples were assessed in duplicate and according to the directions provided by the manufacturer.

Statistical Treatment of Data

Statistical assessments were made via computerized program for analysis of variance (ANOVA); (Statistical Package for the Social Sciences, SPSS, Inc). CPMs from the lymphocyte proliferation assays were converted to stimulation indices in which the average CPM for each mitogen concentration from each animal was divided by the background stimulation level for each animal. Repeated measures analyses of variance (ANOVAS) were conducted using the stimulation indices for each concentration in which the within-subjects factor was concentration of the mitogen or superantigen, and the between subjects factor was the supplement treatment (MGN-3 or control). For the NK cell assay and the cytokine assessments, univariate ANOVAs were performed in which supplement treatment (MGN-3 or con-

FIGURE 2. Mean (± SE) levels IL-2 (pg/ml culture supernatant) produced by stimulated spleen cell suspensions from control (solid bars) and MGN-3 (open bars) treated rats ($n = 12$). Asterisks indicate significant differences between groups ($p < .05$).

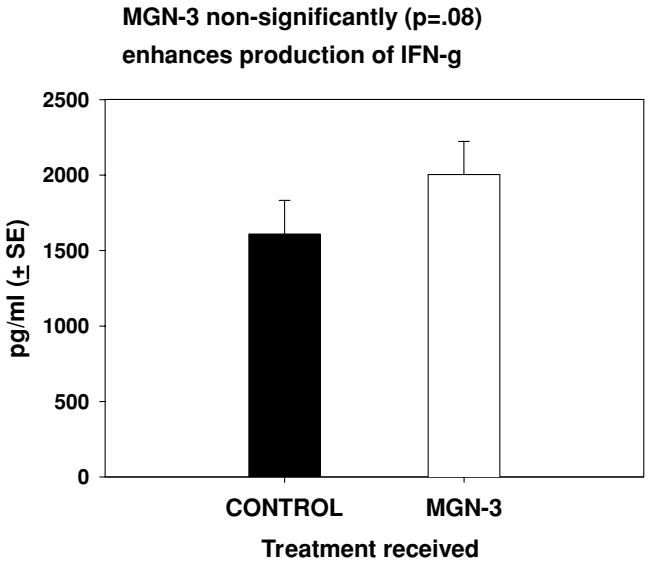


trol) was the variable we were looking at. The level of significance (p value) for the F test was 0.05.

RESULTS

Statistical analyses indicated that dietary supplementation with MGN-3 altered some measures of lymphocyte function and cytokine production. No significant differences in either average daily food consumption or in total body weight gained were observed between control and MGN-3 treated rats. For all reported proliferation assays, repeated measures ANOVA indicated a significant effect of the within-subjects factor of concentration ($p < .01$), with the level of cell proliferation increasing, as the concentration of the mitogens and superantigens increased, demonstrating that the cells were alive and able to proliferate as we expected from these stimuli. This is important because it shows that the laboratory assessments were working and that we had the tools necessary to look at the differences between MGN-3 fed rats and those fed the control diet.

FIGURE 3. Mean (± SE) levels IFN-g (pg/ml culture supernatant) produced by stimulated spleen cell suspensions from control (solid bars) and MGN-3 (open bars) treated rats ($n = 12$).

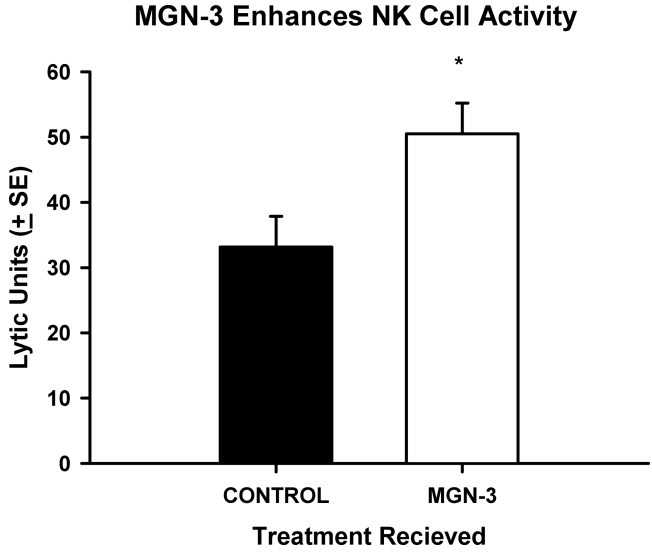


No significant differences in Con-A or PHA-induced proliferation were observed between MGN-3 and control diet-fed rats (data not shown). Figure 1 shows the stimulation indices from spleen cell cultures from rats treated with MGN-3 or the control diet after stimulation with the superantigen TSST-1. MGN-3 treatment significantly increased proliferation of splenocytes to the optimal concentration of TSST-1 as compared to control animals, $F\ (1, 12) = 3.712$, $p < .039$.

Figures 2 and 3 depict levels of IL-2 and IFN-g, respectively, in Con-A stimulated spleen cell suspensions from MGN-3 treated and control rats. Analysis of levels of these cytokines showed a similar pattern to that observed in the proliferation assays. MGN-3 treatment significantly increased production of IL-2, $F\ (1, 12) = 4.385$, $p < .032$, and modestly increased production of IFN-g, although this increase did not achieve statistical significance ($p < .08$).

Figure 4 shows level of NK cell activity in splenocyte cultures from rats treated with control diet or diet containing MGN-3, and includes the data from these assessments. Univariate ANOVA showed a significant

FIGURE 4. Mean (± SE) number of lytic units produced by NK cells from spleen cell suspensions from control (solid bars) and MGN-3 (open bars) treated rats ($n = 12$) against YAC-1 target cells. Asterisks indicate significant differences between groups ($p < .05$).



enhancement of NK cell activity in animals and fed a diet containing MGN-3 as compared to animals fed with the control diet, $F(1, 12) = 3.403$, $p < .045$.

DISCUSSION

Summary of findings: The data presented here provides the first demonstration that short-term ingestion of MGN-3 can modestly enhance aspects of immune cell function in laboratory animals. Rats which ingested MGN-3 for 2 weeks demonstrated significantly enhanced NK cell activity, as well as modest increases in mitogen and superantigen-induced splenocyte proliferation and production of the cytokines interleukin-2 (IL-2) and interferon-gamma (IFN-g) as compared with animals fed only the control diet. These changes did not merely reflect nutritional or body weight differences among the MGN-3 and control groups, as no significant differences in either daily food intake or weight gain were observed, although

the MGN-3 treated rats did gain slightly more weight over the course of the experiment than the control rats. This finding is consistent with previous work suggesting that ingestion of rice bran derivatives encourages weight gain in animals and humans (Annison, Moughan, & Thomas, 1995; Prakash, 1996). Overall, the data generated in the present experiment add to a growing body of evidence that this product may have measurable benefits for immune function, and raises new questions about the mechanisms and significance of these effects. These data show that indeed, basic aspects of immune cell function, including NK cell ability to kill tumor cells and lymphocyte ability to proliferate in response to a challenge and to produce cytokines important for driving the immune response are increased by supplementation with MGN-3 in a controlled study in rats.

Contributions of the present work: None of the previously published studies have assessed the impact of dietary supplementation with MGN-3 in a rat model, and the present study provides not only the first assessment of this type, but importantly, lays the groundwork for future controlled animal studies, investigating both the mechanism of the observed effects, and the possibility that MGN-3 treatment may improve resistance to and/or recovery from infection and illness. Although previous studies have shown similar effects to those reported here in leukocytes from the peripheral blood of humans fed MGN-3 and in mice injected with MGN-3, the present work is the first to explore if ingestion of the compound, consistent with the labeling of the product, can affect immune function in a controlled animal-based study (Ghoneum, 1998b; Ghoneum & Abedi, 2004; Ghoneum, & Jewett, 2000). The present data suggest that it may be possible to explore the potential benefits and possible adverse effects of dietary supplementation with compound for human patients via utilizing an animal model. Future studies could test to see if supplementation with MGN-3 reduces growth of implanted cancers or tumors in rats to begin to determine if the cell-level changes, which we report here, are meaningful for health or not. This is an especially important given study, showing that although many patients consume dietary supplements to treat already diagnosed illnesses such as cancer (Begbie et al., 1996; Oneschuk, Fennell, & Hanson, 1998), others ingest these substances in hope of preventing or protecting against infection and illness (McGinnis, 1991). Moreover, many supplements on the market promise to boost disease resistance and improve health, often with little scientific support to bolster these claims, or with any data on the possible adverse effects of the products advertised. It is important to note that although the present findings suggest that immune function is supported by dietary MGN-3 treatment, they do not demonstrate if these in vitro changes have relevance for disease resistance and recovery in human patients, nor

do they address possible negative side effects of the compound. Additional studies are necessary to determine the real effectiveness of dietary MGN-3 treatment in preventing and treating disease and to determine possible adverse effects of such treatment.

The primary contributions of this article to the existing literature on the efficacy of MGN-3 in enhancing immune function are the concurrent assessment of NK cell activity, lymphocyte proliferation, and cytokine production in the spleen and the finding that 2 weeks of dietary supplementation was sufficient to produce these effects in rats. Previous studies have separately demonstrated enhanced NK cell activity (Ghoneum, 1998b) and modest increases in mitogen-induced stimulation of peripheral blood leukocytes (Ghoneum & Jewett, 2000) in humans following a 2-month regimen of MGN-3 supplementation, but have not assessed these measures concurrently, as reported in the present study. The concurrent observation of these effects in the present study suggests an overall enhancement of cellular immune function associated with MGN-3 supplementation. Moreover, the present data also show concomitant increases in IFNg and IL-2 that complement the findings of increased NK activity and lymphocyte proliferation, further documenting a general enhancement of cellular immunity.

Future Directions: Although the present data and other studies demonstrate enhancement of aspects of immune cell function after treatment with MGN-3, the mechanism and significance of these effects in vivo remains unclear. A previous study by Ghoneum and Jewett suggests that MGN-3 can directly alter function of NK cells and cytokine production in human peripheral blood leukocytes (Ghoneum & Jewett, 2000). Addition of MGN-3 directly to cultured cells dose-dependently increased cytokine production by peripheral blood leukocytes and acted synergistically with IL-2, added to the same cell cultures. Although these data indicate a direct effect of MGN-3 on immune cell function, the actual mechanism for these effects require additional investigation. Moreover, the applicability of these findings to the in vivo system is limited, as MGN-3 ingested by an animal or human will be altered via the digestive tract and will most likely not reach the peripheral immune cells in its intact form (Annison et al., 1995). Several investigators have demonstrated that the components of both rice bran and mushroom extract can alter cell function and affect how biological systems work, and, in some cases, can affect in vitro immune cell function (Mizuno et al., 1992; Qureshi, Mo, Packer, & Peterson, 2000; Shoji et al., 2001). Additional work assessing the effects of administering the components of MGN-3 in the diet is necessary to better identify the ingredient(s) responsible for the observed effects; a critical aspect of

this approach will be determining what components of MGN-3 pass from the digestive tract into the bloodstream to reach peripheral and lymphoid tissues.

The present study showed modest enhancements in immune cell function in rats following only two weeks of MGN-3 supplementation. This timeframe is considerably shorter than previous human studies in which similar effects were observed after 2 months of treatment (Ghoneum, 1998b; Ghoneum, & Jewett, 2000). This discrepancy emphasizes the need for additional studies in both species to assess the required dose and durations of treatment needed to maximize the beneficial effects of MGN-3 treatment. Expansion of the work reported here may shed some light on this issue by determining if longer MGN-3 treatment is more efficacious. Many of the effects reported here did not achieve statistical significance, although they were clearly in the predicted direction. Manipulating the dose of MGN-3, the duration of treatment, and increasing the number of subjects will be likely to clarify these observations and provide a firm set of optimal dosage parameters for future studies of the significance of these effects for in vivo immunity and disease.

Conclusion: In conclusion, the data reported here add to a growing body of evidence that ingestion of MGN-3 can enhance the function of human and animal immune cells, lending modest support to the claims made by the purveyors of this product as immunosupportive. What remains to be seen, however, is if the changes reported here and in other articles have meaningful consequences for health, possible adverse effect of the product, and the mechanism through which these effects occur. Empirical approaches of this type are critical to ensure patient safety and to assure that the claims made by the developers and marketers of these products are justified.

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