AUGMENTATION OF MACROPHAGE PHAGOCYTOSIS BY MODIFIED ARABINOXYLAN RICE BRAN (MGN-3/BIOBRAN)

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MGN-3/BioBran, modified arabinoxylan rice bran, has been shown to be a potent biological response modifier (BRM) as manifested by stimulation of different arms of the immune system such as NK, T and B cells; however, its effect on macrophages has not yet been studied. The effects of MGN-3 on macrophage function was examined in vitro using 3 models: human macrophage cell line U937, murine macrophage cell line RAW264.7, and murine peritoneal macrophages (P-Mφ). Treatment with MGN-3 resulted in an increase in the percentages of attachment and phagocytosis of yeast by macrophages. The effect depends on the type of macrophage and the dose of MGN-3 applied. Macrophages also demonstrated enhancement in their spreading ability, post treatment with MGN-3. Results also showed that MGN-3, in a dose-dependent manner (1, 10, 100 μg/ml), significantly induced high levels of production of cytokines: TNF-α and IL-6. In addition, MGN-3 significantly increased nitric oxide (NO) production. This data demonstrates that MGN-3 is a potent inducer of phagocytic function by macrophage, and may suggest that MGN-3 is a useful agent for fighting microbial infection.

Macrophages may constitute an important arm of defense mechanisms in immune response (1-2). Evidence has been accumulated that confirms the potential importance of macrophages in the host defense against microbial infection (3-8). The antimicrobial activity by macrophages is mediated by secretion of cytokines and nitric oxide (1-4). Therefore, many attempts have been made to augment the antimicrobial and antitumor activities of macrophages. Higher levels of macrophage functions can be induced by a variety of agents such as recombinant interferon gamma (9), nitric oxide (10), Corynebacterium parvum (11-12) and BCG (13).

Since these macrophage activating agents do have severe side effects, we thought it would be of particular interest to examine the effect of a food supplement, MGN-3/BioBran, for augmentation of macrophage phagocytic function. MGN-3 is a denatured hemicellulose that is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from the Shiitake mushrooms (14). The main chemical structure of MGN-3 is an arabinoxylan with a xylene in its main chain and an arabinoxy polymer in its side chain. MGN-3 is a potent biological response modifier (BRM) that enhances human natural killer (NK) cell activity in vivo (15), increases TNF-α and IFN-γ production by human NK cells and by peripheral blood lymphocytes (PBL) (16), and increases T and B cell mitogen response (14). In a double blind study, Tazawa et al (17) found a prophylactic effect of MGN-3 against the common cold syndrome. In addition, MGN-3 reduces the toxicity of conventional chemotherapeutic agents (18-19). Furthermore, recent studies showed MGN-3

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sensitizes human T cell leukemia cells to death receptors (CD95)-induced apoptosis (20) and potentiates apoptosis in cancer cells induced by multiple anti-cancer agents in vitro (21). These findings may suggest that MGN-3 be considered as an additional weapon for fighting cancer. The objectives of the present study were to establish whether or not macrophage functions could be augmented by MGN-3, and define possible mechanisms underlying macrophage activation.

MATERIALS AND METHODS

**MGN-3**

MGN-3 is an arabinosylxylan extracted from rice bran that is treated enzymatically with an extract from Shiitake mushrooms. It contains polysaccharides (1,3-glucan and activated hemicellulose). MGN-3 was freshly prepared by dissolving in CM at concentrations of 1, 10, 100, 500 μg/ml. MGN-3 was provided by Diawa Pharmaceuticals Co. Ltd, Tokyo, Japan.

**Chemicals**

Chemicals and cell culture materials were obtained from the following sources: RPMI 1640, fetal bovine serum (FBS), and penicillin-streptomycin solution (Life Technologies, Inc.); phorbol myristate acetate (PMA) from Sigma, 1α, 25-dihydroxyvitamin D₃ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) thioglycolate medium (Difco Laboratories, Detroit, MI), actinomycin D (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT from Sigma).

**Complete medium (CM)** It consisted of RPMI-1640, and was supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 μg/ml streptomycin and penicillin.

**Macrophage cell lines**

Two macrophage cell lines were used in the present study. These included a human macrophage cell line, U937, and a murine macrophage cell line, RAW264.7 cells. All cell lines were purchased from American Tissue and Culture Collection (ATCC), Manassas, VA, USA. Human U937 cells were suspended at 2 x 10⁶ cells/ml in the CM supplemented with PMA (30 ng/ml), and 1α, 25-dihydroxyvitamin D₃ (0.1 μM). A 0.5-ml aliquot was dispensed into each well of a 48-well culture plate. The cells were washed and the remaining adherent cells were used for stimulation with MGN-3.

RAW264.7 cells were harvested, washed and suspended in the CM at 1 x 10⁶ cells/ml. A 0.5-ml aliquot was dispensed into each well of a 48-well culture plate. After 3 h of culture for adhesion, cells were washed and the remaining adherent cells were used for stimulation with MGN-3.

**Animals and preparation of peritoneal macrophages (P-Mφ)**

C3H/HeN female mice (CLEA Japan Inc., Tokyo, Japan) were used from the age of 6 to 9 weeks. All animal experiments were conducted according to the guidelines of the Laboratory Animal Center, Jichi Medical School. Peritoneal exudate cells (PECs) were taken from mice that had received 2 ml of thioglycolate medium intraperitoneally 4 days in advance. The PECs were washed and suspended in the CM at 1 x 10⁶ cells/ml. A 0.5-ml aliquot was dispensed into each well of a 48-well culture plate. After 3 h of culture, the nonadherent cells were washed off, and the remaining adherent cells were used as P-Mφ.

**Assay for macrophage phagocytosis**

**Preparation of S. cerevisiae:** Commercially available baker’s and brewer’s yeast, S. cerevisiae, was used. Yeast in suspension was washed once with phosphate-buffered saline (PBS). It was then incubated for 1 h at 90 °C to kill yeast and then washed 3 times. Following washing, yeast was quantified using a hemocytometer and cell suspensions were adjusted at 1 x 10⁶ cells/ml.

**Phagocytic assay**

Phagocytosis was assessed by cytoxin preparation as previously described with slight modifications (22-23). In brief, U937 cells and P-Mφ, 2 x 10⁶ cells in 0.1 ml CM, were pipetted to a 24 well flat bottom culture plate (Corning Inc., Corning, NY, USA) and were treated with MGN-3 at concentrations of 100 and 500 μg/ml for 2 days. Cells were then mixed with yeast at a 1:10 ratio. The mixtures were centrifuged in capped plastic tubes (16 x 100 mm; Falcon Plastic, Los Angeles, CA, USA) for 5 min at 50 xg, and incubated at 37 °C and with 5% CO₂. After 2h incubation, the mixtures were thoroughly re-suspended to detach loosely attached yeast from cells. Cell suspensions (200 μl) were used to make cytoxin preparations (Shandon Southern Instruments, Sewickly, PA, USA). Preparations were fixed in 100% methanol, air-dried, and stained with 4% Giemsa. Percentages of attachments and phagocytosis
were examined using oil immersion and a light microscope that was fitted with a 60x objective (Nikon, Tokyo, Japan). Assessment of attachment of yeast by phagocytic cells was calculated as the percentage of 500 phagocytic cells that attached to one or more yeasts. The assessment of uptake of yeast by phagocytic cells was calculated as the percentage of 500 cells that ingested one or more yeasts.

Measurement of the spreading ability
U937 cells and P-Mφ, 2 x 10^5 cells in 0.1ml CM, were pipetted to a 24 well flat bottom culture plate, and were treated with MGN-3 at concentrations of 100 and 500 μg/mL. At 2 days incubation at 37 °C and with 5% CO₂, cell suspensions (200 μl) were used to make cytoospin preparations and stained with Giemsa as described. Percentages of spreading cells were examined using oil immersion and a light microscope fitted with a 60x objective. The spreading ability was expressed as a percentage of spreading cells exhibiting pseudopods in a total of 500 cells.

Measurement of cytokines (TNF-α and of IL-6) and NO production
Measurement of TNF-α: The activity of TNF-α in culture supernatants of macrophages at 4 hr following stimulation was determined by a cytotoxic assay with L-929 cells in the presence of actionimycin D (24). After an overnight culture of L-929 cells with test samples in a 96-well culture plate, viable cells were stained with crystal violet. The blue color was extracted with 30% acetic acid solution and absorbance at 540 nm was measured. The activity of TNF-α (in units/ml) was calculated from the dilution factor of test samples necessary for 50% cell lysis, with correction by an internal standard of a recombinant human TNF-α in each assay.

Measurement of IL-6
The activity of IL-6 in culture supernatants of macrophages at 48 hr following stimulation was determined by a proliferation assay of IL-6-dependent mouse hybridoma MH60.BSF2 cells, (a gift from Dr. N. Nishimoto, Osaka University, Osaka, Japan) (25). The cells were cultured with test samples in 96-well culture plates for 3 days, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) was added for the last 4 hr of culture for formation of formazan blue crystals by viable cells (26). The supernatant was removed and the precipitated formazan crystals were dissolved with an isopropanol solution containing 5% formic acid to measure the absorbance at 540 nm. The activity of IL-6 (in units/ml) was calculated from the dilution factor of test samples required to induce 50% cell growth, with correction by an internal standard of a recombinant human IL-6 in each assay.

Measurement of NO
Production of NO was determined as the amount of nitrite, a stable end-product of NO, in the culture supernatant obtained at 48 h post stimulation. Nitrite was measured by a colorimetric assay using the Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylenediamine dihydrochloride in 2.5% H₃PO₄ solution) (27). The absorbance at 540 nm was measured and the nitrite concentration was quantified (in μM) using sodium nitrite as the standard in each assay.

Apoptic assay
Apoptosis is morphologically defined by cell shrinkage, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptotic cells in cytoospin preparations stained with Giemsa. In brief, U937 cells, 2 x 10^5 cells in 0.1ml CM, were pipetted to a 24 well flat bottom culture plate and were treated with MGN-3 at concentrations of 500 μg/mL for 2 days. The percentage of apoptotic cells was determined by counting the number of apoptotic and non-apoptotic cells of a total 500 cells in triplicate samples. Apoptosis was confirmed by using flow cytometry using propidium iodide (PI) technique. In this technique, dead cells pick up propidium iodide (PI) and fluoresce. Propidium iodide (PI) was briefly added to cells (1 x 10⁥/ml) to give a final PI concentration of (5μg/ml). Cells were stained for 30 m at room temperature in the dark and analyzed by FACScan (Becton Dickinson, San Jose, CA, U.S.A).

Statistical analysis
All experiments were repeated at least three times. Student’s t-test was used to assess the statistical significance of differences. Confidence level of <0.05 was considered significant.

RESULTS

Effect of MGN-3 on stimulation of phagocytosis
Human U937 cells and murine P-Mφ were treated with MGN-3 (100 and 500 μg/ml) for two days. Cells were then cultured with yeast and the percentage of attachment, phagocytosis, and spreading cells was examined at 2 h.
Percent of attachment

Data in Fig. 1 show that treatment with MGN-3 resulted in increased level of attachment by both cell types to yeast. U937 cells demonstrated a high percentage of attachment (27%) post treatment with MGN-3 at concentration of 100 μg/ml; however, when the concentration increased to 500 μg/ml, the level of attachment decreased to 12%. As compared to control untreated cells (10.6). As P-MΦ was treated with MGN-3, the percentage of attachment increased in a dose dependent fashion. MGN-3 at a concentration of 100 g/ml resulted in a 140% increase of attachment, that was further increased to 235%, as the concentration of MGN-3 reached 500 μg/ml, as compared to control untreated cells.

Percent of phagocytosis

Treatment of both cell types with MGN-3 resulted in an increase in the percent of phagocytosis in a fashion similar to that noted for attachment. Treatment of U937 cells with MGN-3 (100 μg/ml) resulted in a 200% increase in the percent of phagocytosis, and 120% as the concentration of MGN-3 reached 500 g/ml, as compared with control cells. With respect to P-MΦ, MGN-3 in a dose dependent manner elicited a notable response. At 100 μg/ml concentration, the percent of phagocytosis climbed to 150%, while a concentration of 500 μg/ml further increased attachment level to 200%, as compared to the control cells (Fig. 2).

Percent of spreading cells

Data in Fig. 3 show that treatment of U937 cells with MGN-3 (100 μg/ml) significantly increased the percentage of spreading cells (12 fold) as compared to the control untreated cells. The higher dose of 500 g/ml registered a 4.2 fold. On the other hand, P-MΦ demonstrated only a 1.7 fold increase in their spreading activity, post treatment with MGN-3 as compared with control cells.

Effect of MGN-3 on stimulation of macrophages for production of cytokines and NO

Three models of macrophages (human U937 cells, P-MΦ and murine RAW264.7 cells) were stimulated with MGN-3 (1, 10 and 100 μg/ml) and production of TNF-α, IL-6, and NO by these cells in response to the stimulation was investigated.

Production of TNF-α

As shown in Fig. 4, all three types of cells produced TNF-α in response to treatment of MGN-3, and in a dose dependent fashion. Increased production of TNF-α (10 U/ml) was noted post treatment with MGN-3 at 1 μg/ml. Higher concentration of 10 μg/ml produced a significant level of TNF-α, that was further increased at 100 μg/ml. Response of RAW264.7 cells to MGN-3 was the highest among the three models. Data in Fig. 4 show that the production of TNF-α by RAW264.7 cells at 10 μg/ml of MGN-3 was still higher than the production of TNF-α by the other two cell types treated with MGN-3 at the greater concentration of 100 μg/ml.

Production of IL-6

Production of IL-6 by the three models in response to MGN-3 was also examined. MGN-3 induced IL-6 production in a dose dependent fashion. (Fig. 5). A significant production of IL-6 (10-80 U/ml) was detected post treatment of the three cell types with 1 μg/ml of MGN-3, and augmented when increasing the concentration of MGN-3 at 10 and 100 μg/ml. Response of human U937 cells to MGN-3 was the highest among the three models.

Production of NO

Production of NO in response to MGN-3 was examined in both murine macrophage models; murine P-MΦ and RAW264.7 cells (Fig. 6). Production of NO was detected post treatment of both cells with 10 μg/ml of MGN-3, however, increasing the concentration to 100 μg/ml resulted in a significant production of NO in both types of cells, but RAW264.7 cells were more responsive than P-MΦ and produced about 5 fold of NO at 100 μg/ml of MGN-3. Production of NO by human U937 cells was examined. Neither MGN-3 nor LPS induced NO production by U937 cells. It is generally known that human cells are slowly responsive in the production of NO, following exposure to various immuno-stimulants.

Action of high dose of MGN-3 (500 μg/ml)

Cell apoptosis

U937 cells were cultured with MGN-3 at concentration of 500 μg/ml for 2 days and cell survival was determined. The results show that the background of dead cells was 2%. Co-culture
**Fig. 1.** Effect of MGN-3 on percent of attachment of two macrophage models to yeast. Human U937 and murine P-MΦ were treated with MGN-3 for 48 hr, and then incubated with yeast for 2 hr in a ratio of 1:10. Data represent the mean SD of 3 different experiments, * P < 0.05; as compared to control untreated cells.

**Fig. 2.** Effect of MGN-3 on percent of phagocytosis of yeast by two macrophage models. Human U937 and murine P-MΦ were treated with MGN-3 for 48 hr, and then incubated with yeast for 2 hr in a ratio of 1:10. Data represent the mean SD of 3 different experiments, * P < 0.05; as compared to control untreated cells.

**Fig. 3.** Effect of MGN-3 on percent of spreading cells of two macrophage models. Human U937 and murine P-MΦ were treated with MGN-3 for 48 hr, and then incubated with yeast for 2 hr in a ratio of 1:10. Data represent the mean SD of 3 different experiments, * P < 0.05, ** P <0.001; as compared to control untreated cells.
**Fig. 4.** Effect of MGN-3 on production of TNF-α by three macrophage models. Murine P-Mφ (A), murine RAW264.7 cells (B) and human U937 cells (C) were stimulated with MGN-3 (1, 10, 100 µg/ml). The culture supernatant obtained at 4 h after the stimulation was subjected to determination of TNF-α by bioassay. The data are the means ± standard errors of the mean (SEM) of triplicate samples. A representative result obtained from three independent experiments is shown.

**Fig. 5.** Effect of MGN-3 on production of IL-6 by three macrophage models. Murine P-Mφ (A), murine RAW264.7 cells (B) and human U937 cells (C) were stimulated with MGN-3 (1, 10, 100 µg/ml). The culture supernatant obtained at 48 hr after the stimulation was subjected to determination of IL-6 by bioassay. The data are the means ± standard errors of the mean (SEM) of triplicate samples. A representative result obtained from three independent experiments is shown.
of cells with MGN-3 resulted in an insignificant change in cell survival as compared with the background of cells.

**NO production**

Action of high dose of MGN-3 (500 μg/ml) on NO production was examined using murine P-Mφ and RAW264.7 cells. Data in Fig. 6 show that treatment with MGN-3 caused only a slight fluctuation in the level of NO by both types of cells as compared with 100 μg/ml.

**DISCUSSION**

MGN-3/Biobran is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushroom (14). The various biological functions of MGN-3, such as anti-HIV activity, increased T and B cell proliferation (14), and NK immunomodulatory function (15, 28) have been previously demonstrated. In this paper, we examined the ability of MGN-3 to activate another arm of the immune system, the macrophages. We tested many important properties associated with macrophages activation, such as production of cytokines (TNF-α and IL-6) and NO from 3 different macrophage models. Data of the present study demonstrated that MGN-3 significantly enhances macrophage phagocytic activity. The effect depends on the type of macrophages and the MGN-3 dose applied. In this study, we used 100 and 500 μg/ml of MGN-3 for studying the effects of this agent on phagocytosis and spreading, while we used a much smaller concentration (1-100 μg/ml) for studying its effects on cytokine and NO production. Earlier studies demonstrated that concentrations ≥100 μg/ml of MGN-3 are necessary to cause significant activation of cells, such as NK cells (15-16) and sensitization of leukemia HUT 78 cells to anti-CD95 antibody-induced apoptosis (20), while induction of cytokines such as IFN-γ secretion by human PBL was noticeable at 1-10 μg/ml of MGN-3 (16). Data from this study showed that treatment with MGN-3 at a higher concentration of 500 μg/ml resulted in an insignificant change in NO production, as compared with 100 μg/ml (Fig. 6).

In addition, this concentration did not cause apoptosis of U937 cells. Several investigators examined the enhancement of macrophage phagocytosis by natural agents; these include ginsan (2), Panax ginseng (29), shi-ka-ron and Chinese herbs (30), Perilla frutescens var. crispa (31), Platycodon grandiflorum (32-33), and fermented papaya preparation (34).

MGN-3-induce macrophage phagocytic activity was paralleled with an increase in cytokine secretion. Significant levels of TNF-α secretion were triggered by the presence of MGN-3 in a dose dependent manner. TNF-α is a multifunctional cytokine. It is produced by macrophage in order to destroy microorganisms (35-37) and is involved in the early phase of the cytokine cascade; it induces NO and IL-6 production (38). In addition, TNF-α promotes the generation of T cell-mediated antitumor cytotoxicity (39), the generation of lymphokine-activated killer cells (LAK) (40), and

![Fig. 6. Effect of MGN-3 on production of NO in two macrophage models. Murine P-Mφ (A) and murine RAW264.7 cells (B) were stimulated with MGN-3 (1, 10, 100, 500μg/ml). The culture supernatants obtained at 48 hr after the stimulation was subjected to determination of NO by Griess reagent. The data are the means ± standard errors of the mean (SEM) of triplicate samples. A representative result obtained from three independent experiments is shown.](image-url)
also regulates interleukin-2-mediated activation of immature human NK cells (41-43). Previously we have reported that MGN-3 is a potent inducer of TNF-α from human peripheral blood lymphocytes (PBL) and also increases levels of TNF-α from NK cells (16); this may suggest a possible involvement of this cytokine in the enhancement of NK activity in healthy subjects (15) and in cancer patients (28,44-45) post treatment with MGN-3.

Interleukin-6 (IL-6) is another cytokine produced by macrophage in order to destroy microorganisms. Data in the present study reveals a consistent increase in the level of IL-6, post treatment with MGN-3. The effect, in a dose dependent manner, was noted in the three macrophage models being studied. IL-6 has multiple biological activities in various cell types: IL-6 synergizes with IL-1 and promotes T cell proliferation, T helper cells differentiation, and the development of T cell-mediated cytotoxicity by CD8+ cells (46-47).

It has been shown also that nitric oxide (NO) is produced by activated macrophages. NO is synthesized endogenously by the enzyme nitric oxide synthase (NOS) in activated macrophages. It contributes to immune function, and in particular to ‘non-specific host defense’. In addition, NO plays an important role in the killing of intracellular microbial pathogens, and possesses tumoricidal activity (48). Treatment with MGN-3 increased NO levels in the two murine types of macrophages used. The effect was dose dependent. Increased production of NO was detected at a low concentration of MGN-3 (10 μg/ml), and further increased at 100 μg/ml. However, augmenting the concentration to 500 μg/ml caused only a slight fluctuation in the level of NO production by both types of cells, as compared with 100 μg/ml. Production of NO by human U937 cells post treatment with MGN-3 was also examined. Neither MGN-3 nor LPS induced NO production by U937 cells. It is known that human cells have a slow response in the production of NO, following exposure to various immunostimulants such as bacterial lipopolysaccharide (LPS) and interferon-γ (49). It was reported that interferon is the key cytokine for the induction of NOS2 in macrophages (50-51). The cytotoxic actions of NO against tumor cells appear to be related to inhibition of several heme-containing enzymes which are found in the mitochondrial electron transport complex and the citric acid cycle (52).

Data in the present study reveal a differential response among Mφ models towards the augmentory effect of MGN-3 on enhancing phagocytosis and secretion of cytokines and NO. The reason for this phenomenon is not known, but it could be attributed to the difference in the type of macrophage model: human versus murine, cell line versus murine P-Mφ, or due to the mechanisms of activation of macrophage models (i.e difference in the pathways of activation and possible interference of different cytokines of the activation process) (53-54). In conclusion, we have presented evidence for the role of MGN-3 in the enhancement of phagocytic activity, and in the production of cytokine and NO within the three macrophage models. This data suggests that MGN-3 may be a useful agent for fighting against microbial infection; it also indicates that MGN-3 is a potent BRM that is not restricted to specific immune cells, but may cause an overall activation of the immune system.

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