Augmentation of Macrophage Phagocytosis by Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

M. GHONEUM
Department of Otolaryngology, Drew University of Medicine and Science,
Los Angeles, CA, USA
M. MATSUURA
Jichi Medical School, Department of Infection and Immunity, Tochigi, Japan

Key words:
MGN-3, TNF- α , IL-6, Nitric Oxide, Macrophage, Macrophage Cell Line

Abstruct

BioBran/MGN-3, modified arabinoxylan from 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 effect of BioBran /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-M ϕ). Treatment with BioBran/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 BioBran/MGN-3 applied. Macrophages also demonstrated enhancement in their spreading ability, post treatment with BioBran /MGN-3. Results also ahowed that BioBran/MGN-3, in a dose dependent manner (1, 10, 100 μg/m*l*), sig-

This paper is published upon partial modification of an article in *International Journal of Immunopathology and Pharmacology*, Vol. 17 (3), pp.283-292, 2004.

nificantly induced high levels of production of cytokines : TNF-α and IL-6. In addition, BioBran/MGN-3 significantly increased nitric oxide (NO) production. This data demonstrates that BioBran/MGN-3 is a potent inducer of phagocytic function by macrophage, and may suggest that BioBran/MGN-3 is a useful agent for fighting microbial infection.

Introduction

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, BioBran/MGN-3, for augmentation of macrophage phagocytic function. BioBran/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 BioBran/MGN-3 is an arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain. BioBran/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 BioBran/MGN-3 against the common cold syndrome. In addition, BioBran/MGN-3 reduces the toxicity of conventional chemotherapeutic agents[18, 19]. Furthermore, recent studies showed BioBran/MGN-3 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 BioBran/MGN-3 be considered as an additional weapon for fighting cancer. The objectives of the present study were to establish whether or not macroph-age functions could be augmented by BioBran/MGN-3, and define possible mechanisms underlying macro-phage activation.

Materials and Methods

1. BioBran/MGN-3

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

2. 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, 1 α, 25-dihydroxyvitamin D_3 (Wako Pure Chemical Industries, Ltd., Osaka, Japan)

thioglycolate medium (Difco Laboratories, Detroit, MI), actionmycin 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 percent fetal calf serum (FCS), 2 mM glutamine, and 100 μg/m*l* streptomycin and penicillin.

3. 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 2×10^5 cells/m*l* in the CM supplemented with PMA (30 ng/m*l*), and 1 α, 25-dihydroxyvitamin D_3 (0.1 μM). A 0.5-m*l* aliquot was dispensed into each well of a 48-well culture plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), and cultured for 3 days to induce differentiation into macrophage-like cells. The cells were washed and the remaining adherent cells were used for stimulation with BioBran/MGN-3.

RAW264.7 cells were harvested, washed and suspended in the CM at 1×10^6 cells/m*l*. A 0.5-m*l* 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 BioBran/MGN-3.

4. Animals and preparation of peritoneal macrophages (P-M ϕ)

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 m*l* of thioglycolate medium intraperitoneally 4 days in advance. The PECs were washed and suspended in the CM at 1×10^6 cells/m*l*. A 0.5-m*l* 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-M ϕ.

5. 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℃ to kill yeast and then washed 3 times. Following washing, yeast was quantified using a hemocytometer and cell suspensions were adjusted at 1×10^7 cells/m*l*.

6. Phagocytic assay

Phagocytosis was assessed by cytospin preparation as previously described with slight modifications[22, 23]. In brief, U937 cells and P-M ϕ, 2×10^5 cells in 0.1 m*l* CM, were pipetted to a 24 well flat bottom culture plate (Corning Inc., Corning, NY, USA) and were treated with BioBran/MGN-3 at concentrations of 100 and 500 μg/m*l* for 2 days. Cells were then mixed with yeast at a 1:10 ratio. The mixtures were centrifuged in capped plastic tubes (16×100 mm; Falcon Plastic, Los Angeles, CA, USA) for 5 min at 50 ×g, and incubated at 37℃ and with 5% CO_2. After 2h incubation, the mixtures were thoroughly re-suspended to detach loosely attached yeast from cells. Cell suspensions (200 μl) were used to make cytospin 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). Assess-

ment of attachment 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.

7. Measurement of the spreading ability

U937 cells and P-M ϕ, 2×10^5 cells in 0.1 m*l* CM, were pipetted to a 24 well flat bottom culture plate, and were treated with BioBran/MGN-3 at concentrations of 100 and 500 ug/m*l*. At 2 days incubation at 37℃ and with 5% CO_2, cell suspensions (200 μl) were used to make cytospin 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.

8. 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 actionmycin 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/m*l*) 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.

9. 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-diphenyltetrazolium 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/m*l*) 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.

10. 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-naphtylethylendiamine dihydrochloride in 2.5% H_3PO_4 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.

11. Apoptic assay

Apoptosis is morphologically defined by cell shrinkage, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptic cells in cytospin preparations stained with Giemsa. In

brief, U937 cells, 2×10^5 cells in 0.1 m*l* CM, were pipetted to a 24 well flat bottom culture plate and were treated with BioBran/MGN-3 at concentrations of 500 μg/m*l* for 2 days. The percentage of apoptic cells was determined by counting the number of apoptic and non-apoptic 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 (1×10^6/m*l*) to give a final PI concentration of (5 μg/m*l*). Cells were stained for 30 m at room temperature in the dark and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

12. 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

1. Effect of BioBran/MGN-3 on stimulation of phagocytosis

Human U937 cells and murine P-M ϕ were treated with BioBran/MGN-3 (100 and 500 μg/m*l*) for two days. Cells were then cultured with yeast and the percentage of attachment, phagocytosis, and spreading cells was examined at 2 h.

2. Percent of attachment

Data in **Fig.1** show that treatment with BioBran/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 BioBran/MGN-3 at concentration of 100 μg/m*l*, however, when the concentration increased to 500 μg/m*l*, the level of attachment decreased to 12.1%, as compared to control untreated cells (10.6). As P-M ϕ was treated with BioBran/MGN-3, the percentage of attachment increased in a dose dependent fashion. BioBran /MGN-3 at a concentration of 100 g/m*l* resulted in a 140% increase of attachment, that was further increased to 235%, as the concentration of BioBran/MGN-3 reached 500 μg/m*l*, as compared to control untreated cells.

3. Percent of phagocytosis

Treatment of both cell types with BioBran/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 BioBran/MGN-3 (100 μg /m*l*) resulted in a 200% increase in the percent of phagocytosis, and 120% as the concentration of BioBran /MGN-3 reached 500 g/m*l*, as compared with control cells. With respect to P-M ϕ , BioBran/MGN-3 in a dose dependent manner elicited a notable response. At 100 μg/m*l* concentration, the percent of phagocytosis climbed to 150%, while a concentration of 500 μg/m*l* further increased attachment level to 200%, as compared to the control cells (**Fig.2**).

4. Percent of spreading cells

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

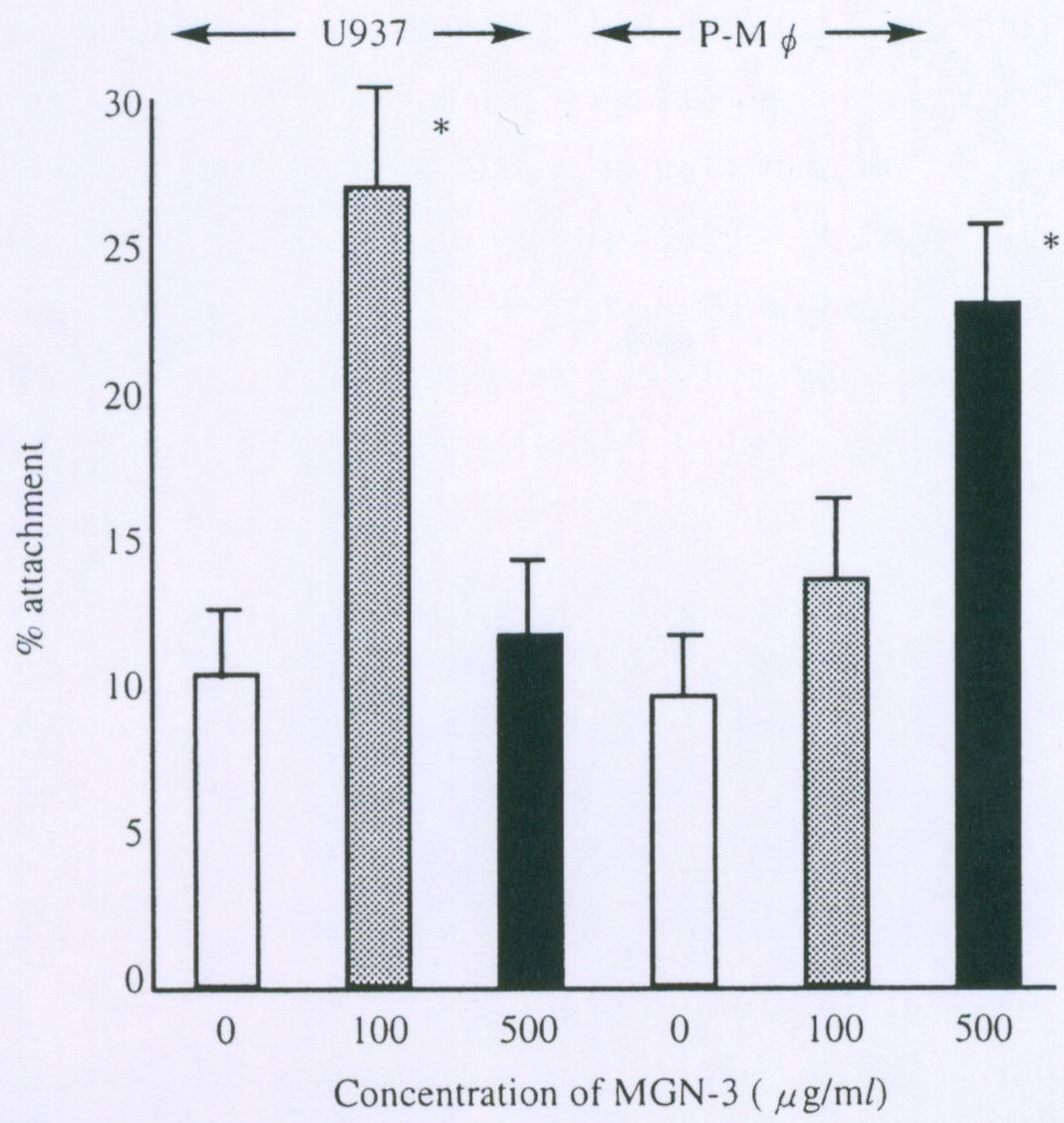

Fig. 1 Effect of MGN-3 on percent of attachment of two macrophage models to yeast.

Human U937 and murine P-M ϕ were treated with MGN-3 for 48 hr, and then incubated with yeast for 2 hr in a ratio of 1: 10.

Data repredent the mean SD of 3 different experiments, $*P<0.05$; as compared to control untreated cells.

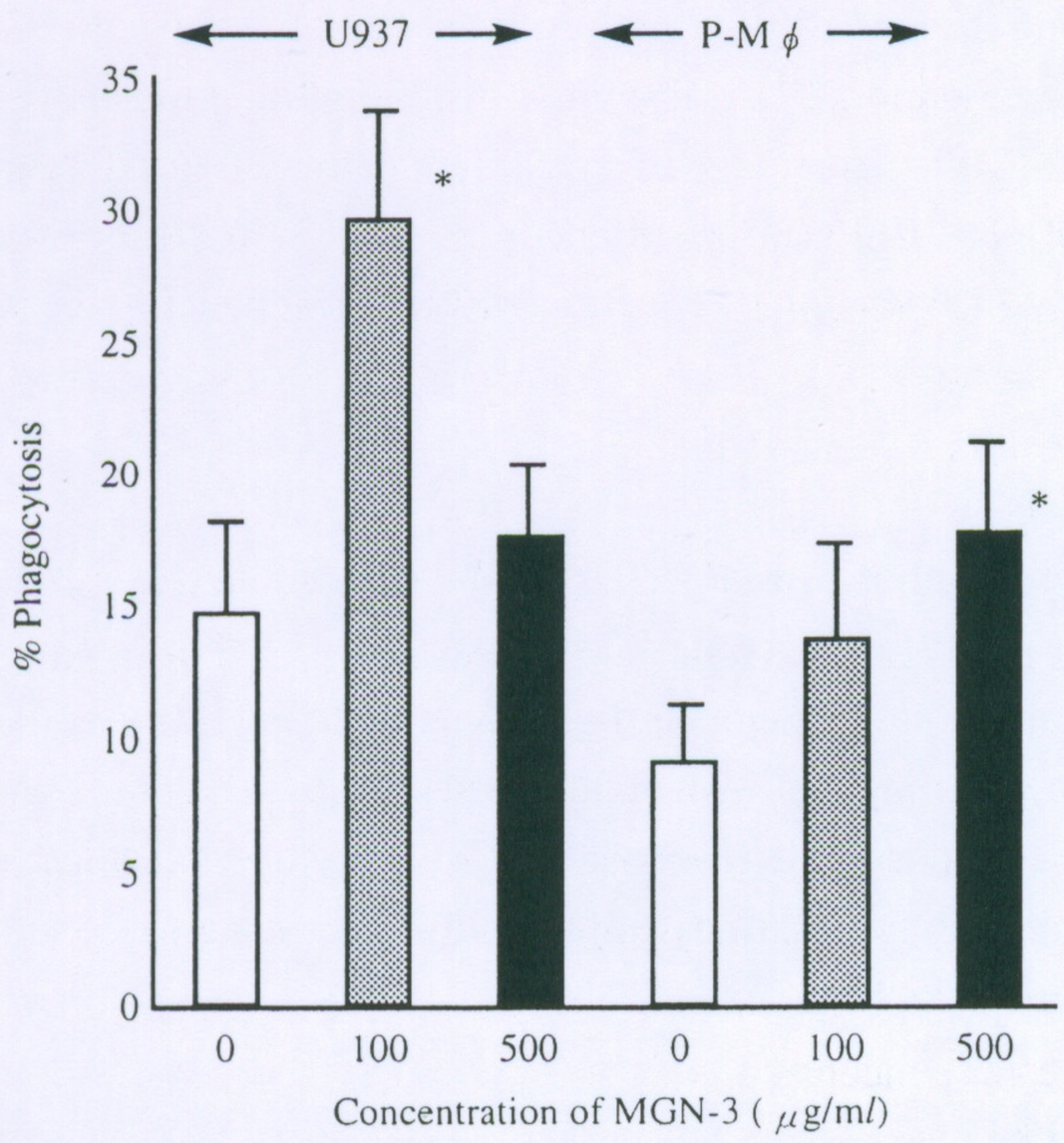

Fig. 2 Effect of MGN-3 on percent of phagocytosis of yeast by two macrophage models.

Human U937 and murine P-M ϕ 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.

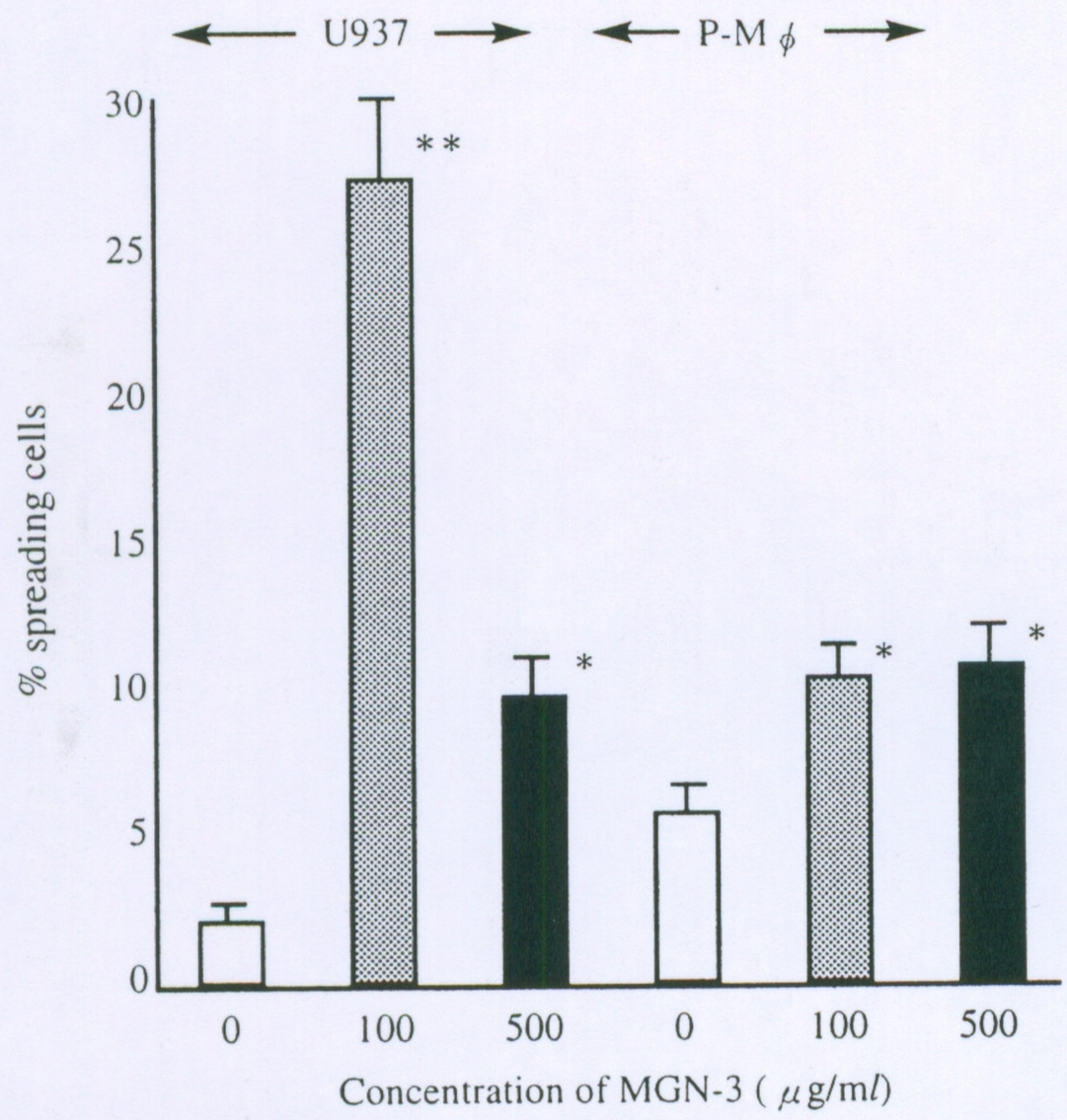

Fig. 3 Effect of MGN-3 on percent of spreading cells of two macrophage models.

Human U937 and murine P-M ϕ 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.

spreading activity, post treatment with BioBran/MGN-3 as compared with control cells.

5. Effect of BioBran/MGN-3 on stimulation of macrophages for production of cytokines and NO

Three models of macrophages (human U937 cells, P-M ϕ and murine RAW264.7 cells) were stimulated with BioBran/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.

6. Production of TNF-α

As shown in Fig.4, all three types of cells produced TNF-α in response to treatment of BioBran/MGN-3, and in a dose dependent fashion. Increased production of TNF-α (10 U/ml) was noted post treatment with BioBran/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 BioBran/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 BioBran/MGN-3 was still higher than the production of TNF-α by the other two cell types treated with BioBran/MGN-3 at the greater concentration of 100 μg/ml.

7. Production of IL-6

Production of IL-6 by the three models in response to BioBran/MGN-3 was also examined. BioBran /MGN-3 induced IL-6 production in a dose dependent fashion (**Fig.5**). A significant production of IL-6