II The basic studies of BioBran

II-review-1

Immunomodulation and Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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The phenomenon in which exposure to an infection once prevents the possibility of catching it again is called immunity. Jenner first applied this concept to vaccination against smallpox. Nowadays, immunity is understood in a broader sense, to mean the ability to distinguish between self and not-self, which is essential for maintaining homeostasis. If a harmful pathogen enters the body or a cancer cell develops, the body tries to eliminate it in a selective manner. Immunity is a basic function possessed by animals which is essential for survival, and is characterised by its selective response and specific memory. The immune organs include the spleen, lymph nodes, thymus, and the bone marrow, where lymphocytes and macrophages interact with each other to develop immune responses. Lymphocytes comprise B cells, T cells and natural killer cells, all of which have several subsets. Immunity can be categorised as humoral immunity, which involves immunoglobulin, and cellular immunity, which involves sensitised lymphocytes. There are innate immune responses, which are inherent, and acquired immune responses, which lead to immunity induced by the different pathogens and substances derived from plants or animals that enter the body after birth. Immune function is known to be affected by various environmental factors, including nutrition, exercise, sleep, environmental hormones, radiation, and stress. Nutrition is one of the most

important of these factors. Some functional foods and health foods can exert an immunomodulatory effect. When the immune system is compromised, it may be possible to strengthen biological defense and to work towards the prevention of diseases by taking those functional foods.

A variety of health foods have recently entered the market, including oligosaccharides, chitin, chitosan, yoghurt, colostrum, agaricus, salt, and soybean products. This is an indication of health awareness, showing that people's needs have diversified. In Western countries, the number of people who are using health foods and supplements has greatly increased. Many users want to be informed about the effects of these products, based on reliable data. However, the current flood of information is often confusing for many people. There is a need to evaluate health food products properly and produce data. It is likely that health foods where the effect is proven would be useful in preventing diseases and improving health.

Functional food is defined scientifically as "food designed and processed so that its functions can be used efficiently." The requirements for a food to qualify as a functional food are: 1. it must be produced for a clear purpose, 2. it must be orally effective, 3. the chemical structure of the functional factor in the food must be clear, 4. its form within the food (whether it is bound or free) must be clear, 5. the mechanism by which the functional factor acts must be known, 6. it must be very safe (i.e. the ratio of the unsafe dose to the effective doses must be high), 7. the functional factor must be stable in the food, and 8. it must be acceptable as a food. Food has the the following three types of function: 1. nutritional, providing the components of the body's materials and energy sources (the primary function), 2. sensory, including possession of the taste and texture suitable for a food (the secondary function), and 3. bioregulatory, fulfilled by special groups of substances originating from food (the tertiary function). Functional foods are designed such that these functions can be used efficiently and appropriately for the purposes intended. The following is a summary of the bioregulatory functions of food. The functions with respect to the digestive system include inhibiting the absorption of bile acid by peptides, lowering serum cholesterol, inhibiting the secretion of gastric acid, and increasing bifidobacteria. The functions within the cardiovascular system are lowering blood pressure, inhibiting platelet aggregation, and preventing cerebral strokes. The functional factors for the endocrine system include the various hormones involved in lactation, hormones derived from animals, factors to increase the secretion of insulin, and glucagon-like peptides. The functions related to the nervous system are associated with intestinal peristalsis and memory improvement. Functional factors in the biological immune defence system involve immunoglobulins (antibodies), transferrin, polysaccharides, and linoleic acid, which are associated with immunomodulation, host defence, and decreasing the action of allergens.

BioBran/MGN-3 has been reported to activate NK cells and macrophages. It also promotes the production of interferon γ and cytokines in lymphocytes. The following is an explanation of the immunomodulatory action of BioBran/MGN-3.

BioBran Beneficial for Our Health Defense System

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Our health defense system is an integrative one for maintaining wellness of our body. Basically speaking, it is composed of our defense system against foreign bodies (not-self) and our altered bodies themselves. Reality of our health defense system is relevant to a mode of inflammatory response. In this situation, immune-committed cells are main role in natural healing power. BioBran shows immunologically excellent function observed in animal models showing anti-inflammatory result as well as anti-allergic phenomenon.

Generally speaking, it is recognized that inflammation is often caused by any infection of bacteria or viruses so that causative factors of inflammation must come from outside of the body. Nevertheless, almost all phenomena of inflammation must come from inside of the body itself without any infection. For instance, sun burn after bathing or a bump at the forehead without wound must lead to inflammatory phenomenon in the skin. Primary trigger of inflammation have been recognized to come out from our cells themselves, such as a specific fatty acid, namely arachidonic acid basically integrated into the lipid bilayer of the cell wall. Arachidonic acid is one of the most fundamental substances causing a main cascade chain system of inflammatory chemical mediators involving prostaglandins and leukotrienes. In other words, cell wall of every cells in our body, whatever it is, must involve inflammatory cascade system, which could lead and potentiate inflammatory process, whenever any cell is damaged in the form of passive process of cell death (so-called necrosis, a technical term in pathology) due to whatever it is going to be. On the contrary, apoptosis, in other words programmed cell death, does not cause inflammation.

Both of the two are strictly different each other from the biological point of view.

Diseases from the outside, and those from the inside

Causative factors of representative diseases from the outside are bacteria, fungi, parasites or viruses, which have influenced enormous impact to human history in terms of catastrophic and horrible objectives. Infectious diseases are fundamental causes to kill human beings and to shorten life expectancy of them. Overcome of infectious diseases had been by all means the primary purpose of human history until discovery of antibiotics. Battles to overcome infectious diseases were basically started and have given people more fruitful progress than ever by practical and widespread use of light microscopy, which has shown people a micron-meter world by magnification of 1,000 times of microorganisms and cells.

This tool enabled to boost scientific progress to open a new world of modern medicine on the basis of establishment of pathology and microbiology especially in Germany and western world. In the middle of the 20th century, penicillin and other antibiotics were discovered and distributed world wide followed by of overcome of infectious diseases. Drs. Fleming and Waxman became heroes and deserved Nobel Prize. Thereafter, life expectancy of people in the developed countries has prolonged revolutionary and then diseases from the inside such as life-style related diseases such as diabetes mellitus, arteriosclerosis (hardening of arterial wall) due to hypertension and/or diet overload of lipid, and cancer, has emerged.

Diseases from the inside

Alteration of self body is closely related to nutritional abnormalities, allergy or cancer. These disorders are appeared insidiously and not easily so far cured by any modern treatment. We have not been able to discover any specific drug like antibiotics against bacterial infection. Every specific drug has tendency to cause, more or less, inevitable adverse effect, because of the necessity of long-term treatment using chemical substances. Therefore, the point to discuss health defense system of our body is to focus on the matter to protect or relieve diseases from the inside. Among others, cancer is to be discussed in this article from the point of protective efficacy of BioBran in the consequence of chronic inflammation as a major promoter in cancer formation.

What is cancer

Cancer is the disease of DNA. DNA is involved in the nucleus of every cell in our body. DNA is reality of cell generation, such as promotion or inhibition of cell growth of apoptosis (programmed cell death). These complicated and well-organized mechanisms are regulated by fundamental genome, so-called functional DNA sequence, namely exon. Irreversible alteration is occurred in cancer cells. Cancer cells are defined as autonomic growth not controlled by physiologically ordinary system. Therefore, regardless to say, cancer cells are originated from own cells and have characteristics nearly close to "self" property. Even if cancer cells are characterized as something foreign in nature, namely "not-self" just the same as bacteria or viruses for immune competent cells, cancer cells must be a target of immune cells in terms of rejection. However, this is in reality not the case. Nonetheless, transformed cells are assumed to be subtly different from self cell in immunological features. If ever, not-self part of cancer cells must be target epi-

topes in terms of vaccine potentiality in cancer cell. It is assumed that cancer cells in advanced stage must be quite different from self characteristics so that they must be target cells in cancer immunotherapy. Unfortunately, it is still on going in scientific and clinical research as one of the greatest goals in place of anti-cancer drugs.

Cancer in adulthood is thought to appear after a long term of latent period, more than 20 years. During the latent period, DNA may be damaged step by step by toxic effect closely related to life-style or environmental influence. Life-style related cancer occur only in adulthood and most frequently over 50 years of age. Incidence of life-style related cancer have been increased much more than expected, even though it is considered that life expectancy has been getting longer in order to a common sense saying that the longer the life expectancy is, the more chance to occur cancer in adulthood is increasing. It is stressed to say that cancer site in adulthood is quite different from that in children. Thus, cancer site in adulthood is thought to be closely related to life-style and environmental influence.

Chronic inflammation as a major promoter of cancer formation

As far as cancer formation is concerned, three theories as follows are accepted on the global standard.

1. Genomic abnormalities of cell growth, formerly oncogenes
2. Genomic abnormalities of cell growth inhibition, formerly tumor suppressor genes
3. Genomic abnormalities of apoptotic processes

Gene mutations must occur step by step at random along the double stranded chains of DNA in the cell nucleus mainly by oxidant production closely linked to oxygen consumption during metabolic processes every second. In accordance with the widespread theory of cancer formation, multistep theory of cancer is composed of initiation, promotion and progression of DNA damages and gene mutations. Author has considered chronic inflammation as one of the most potent promoters. Inflammatory cells such as neutrophils and macrophages have tendency to migrate and produce oxidant in the inflammatory area of the body. When tons of oxidant produced in the inflammatory area must attack bacteria, they would be beneficial for us. When they attack our own cells, they become enemy and they are damaged. Tissue are repaired by regenerating cells so that cell cycle are accelerated in the inflammatory area and DNA of regenerating cells must be a target of oxidant. Under these circumstances, epithelial cells are vulnerable in the inflammatory area and frequently damaged in terms of erosion or ulcer formation in the digestive tract or respiratory tract. Therefore, author would like to suggest that chronic inflammation is the most fundamental factor as promoter of cancer formation in adulthood.

Following evidence is pointed out to convince the above-mentioned hypothesis.

1. Stomach cancer in the antral area of chronic inflammation (chronic atrophic gastritis) due to *Helicobacter pylori*.
2. Decreased incidence of colon cancer due to anti-inflammatory drugs.
3. Skin cancer and sun burn (ultraviolet light dermatitis).
4. Higher incidence of lung cancer and chronic inflammation (chronic interstitial pneumonia) by cigarette smoking than those of smokers.
5. Cervical cancer and chronic contact cervicitis associated with human papilloma virus infection.
6. Hepatocellular carcinoma associated with liver cirrhosis and inflammation due to hepatitis B or C virus infection.

Summary

Key-word of this hypothetic scheme is oxidant and anti-oxidant. Anti-oxidant as well as anti-inflammatory effect of BioBran must be a potent candidate against cancer protection. Oxidant produced in the inflammatory area is a potent promoter and might attack DNA of regenerating epithelial cells which must fasten cell cycle. BioBran can protect oxidant attack as an anti-oxidant substance and inflammatory processes as anti-inflammatory effect so that it has prophylactic effect against cancer formation in adulthood. In advanced stage of cancer, BioBran can support immune function of natural healing power, which must accentuate immune system against advanced cancer cells in terms of less-self cancer cells as a major target of immunological rejection.

Moreover, this strategy must expand to apply to protect thrombotic reaction on the basis of chronic inflammation at site by BioBran. Initial step of atherosclerosis or thrombosis of the deep veins must be oxidant damage of endothelial cells along the arterial and venous system. Tar components of cigarette is one of the most potent oxidant effects so that they have to be avoided or suppressed by anti-oxidant. Thus, BioBran must be a versatile tool against various modes of life-style related diseases.

Enhancement of Human Natural Killer Cell Activity by Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Summary

Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) was examined for its augmentory effect on human NK (NK) cell activity *in vivo* and *in vitro*. Twenty-four individuals were given BioBran/MGN-3 orally at three different concentrations: 15, 30 and 45 mg/kg/day for 2 months. Peripheral blood lymphocyte-NK cell activity was tested by ^{51}Cr release assay against K562 and Raji tumor cells at 1 week, 1 month and 2 months post-treatment and results were compared with baseline NK activity. Treatment with BioBran/MGN-3 enhanced NK activity against K562 tumor cells at all concentrations used. In a dose-dependent manner, BioBran/MGN-3 at 15 mg/kg/day increased NK activity after 1 month posttreatment (twofold over control value), while significant induction of NK activity at 30 mg/kg/day was detected as early as 1 week posttreatment (three times control value). NK cell activity continued to increase with continuation of treatment and peaked (fivefold) at 2 months (end of treatment period). Increasing the concentration to 45 mg/kg/day showed similar trends in NK activity, however the magnitude in values was higher

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than for 30 mg/kg/day. After discontinuation of treatment, NK activity declined and returned to baseline value (14 lytic units) at 1 month. Enhanced NK activity was associated with an increase in the cytotoxic reactivity against the resistant Raji cell line. BioBran/MGN-3 at 45 mg/kg/day showed a significant increase in NK activity after 1 week (eightfold) and peaked at 2 months posttreatment (27 times that of baseline). Culture of peripheral blood lymphocytes (PBL) with BioBran/MGN-3 for 16 h demonstrated a 1.3 to 1.5 times increase in NK activity over control value. The mechanism by which BioBran/MGN-3 increases NK activity was examined and showed no change in cluster of differentiation (CD) 16^+ and $CD56^+$, $CD3^-$ of BioBran/MGN-3-activated NK cells as compared with baseline value; a four fold increase in the binding capacity of NK to tumor cell targets as compared with baseline value; and a significant increase in the production of interferon- γ (340-580 pg/m*l*) postculture of PBL with BioBran/MGN-3 at concentrations of 25-100 μg/m*l*. Thus, BioBran/MGN-3 seems to act as a potent immunomodulator causing augmentation of NK cell activity, and with the absence of notable side-effects, BioBran/MGN-3 could be used as a new biological response modifier (BRM) having possible therapeutic effects against cancer.

Introduction

There is an increasing body of evidence implicating the NK phenomenon as a discrete subpopulation of lymphocytes capable of mediating lysis of a variety of tumor target cells regardless of major histocompatibility components[1-5]. For example, it has been shown that patients with Chediak-Highashi syndrome who display selective natural killer (NK) deficiency are also prone to develop lymphoproliferative disorders[6]; defective NK cell activity predisposes patients to develop lymphomas[7]; genetic abnormalities associated with impaired NK activity may predispose animals to develop lymphoid malignancies[8,9]; adoptive transfer of NK clone to beige mice provided resistance to radiation-induced thymic leukemia[10]; and data from our laboratory show that female mice that have lower NK activity compared with males are also more susceptible to tumor development than males[2]. These data taken together provide compelling evidence supporting the role of NK cells in host surveillance.

The various immunological functions of NK cells make them prime candidates as therapeutic agents. Interleukin-2 (IL-2) has been shown to boost NK activity in peripheral blood, both *in vitro* and *in vivo*. These activated NK cells have broader antitumor cytolytic capabilities, including lysis of fresh, uncultured, human tumor cells as well as a wide variety of tumor cell lines[11,12]. Activated NK cells are defined as lymphokine activated killer (LAK) cells. Together, IL-2 and LAK cells have been used as adoptive immunotherapy against cancer[13]. However, although IL-2 has been reported to have promising results when administered to patients with advanced malignancies[14-16], the overall clinical success of IL-2 has been limited due to its severe side-effects.

In this study, we tested the ability of a new immunomodulator called BioBran/MGN-3, an enzymatically modified arabinoxylan from rice bran with no notable side-effects, to enhance human NK cell activity both *in vivo* and in *vitro*.

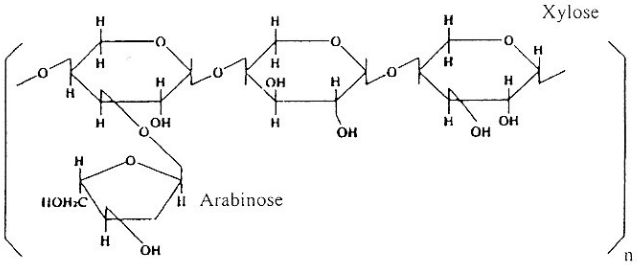

Fig.1 Main chemical structure of MGN-3.
It is an arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain

Materials and methods

1. Human subjects and treatment with BioBran/MGN-3

Twenty four healthy control subjects (15 females and nine males) participated in this study. Subjects ranged in age from 20-46 years, with a mean age of 34 years. They had not ingested any medications or vitamins for at least 2 weeks prior to their participation. Moreover, the subjects did not have a history of chronic diseases. During the course of the experiment that extended for 2 months, the women were not menstruating nor taking oral contraceptives—both of which can affect the level of NK cell activity.

Subjects were divided into three groups of eight individuals and were given BioBran/MGN-3 orally for 2 months. Group 1, group 2 and group 3 received BioBran/MGN-3 in doses of 15 mg/kg/day, 30 mg/kg/day and 45 mg/kg/day, respectively. Twenty m*l* of blood was drawn from each individual before treatment (day zero) and at different intervals posttreatment—1week, 1month and 2 months.

2. BioBran/MGN-3

BioBran/MGN-3 is an arabinoxylan from rice bran, a polysaccharide that contains β-1,4-xylopyranose hemicellulose, that has been enzymatically treated with an extract from hyphomycetes mycelia and was prepared in 500 mg tablets (Daiwa Pharmaceutical Co., Ltd., Tokyo, Japan). **Fig.1** shows the main chemical structure of BioBran/MGN-3.

3. Complete medium

Complete medium consisted of RPMI-1640 supplemented with 10% fetal calf serum and 1% antibiotic (100 U penicillin and 100 μg/ml streptomycin).

4. Tumor cell lines

Two human tumor cell lines were used as targets: sensitive K562, an erythroleukemic cell line, and resistant Raji, a Burkett cell lymphoma.

5. Preparation of peripheral blood lymphocytes (PBLs)

PBLs were prepared from fresh heparinized peripheral venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were washed three times with Hanks balanced salt solution and resuspended to 10×

10^6 cells/ml in complete medium.

6. Culture of PBLs with BioBran/MGN-3

PBLs from six healthy control subjects were adjusted to 1×10^6 cells/m*l* in complete medium and cultured with BioBran/MGN-3 at concentrations of 25 and 100 μg/m*l* for 16 h. PBLs were then washed twice and examined for NK activity at effector: target (E:T) ratios of 100: 1.

7. ^{51}Cr-release assay for measuring NK activity

NK activity was measured by a standard 4-h ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr-labeled tumor target cells in 0.1 m*l* complete medium were added to different wells of a 96-well microtiter plate. Effector cells were then pipetted into quadruplicate wells to give E:T ratios of 12:1, 25:1, 50:1 and 100:1. After a 4-h incubation (37℃), the plates were centrifuged (1,400 rpm for 5 min) and 0.1 m*l* of supernatant from each well was collected and counted in a gamma counter (Beckmann G50, Beckmann Instruments).

The percentages of isotope released were calculated by the following formula:

$$\%\ \text{lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

Spontaneous release from target cells was no more than 8-10% of total release. Total release was measured by adding 0.1 m*l* Triton X-100 (Sigma Chemical Co.) to designated wells. Lytic units were calculated from effector titration curves with one lytic unit defined as the number of effector cells required to achieve 30% lysis for K562 and 8% lysis for Raji cells. This serial performance of NK cytotoxic assay was based on criteria for a reproducible NK test and was intended to help minimize associated errors[17].

8. NK subpopulations

NK cell subset enumeration was carried out with PBLs from individuals for baseline and after 1 month treatment with BioBran/MGN-3. A single laser flow cytometer (Epics Profile, Coulter Epics, Inc., Hialeah, FL), which discriminates forward and right-angle light scatter, as well as two colors, was used with a software package (Quad Stat, Coulter). Mononuclear cell populations were determined by two-color direct immunofluorescence, by using a whole-blood staining technique with the appropriate monclonal antibody and flow cytometry[18]. Fluorescein isothiocyanate-(FITC, CD3-FITC), or phycerythrin (PE, CD56-PE)-conjugated monclonal antibodies (Coulter Immunology) were selected for the determination of NK cell subsets. To monitor lymphocyte markers, bitmaps were set on the lymphocyte population of the forward-angle light scatter versus a 90° light scatter histogram. The percentage of positively stained cells for each marker, as well as the percentage of double-stained lymphocyte positive for the respective surface markers, were determined.

9. Effector: target cell conjugate assay

The capacity of NK effector cells from different treatment conditions to form conjugates with K562 targets was measured as previously described[19]. Briefly, 1×10^5 lymphocytes were incubated with 1×10^6 K562 target cells in 1.0 m*l* complete medium in 12×75 mm glass tubes, pelleted at 130 G for 5 min and incubated for 1 h at 4℃. The pellets were gently resuspended and cytocentrifuged. Smears were prepared

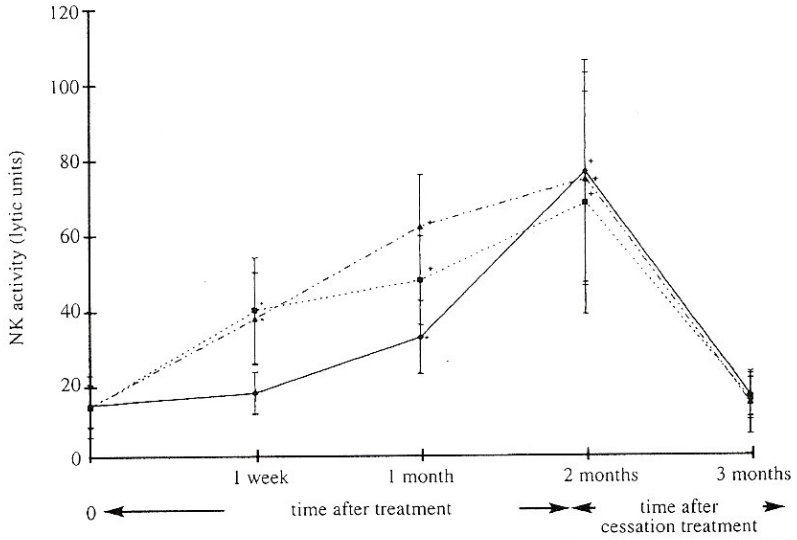

Fig. 2 Dose range and time course of natural killer (NK) cell activation by MGN-3 against K562 tumor cells

Activity is expressed as number of lytic units at 30%.
MGN-3 at 15 mg/kg/day (●), 30 mg/kg/day (◆) and 45 mg/kg/day (▲).
NK activity was examined at baseline zero day, 1 week, 1 month, 2 months and 3months.
Means±SD of eight different individuals in each dose. †$p<0.001$

using a cytospin cytocentrifuge (Shandon Instruments) and stained with Giemsa. The percentage of conjugates was determined by counting 200 lymphocytes (bound and free) in triplicate samples.

10. Interferon-γ production

Peripheral blood mononuclear cells from five individuals were collected, adjusted to 10×10^6 cells/m*l*, and cultured with BioBran/MGN-3 at different concentrations (0, 5, 50 and 100 μg/m*l*) for 16 h. Culture supernatants were then collected and analyzed for interferon (IFN)-γ using enzyme-linked immunoabsorbent assay.

11. Blood chemistry

Five individuals were given BioBran/MGN-3 (45 mg/kg/day) for 1 month. At day zero and the end of treatment, 5 m*l* of blood was drawn from each subject for blood chemistry analysis using Panel 20 which includes liver enzymes (serum glutamic-oxaloacetic transaminase[SGOT], and serum pyruvic-oxaloacetic transaminase [SGPT]).

12. Statistical analysis

The statistical analysis software (SAS) procedure of analysis of variance was used to examine the effects before and after treatment with BioBran/MGN-3 in different determinations; the effect of changing the ratios between E:T cells; and the interaction of the two effects.

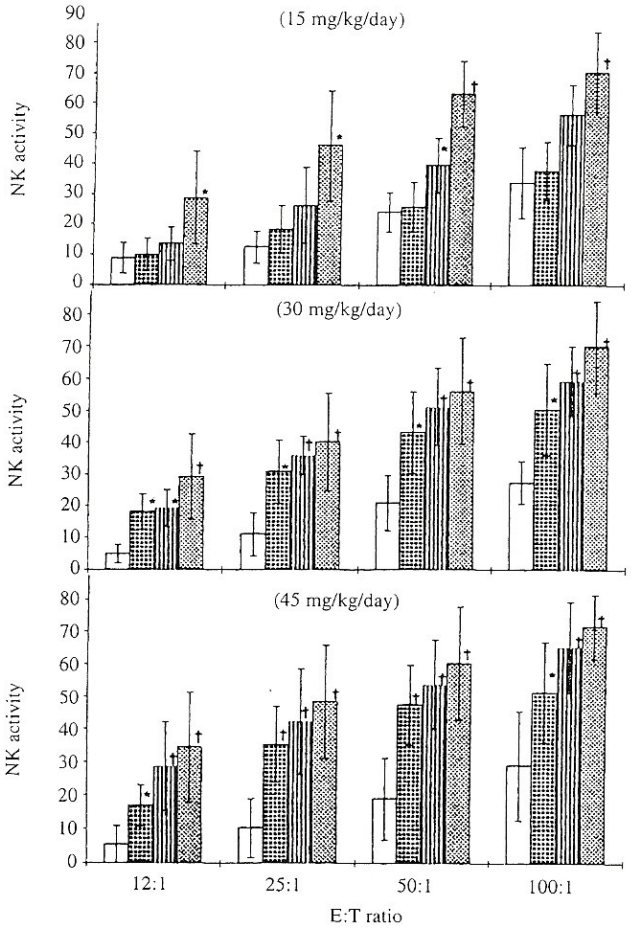

Fig. 3 Natural killer (NK) cell activation by MGN-3 against K562 at different effector: target (E:T) ratios
MGN-3 at 15, 30 and 45 mg/kg/day.
NK activity was examined at baseline, 1 week, 1 month, 2 months and 3 months posttreatment.
Mean±SD of eight different individuals in each dose. *$p<0.005$, †$p<0.001$

Results

1. Augmentation of NK activity by ingestion of BioBran/MGN-3

Results of NK activity enhancement by ingestion of BioBran/MGN-3 were expressed with respect to dose-response and time-course of changes in NK activity; E:T ratios; quantification of NK cells; and percentage of conjugate formation.

To test the reproduceability of the results, the present study was repeated with the same individuals after 4-6 month intervals, and by assaying NK function at different E: T ratios (12:1, 25:1, 50:1 and 100:1). These results were similar with regard to enhancement of NK function by BioBran/MGN-3 (second trial

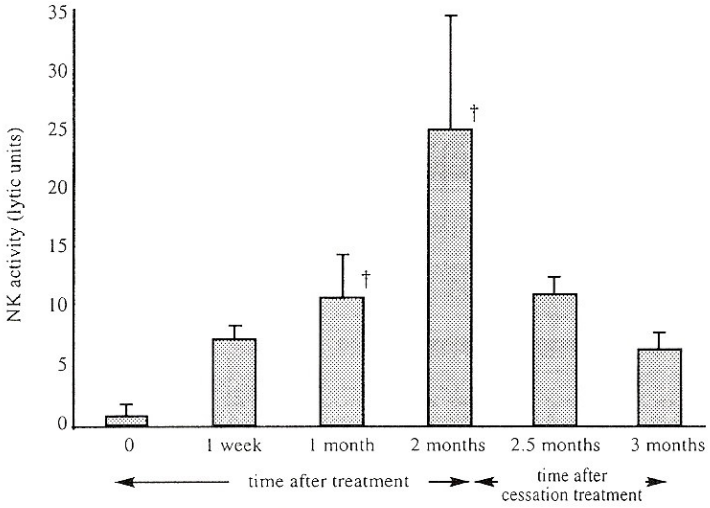

Fig. 4 Time course of natural killer (NK) activation by MGN-3 (45 mg/kg/day) against Raji cells
Activity was expressed as number of lytic units at 8% NK activity at baseline (zero day), 1week, 1month, 2 months, 2.5 months and 3 months.
Mean±SD of eight different individuals. † $p<0.001$

data not shown).

2. Effect against K562

Dose-range and time-course of NK activation by BioBran/MGN-3 was examined. **Fig. 2** shows the augmenting effect of BioBran/MGN-3 on NK cell activity against K562 tumor cells (activity expressed as number of lytic units). BioBran/MGN-3 at a dose of 15 mg/kg/day showed no changes at 1 week as compared with baseline values, however a twofold increase in NK cytotoxicity was detected after 1 month of treatment. Increasing the dose to 30 mg/kg/day resulted in a significant enhancement of NK activity (310% over baseline) that was detected as early as 1 week. The activity of NK cells continued to increase with continuation of treatment. The peak response was observed at the end of the treatment period (2 months) where NK activity increased fivefold (68.2 lytic units in comparison with 13.6 lytic units for baseline). Increasing the dose to 45 mg/kg/day demonstrated a similar increasing trend in NK activity but the values were higher in magnitude than those for 30 mg/kg/day. Discontinuation of treatment resulted in a decline of NK activity and at 1 month, NK activity returned to baseline. **Fig. 3** shows NK cell activity against K562 at different E:T ratios. All subjects demonstrated enhancement in their NK activity, however, there was a differential response among different individuals toward the augmentory function by BioBran/MGN-3. An increse in activity was detected for all E:T ratios, but the level of enhancement was higher at lower E:T ratios (12:1, 25:1) than at higher ratios (50:1, 100:1).

3. Effect against Raji cells

Fig. 4 summarizes NK activation against Raji cells. The augmentory effect of BioBran/MGN-3 was examined at 45 mg/kg/day taken for 2 months. Activated NK cells were able to kill Raji cells as early as

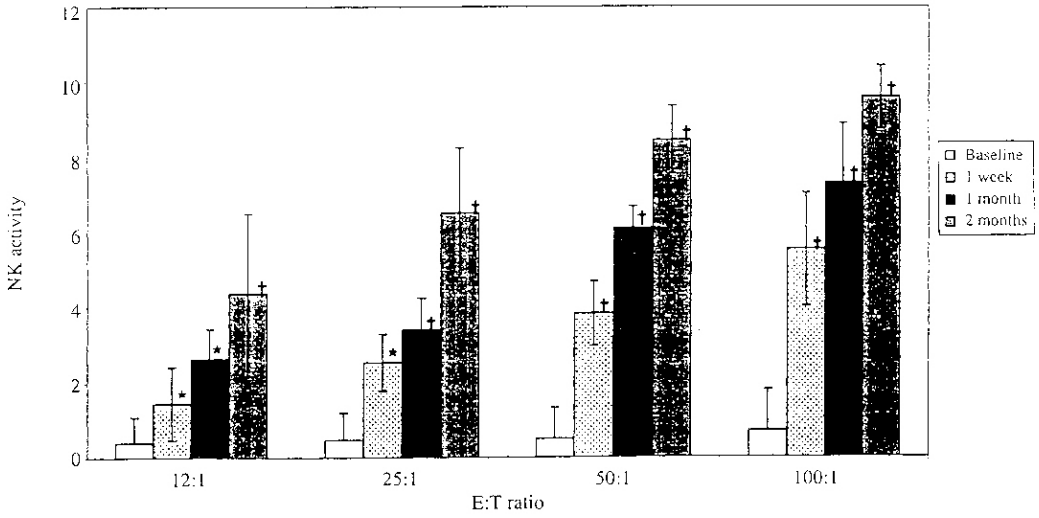

Fig. 5 Action of MGN-3 on natural killer (NK) activity against Raji cells at different effector : target ratios

Activity was examined at baseline, 1 week, 1 month and 2 months.
Mean±SD of eight different individuals. *p<0.005, †p<0.001

Table 1 Total NK cells posttreatment with MGN-3

Dose (mg/kg/day)	Baseline		1month after treatment	
	$CD56^-/CD3^-$	$CD16^+$	$CD56^+/CD3^-$	$CD16^+$
15	4.7±3.1	not tested	4.3±2.4	not tested
30	6.4±1.8	12.2±1.7	8.2±3.6	12±5.3
45	4.2±2.6	10±2	6.4±2.4	9±2.4

1 week (7.4 lytic units as compared with 0.9 lytic units for baseline). Activity continued to increase and peaked at 2 months after treatment (24.6 lytic units). After discontinuation of treatment, NK activity gradually declined to 10.9 lytic units at 2 weeks and 6.3 lytic units after 1 month. Enhancement of NK activity was detected at all E:T ratios (**Fig. 5**).

4. Quantification of total NK cells

Flow cytometry was used to analyze changes in the total NK cells cultured with BioBran/MGN-3 (at all doses). **Table 1** demonstrates that treatment with BioBran/MGN-3 at different concentrations had no significant effect on the percentages of total NK cell population as identified by $CD56^+$ $CD3^-$ and $CD16^{+-}$ monoclonal antibodies, respectively.

5. Percent conjugates

The binding capacity of NK cells to K562 tumor targets was examined after 1-month treatment (45 mg

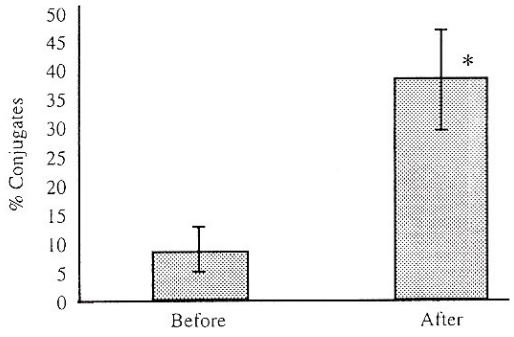

Fig. 6 Percentage of conjugate formation between natural killer (NK) cells and K562 target cells

Conjugate formation was examined at baseline (zero day) and at 1 month, posttreatment.
Mean±SD of five different individuals. *$p<0.005$

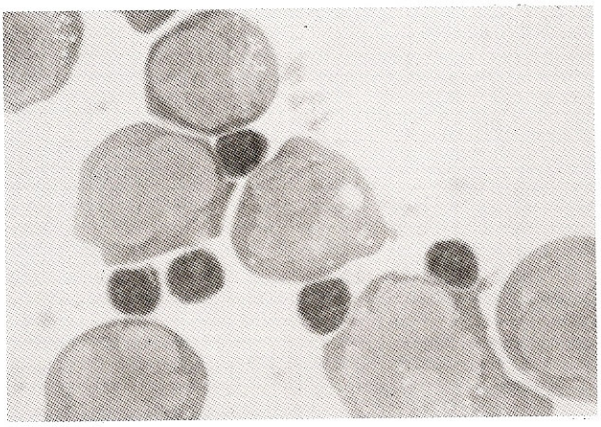

Fig. 7 Natural killer (NK) effector-tumor targets conjugate formation

Peripheral blood lymphocyte from an individual treated with MGN-3 (45 mg/kg/day) for 1 month and cultured with K562.
Note increased binding of NK cells to tumor cells and 1 NK cell binding to 3 tumor cells.
Giemsa stained.

/kg/day) with BioBran/MGN-3. **Figs. 6** and **7** show that the percentage of conjugate formation increased significantly posttreatment (38.5%), compared with baseline (9.4%).

In vitro studies

1. NK activity post culture with BioBran/MGN-3

Culture of PBL with BioBran/MGN-3 for 16 h resulted in an enhancement of NK cell activity that was dose-dependent. BioBran/MGN-3 at a concentration of 25 μg/m*l* increased NK activity by 130%. NK

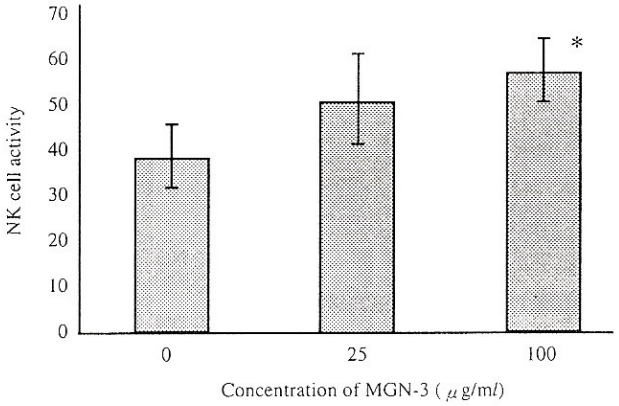

Fig. 8 *In vitro* **effect of MGN-3 on natural killer (NK) activity**
PBL were cultured for 16 h with MGN-3 at two different concentrations; 25 and 100 μg/ml.
NK activity examined at 100:1.
Mean±SD of five different individuals in each cencentration. *p<0.005

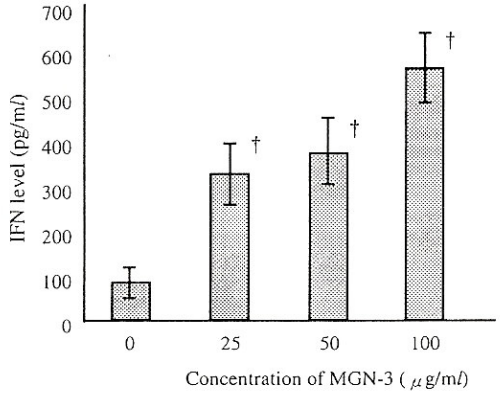

Fig. 9 *In vitro* **action of MGN-3 on interferon (IFN)-γ production**
PBL were cultured with MGN-3 at different concentrations: 25, 50 and 100 μg/ml
Mean±SD of five different individuals in each concentration. †p<0.001

activity was further enhanced (150%) when the BioBran/MGN-3 concentration was increased to 100 μg /ml (**Fig. 8**).

2. Production of IFN-γ

Fig. 9 summarizes results of the effect of BioBran/MGN-3 on the production of IFN-γ. PBL cultured without BioBran/MGN-3 showed little IFN-γ. BioBran/MGN-3 treatment resulted in a significant increase in IFN-γ production that was dose-dependent. Treated PBL at BioBran/MGN-3 concentrations of 25, 50 and 100 μg/ml demonstrated 340, 390 and 580 pg/ml of IFN-γ production, respectively.

Discussion

In this study a new biological response modifier, BioBran/MGN-3, was examined for its ability to enhance human NK cell activity *in vivo* and *in vitro*. The results showed that BioBran/MGN-3 is a potent biological response modifier (BRM) as manifested by significant induction of NK cytotoxicity upon BioBran/MGN-3 ingestion—the augmentory action was detected not only against sensitive K562 targets, but also against cell lines known to be highly resistant to NK activity, such as Raji, a Burkitt lymphoma, and Daudi another Burkitt cell line (data not shown). That BioBran/MGN-3 potent biological response modification is also shown by the decline of NK activity after discontinuation of treatment, as well as an increase in NK activity postculture with BioBran/MGN-3 for 16 h. The immunomodulatory function of BioBran/MGN-3 depended on two factors. Firstly, the concentration. As shown in **Figs. 2** and **3**, BioBran /MGN-3 increases NK activity in a dose-dependent manner. BioBran/MGN-3 at 15 mg/kg/day demonstrated an increase in NK cytotoxicity against K562 tumor cells after 1-month treatment, while higher concentrations of 30 and 45 mg/kg/day resulted in a significant induction of NK activity after 1-week treatment. Activity continued to increase and peaked at 2 months. Secondly the augmentory function of BioBran /MGN-3 is differential among subjects.

The type of BioBran/MGN-3-activated killer cells is not fully defined, but it may be similar to the IL-2-induced LAK cell phenomenon with respect to heterogeneity. Both types of cells are associated with increased conjugate formation between peripheral blood mononuclear cells and tumor targets[12]. Consequently, various effector cells may be involved. While NK cells may represent a major component of BioBran/MGN-3-activated cells, as seen in increased lysis of the classical NK-sensitive K562, lysis of resistant tumor cell lines (Raji and Daudi) may involve either NK cells and/or other effector cells having anti-cancer activity such as cytotoxic T-lymphocytes. Another similarity between the two induced cell types (BioBran/MGN-3 and IL-2) is the upregulation of $CD25^+$ and $CD69^+$ receptors[20].

The mechanism by which BioBran/MGN-3 boosts NK activity appears to be via its ability to induce IFN production. Most agents that can activate NK cells appear to be acting by their ability to induce, either *in vivo* or *in vitro*, IFN production[21]. Several bacterial immunomodulators, such as *bacilli* Calmette-Guérin and *Corynebacterium parvum*, have been found to induce IFN and augment NK activity[22]. Among the IFN stimulators studied so far, bacteria appear to have specificity for induction in large granular lymphocytes (LGLs) alone[23]. Other biological agents can induce rapid IFN production from LGLs and it is the production of IFN which produces the self-activation of NK activity in LGLs. Our work shows that BioBran /MGN-3 (25-100 μg/m*l*) induces PBL IFN-γ production (340-580 pg/m*l*) in culture. This suggests that BioBran/MGN-3 enhances IFN production, which in turn may augment NK activity. Our work also shows the ability of BioBran/MGN-3 to stimulate the production of other cytokines such as TNF-α [20].

It is possible that the increase in NK cell activity from ingestion of BioBran/MGN-3 may be due to an increase in the activity per cell and not due to an increase in the actual NK cell number. This was confirmed by noting that lower E:T ratios (12:1 and 25:1) achieved a maximum induction in NK activity (in comparison with 100:1), while flow cytometry analysis showed no significant changes in total NK cells after treatment (compared with baseline values). The results also showed an increase in the binding capacity of BioBran/MGN-3-cultured effector cells to tumor targets (four fold). The increased number of NK cells in conjugates with no increase in NK cell numbers in PBLs after treatment suggests that BioBran

/MGN-3 increases the binding capacity of NK cells as well as other cell populations to tumor targets. It is well established that the NK-tumor cell interaction proceeds through several discrete stages[24] including E: T cell recognition and binding; triggering and activation of the NK cells; release of the granules from NK cells and binding to receptor sites on the tumor cell surface; and tumor target cell death. This binding process is a key event in the activation of NK cells.

The anticancer activity of natural BRMs may involve activation of different arms of the immune system. For example, killed bacteria (*Corynebacterium parvum* and *Bacilli* Calmette-Guérin) may involve NK activation[25-28]; lectins from plants such as *griffonia simplicflial-*β4 isolectin may involve activation of macrophage[29]; bitter melon protein may involve neutrophil activation[30]; and lentinan isolated from edible mushroom, *Lentius edodes* (Berk) Sing. possesses augmentory effects on NK and killer T-cell activity[31-35]. With respect to BioBran/MGN-3, activated cells are mainly NK cells although cytotoxic T-lymphocytes may be involved in the activation process. Extracted hemicellulose from rice bran fiber has known unique biological effects; for example, α-glucan from rice bran shows potent antitumor activity in mice[36], and arabinose and xylose from rice bran fiber shows defensive effects against bis(tri-*n*-tributyltin)oxide-induced thymic atrophy in rats[37]. Unprocessed rice bran fiber and cholestyramine have been observed to increase peripheral blood leukocytes in humans[38]. The product used in this study is a modified arabinoxylan from rice bran. Modification occurs by enzymatic treatment with an extract from *Hyphomycetes mycelia*, which shows a high augmentory effect on human NK activity *in vivo* and *in vitro*, as well as in mice and rats (data not shown). BioBran/MGN-3 was examined for toxicity using blood chemistry analysis utilizing Panel 20 which includes liver enzymes (SGOT and SGPT). After 1-month treatment, no abnormalities were detected for these parameters as compared with baseline. We conclude that the high augmentory effect of BioBran/MGN-3 and the absence of notable side-effects make this material a promising immunotherapeutic agent for treating cancer patients. Preliminary studies in this regard are very encouraging. NK immunomodulatory function by BioBran/MGN-3 was detected in 27 patients afflicted with different types of malignancies[39], additionally, the relationship between the immunomodulatory and anticancer properties of BioBran/MGN-3 was assayed in five patients with breast cancer[40].

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

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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-

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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, 500μg/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, 1α, 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 μ g/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 μ g/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 μ g/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 μ g/m*l*, however, when the concentration increased to 500 μ g/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 μ g/m*l* concentration, the percent of phagocytosis climbed to 150%, while a concentration of 500 μ g/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

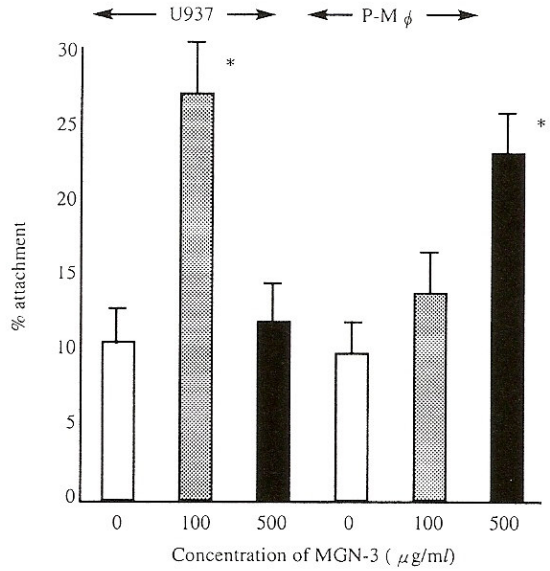

Fig. 1 Effect of MGN-3 on percent of attachment of two macrophage models to yeast.

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.

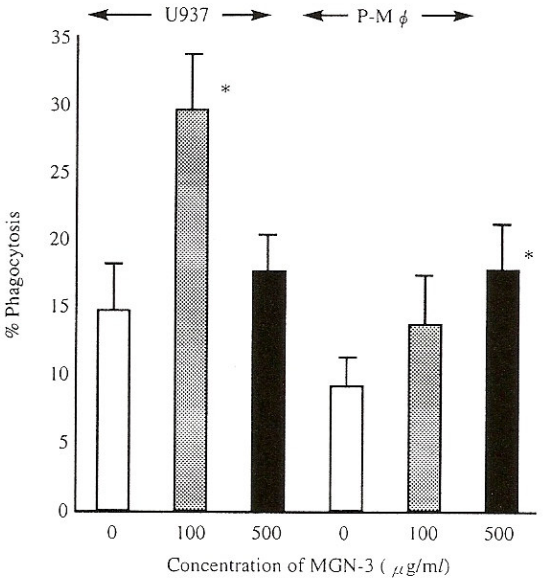

Fig. 2 Effect of MGN-3 on percent of phagocytosis of yeast by two macrophage models.

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.

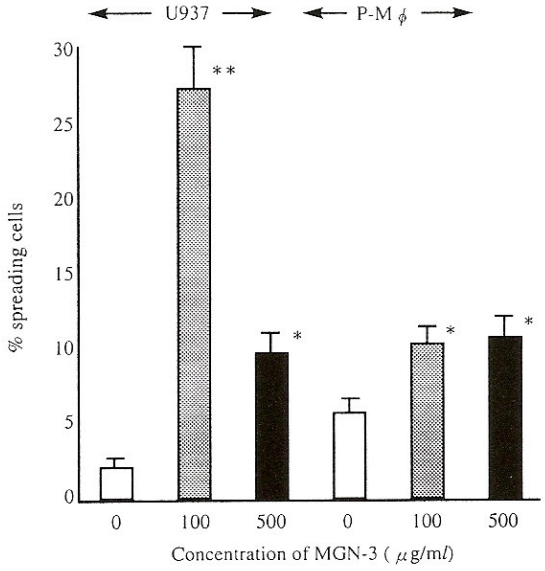

Fig. 3 Effect of MGN-3 on percent of spreading cells of two macrophage models.
Human U937 and murine P-M ϕ were treated with MGN-3 for 48 hr, and then incubated with yeast for 2 hr in a ratio of 1: 10.
Data represent the mean SD of 3 different experiments, $*P<0.05$, $**P<0.001$; as compared to control 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/m*l*) 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/m*l*) was noted post treatment with BioBran/MGN-3 at 1 μg/m*l*. Higher concentration of 10 μg/m*l* produced a significant level of TNF-α, that was further increased at 100 μg/m*l*. 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/m*l* 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/m*l*.

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

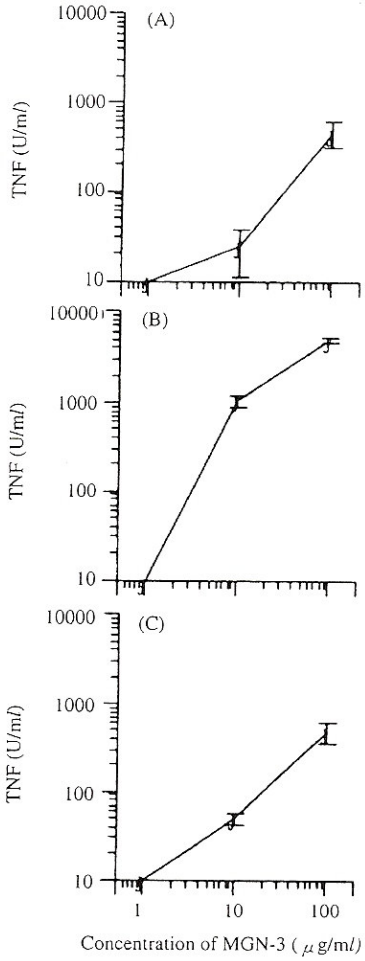

Fig. 4 Effect of MGN-3 on production of TNF- α by three macrophage models.

Murine P-M φ (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.

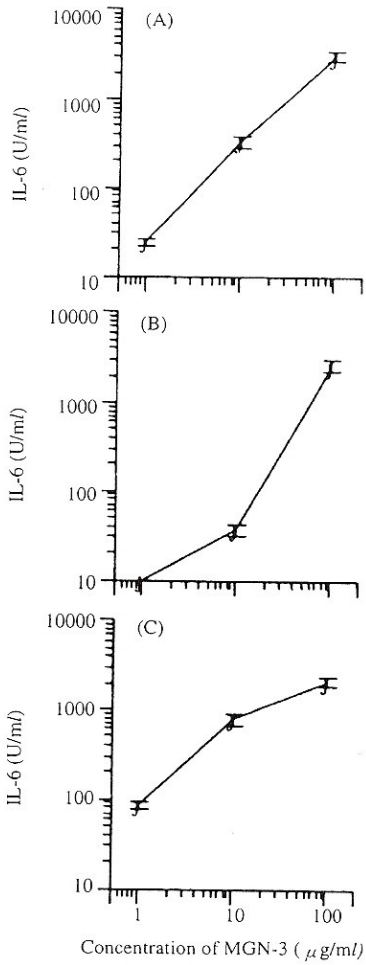

Fig. 5 Effect of MGN-3 on production of IL-6 by three macrophage models.

Murine P-M φ (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.

(10-80 U/ml) was detected post treatment of the three cell types with 1 μg/ml of BioBran/MGN-3, and augmented when increasing the concentration of BioBran/MGN-3 at 10 and 100 μg/ml. Response of human U937 cells to BioBran/MGN-3 was the highest among the three models.

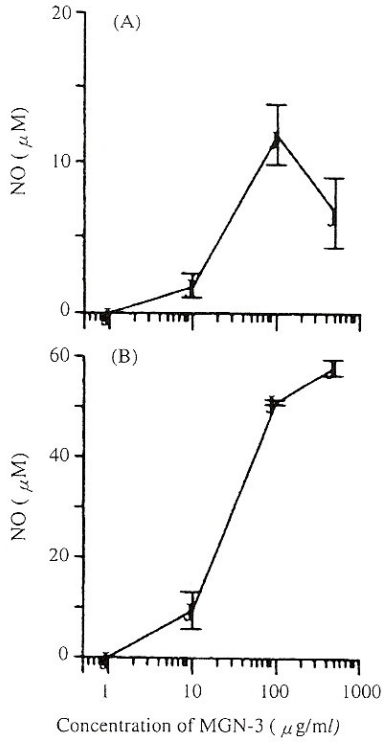

Fig. 6 Effect of MGN-3 on production of NO in two macrophage models.

Murine P-M ϕ (A) and murine RAW264.7 cells (B) were stimulated with MGN-3 (1, 10, 100, 500 μg/m*l*). The culture supernatant 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.

8. Production of NO

Production of NO in response to BioBran/MGN-3 was examined in both murine macrophage models; murine P-M ϕ and RAW264.7 cells (**Fig.6**). Production of NO was detected post treatment of both cells with 10 μg/m*l* of BioBran/MGN-3, however, increasing the concentration to 100 μg/m*l* resulted in a significant production of NO in both types of cells, but RAW264.7 cells were more responsive than P-M ϕ and produced about 5 fold of NO at 100 μg/m*l* of BioBran/MGN-3. Production of NO by human U937 cells was examined. Neither BioBran/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.

9. Action of high dose of BioBran/MGN-3 (500 μg/m*l*) cell apoptosis

U937 cells were cultured with BioBran/MGN-3 at concentration of 500 μg/m*l* for 2 days and cell survival was determined. The results show that the background of dead cells was 2%. Co-culture of cells with BioBran/MGN-3 resulted in an insignificant change in cell survival as compared with the background of

cells.

10. NO production

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

Discussion

BioBran/MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushroom[14]. The various biological functions of BioBran/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 BioBran/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 BioBran/MGN-3 significantly enhances macrophage phagocytic activity. The effect depends on the type of macrophages and the BioBran/MGN-3 dose applied. In this study, we used 100 and 500 μg/m*l* of BioBran/MGN-3 for studying the effects of this agent on phagocytosis and spreading, while we used a much smaller concentration (1-100 μg/m*l*) for studying its effects on cytokine and NO production. Earlier studies demonstrated that concentrations $\geqq$100 μg/m*l* of BioBran/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/m*l* of BioBran/MGN-3[16]. Data from this study showed that treatment with BioBran/MGN-3 at a higher concentration of 500 μg/m*l* resulted in an insignificant change in NO production, as compared with 100 μg/m*l* (**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].

BioBran/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 BioBran/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 also regulates interleukin-2-mediated activation of immature human NK cells[41-43]. Previously we have reported that BioBran/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 BioBran/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 BioBran /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 BioBran/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 BioBran/MGN-3 (10 μg/m*l*), and further increased at 100 μg/m*l*. However, augmenting the concentration to 500 μg/m*l* caused only a slight fluctuation in the level of NO production by both types of cells, as compared with 100 μg/m*l*. Production of NO by human U937 cells post treatment with BioBran/MGN-3 was also examined. Neither BioBran/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 immuno-stimulants 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 M ϕ models towards the augmentory effect of BioBran/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 BioBran/MGN-3 in the enhancement of phagocytic activity, and in the production of cytokine and NO within the three macrophage models. This data suggests BioBran/MGN-3 may be a useful agent for fighting against microbial infection; it also indicates that BioBran/MGN-3 is a potent BRM that is not restricted to specific immune cells, but may cause an overall activation of the immune system.

Acknowledgement

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Immunostimulation and Cancer Prevention

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Several studies have established the excessive cancer risk for workers exposed to several widespread workplace chemicals. The present study was designed to examine the immune alteration associated with exposure to toxic chemicals and possible counteracting of chemical toxicity using BioBran/MGN-3. BioBran/MGN-3 is a new biological response modifier (BRM) that consists of arabinoxylan compound, which is a polysaccharide that contains hemicellulose B extract of rice bran, modified by enzymes from shiitake mushrooms.

Eleven individuals who had been exposed to chemicals in the workplace participated in the study. The participants demonstrated immune dysfunction as indicated by: low levels of natural killer (NK) cell activity (10.2±4.2 LUs), lymphocyte blastogenic responses to T-cell mitogens (PHA, 39060±12517 cpm and CONA, 36224±11922 cpm) and B-cell mitogen (PWM, 16550±6330 cpm), compared to control responses. Subjects received BioBran/MGN-3 at a dose of 15 mg/kg/d for four months. Treatment with BioBran/MGN-3 increased NK cell activity 4 and 7 fold at two and four months respectively, while T and B-cell functions were 130-150% higher than base line values.

This article is a revision of a presentation given at 7th International Congress on Anti-Aging & Biomedical Technologies.

II-3-1 Immunostimulation and Cancer Prevention

NK cell immunomodulatory function by BioBran/MGN-3 was also examined in ninety (90) patients with different types of malignancies: prostate (22), breast (20), multiple myeloma (16), ovary (5), and other organs (27). Patients received and completed conventional therapy such as surgery, chemotherapy, radiation or hormonal therapy prior to participation in the study. NK cell activity was serially monitored in a monthly basis using K562, a human erythroleukemic cell target. Patients received BioBran/MGN-3 at dose 45 mg/kg/d for 2-5 years. In clinical BioBran/MGN-3 trials, 95.5% of patients (86/90) demonstrated an augmentation of NK activity (2-10 fold increase of baseline) at 1-2 weeks post treatment. The activity was maintained at a high level for up to 5 years.

The effect of stress during human NK cell immunomodulation by BioBran/MGN-3 is worth investigation, since stress in known to be a strong immune depressant. During the course of treatment with BioBran/MGN-3, 11.6% of the responders (10/86) suffered from different kinds of stressors, such as: sleep deprivation, bereavement following the loss of a spouse or close relative, work overload or financial problems. These patients evidence 40-75% decrease in their NK activity as compared to the previous level. After elimination of some stressors, the NK activity increased back to the former value.

We conclude that 1) BioBran/MGN-3 is a new BRM that does offer a new immunotherapeutic approach to cancer by increasing host anti-tumor responses through an augmentation of the NK cell activity. 2) Suppression in NK activity by chemical exposure can be fully restored with BioBran/MGN-3. 3) Our preliminary results suggest that stress cause immune suppression during the course of BioBran/MGN-3 treatment. Monitoring NK activity on a monthly basis will be able to detect this suppression which requires correction, otherwise it will have definite future clinical ramifications.

Production of Tumor Necrosis Factor-α and Interferon-γ from Human Peripheral Blood Lymphocytes by a Modified Arabinoxylan from Rice Bran (BioBran/MGN-3), and Its Synergy with Interleukin-2 *In Vitro*

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Key words:
Arabinoxylan, interleukin-2, MGN-3, natural killer cell, synergy, TNF-α

Abstract

Recently, we presented evidence for the role of BioBran/MGN-3, an enzymatically modified arabinoxylan extracted from rice bran, in potent activation of human natural killer (NK) cell function *in vivo* and *in vitro*[1]. In the current study, we examined the mechanism by which BioBran/MGN-3 elevated NK cytotoxic activity. We did this by testing the action of BioBran/MGN-3 on the levels of both tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) secretions and BioBran/MGN-3 function on the expression of key cell surface receptors. Peripheral blood lymphocytes were treated with BioBran/MGN-3 at concentrations of 0.1 mg/m*l* and 1 mg/m*l*, and supernatants were subjected to enzymelinked immunosorbent assay. Result showed that BioBran/MGN-3 is a potent TNF-α inducer. The effect was dose-dependent. BioBran/MGN-3 concentration at 0.1 and 1 mg/m*l* increased TNF-α production by 22.8- and 47.1-fold, respectively.

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BioBran/MGN-3 also increased production of IFN-γ but at lower levels as compared to TNF-α. With respect to key cell surface receptors, BioBran/MGN-3 increases the expression of CD69, an early activation antigen at 16 hours after treatment. Furthermore, the interleukin-2 receptor CD25 and the adhesion molecule ICAM-1 (CD54) were upregulated after treatment with BioBran/MGN-3. Treating highly purified NK cells with BioBran/MGN-3 also resuled in increased levels of TNF-α and IFN-γ secretion in conjunction with augmentation of NK cell cytotoxic function. Furthermore, addition of BioBran/MGN-3 to interleukin-2-activated NK cells resulted in a synergistic induction of TNF-α and IFN-γ secretion. Overall, our data suggest that BioBran/MGN-3, a novel biological response modifier, can be used as a safe alternative or as an adjuvant to the existing immunotherapeutic modalities.

Introduction

Natural killer (NK) cells play an important function in the primary host defense against infection and neoplasm, and defective NK cell function has been attributed to the pathogenesis of infectious diseases, such as acquired immunodeficiency syndrome and various malignancies[2-7]. Thus, increase in the NK function or restoration of defective NK function should result in the enhancement of immunity in the diseases mentioned.

Several biological response modifiers (BRMs) of fungal and bacterial origin have been developed for the enhancement of NK cells' anticancer activity. These BRMs include PSK protein bound polysaccharide (Krestin), *Lentinus edodes* mycelia (LEM), and killed streptococcal preparations (OK432), *Corynebacterium parvum*, and bacille Calmette-Guérin[8-12]. Recently, we developed a new BRM called BioBran/MGN-3, an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushrooms. The various biological functions of BioBran/MGN-3, such as anti-human immunodeficiency virus activity[13] and NK immunomodulatory function[1,14,15] have been demonstrated previously. In this study, we examined the mechanism by which BioBran/MGN-3 increases NK cell activity; we tested many important properties of NK cell function, such as production of cytokines and cell surface receptors; CD69, an early activation antigen; the interleukin-2 (IL-2) receptor CD25; and the intracellular adhesion molecule-1 (ICAM-1), CD54.

Further studies were designed also to examine possible synergistic immune activating function of BioBran/MGN-3 in the presence of a low concentration of IL-2 (500U). Conventional immunotherapeutic modalities, such as IL-2 treatment, are shown to produce undesirable side effects at high doses in cancer patient[16-18]. Further studies are needed to apply this regimen in multiple clinical trials. Thus, BioBran /MGN-3 might be a safe alternative to the conventional immunotherapeutic regimens used in the activation of immune function in patients suffering from human immunodeficiency virus and malignancies.

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 is a polysaccharide that contains β-1,4-xylopyronase hemicellulose. BioBran /MGN-3 is commercially known as BioBran/MGN-3 (Daiwa Pharmaceutical Co. Ltd., Tokyo, Japan).

2. Cell Lines and Reagents

K562 target cells were cultured in RPMI 1640 supplemented with 1% penicillin-streptomycin (Life Technologies, Grand Island, NY) and 10% fetal calf serum (Irvine Scientific, Santa Ana, CA).

Recombinant IL-2 was obtained from Hoffman La Roche (Paramus, NJ). Interferon-γ (IFN-γ) was obtained from Peprotech (Piscataway, NJ). NK purification kits were obtained from Miltenyi Biotech (Auburn, CA). FITC-conjugated anti-CD54, anti-CD16, and PE-conjugated anti-CD69 antibodies were obtained from Coulter/Immunotech (Miami, FL). Anti-tumor necrosis factor-α (anti-TNF-α) monoclonal antibodies (mAB) were prepared in our laboratory. Enzyme-linked immunosorbent assays (ELISAs) for TNF-α and IFN-γ have been described[4].

3. Purification of Peripheral Blood Lymphocytes and NK Cells

Twenty-five healthy control subjects (12 female, 13 male) were selected for participation in this study. The subjects ranged in age from 17 to 42 years, with a mean of 26 years. Peripheral blood mononuclear cells were isolated as described elsewhere[4,19]. Peripheral blood lymphocytes (PBLs) were obtained after Ficoll-Hypaque centrifugation. Purified NK cells were selected negatively using an NK isolation kit (Miltenyi) and consisted of more than 95% of $CD16^+$ cells, whereas the percentage of $CD3^+$ and $CD19^-$ contaminant cells were 2.3 ± 3.2 and 3 ± 4, respectively.

4. Culture Conditions

PBLs and highly purified NK cells were cultured overnight with BioBran/MGN-3 (1-1,000 μg/m*l*) and IL-2 (500 U/m*l*) and the combination of BioBran/MGN-3 and IL-2. The supernatants were removed for ELISA assay, and the cells were washed and used in ^{51}Cr release assay and cell surface staining experiments.

5. ELISA for TNF-α and IFN-γ

mABs to two distinct epitopes of TNF-α —B154.9.1 and B154.7.1—were provided generously by Dr. G. Trinchieri (Wistar Inst, Philadelphia, PA), and polyclonal rabbit anti-TNF antibodies were prepared in our laboratory. Monoclonal antibody to IFN-γ was purchased from Genzyme (Cambridge, MA), and the polyclonal antibody to IFN-γ was prepared in our laboratory. The ELISA for TNF-α and IFN-γ was described previously[4]. Briefly, wells of ELISA plates were coated with 50 μl of mAB specific for TNF-α and IFN-γ. Plates coated with anti-TNF-α and anti-IFN-γ mABs were kept for 1 day before use, were washed three to four times, and were blocked with ELISA phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 30 minutes. Then plates were washed twice, and 50 μl of supernatants from treated NK samples was added to each well. After overnight incubation at 37℃, plates were washed four times, 50 μl of polyclonal anti-TNF-α and anti-IFN-γ antibodies at 1 : 1000 dilution was added, and the incubation continued for 2 hours. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA) at a dilution of 1 : 2000 was added to the plates that were incubated for an additional 2 houres. Finally, the plates were washed and incubated with an alkaline phosphatase substrate (Sigma 104, St. Louis, MO) and read after 2 hours in a titrated Multiskan MCC/240 ELISA reader using a 405-nm filter.

6. Surface Staining of NK Cells

NK cells were washed twice with ice-cold PBS containing 1% BSA and 0.01% sodium azide.

Predetermined optimal concentrations of specific mAbs were added to 5×10^4 cells in 50 μl of cold PBS-BSA and were kept on ice for 30 minutes. Thereafter, cells were washed twice in cold PBS-BSA and were brought up in 1 : 50 dilution of PE-GAM. After 30 minutes of incubation on ice, cells were washed twice and fixed in 1% paraformaldyhyde solution. For staining, cells were incubated with PE-conjugated antibodied for 30 minutes, after which they were washed and fixed as outlined. An Epics C (Coulter Electronics, FL) flow cytometer was used for analysis.

7. ^{51}Cr-Release Assay for Measuring NK Activity

NK activity was measured by ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr-labeled tumor cells in 0.1 m*l* CM were added to different wells of a 96-well microtiter plate. Effector cells then were pipetted into quadruplicate wells to give effector : target (E : T) ratios of 12 : 1, 25 : 1, 50 : 1, and 100 : 1. After a 4-hour incubation (at 37℃), the plates were centrifuged (1,400 rpm for 5 minutes), and 0.1 m*l* of supernatant from each well was collected and counted in a gamma counter (Beckmann G50, Beckmann Instruments, Fullerton, CA). The percentages of isotope released were calculated by the follwing formula:

$$\% \text{ Lysis} = (\text{Exp. rel.} - \text{sp. rel.}) \times 100 / \text{Total rel.} - \text{sp. rel.}$$

where *exp. rel.* is experimental release, *sp. rel.* is spontaneous and *total rel.* is total release. Spontaneous release (from target cells was no more than 8% to 10% of total release. Total release was measured by adding 0.1 Triton X-100 (Sigma) to designated wells. Lytic units (LU) were calculated from effector titration curves, with one LU defined as the number of effector cells required to achieve 40% lysis for K562 cells.

8. Statistical Analysis

The student's *t* test was performed to determine the significance levels between control and BioBran /MGN-3 treated cells, and the level of significance was set at a probability equal to 0.05.

Results

1. Increase in *InVitro* NK Cell Cytotoxicity by BioBran/MGN-3 and IL-2

PBLs from healthy individuals were treated with BioBran/MGN-3 in the presence and absence of IL-2 and were examined for NK activity. **Figure 1A** shows that the addition of BioBran/MGN-3 at a concentration of 0.5 mg/m*l* to PBLs significantly increased the cytotoxic function of NK cells ($P<0.001$). Furthermore treatment of PBLs with the combination of IL-2 and BioBran/MGN-3 resulted in a higher augmentation of NK cell cytotoxicity as compared to treatment with either agent alone. Addition of BioBran/MGN-3 to highly purified NK cells increased NK cell cytotoxic function (see **Figure 1B**). Data also show that the level of NK activation is maximized after treatment with IL-2 and that NK activity did not increase further after the combined treatment of BioBran/MGN-3 and IL-2, as compared to use of either agent alone.

2. Increase in TNF-α Secretion

1) Titration Experiments

PBLs were incubated with BioBran/MGN-3 for 16 hours, and supernatants were recovered and subjected to a specific and sensitive ELISA assay. First, titration experiments were carried out to examine the effect

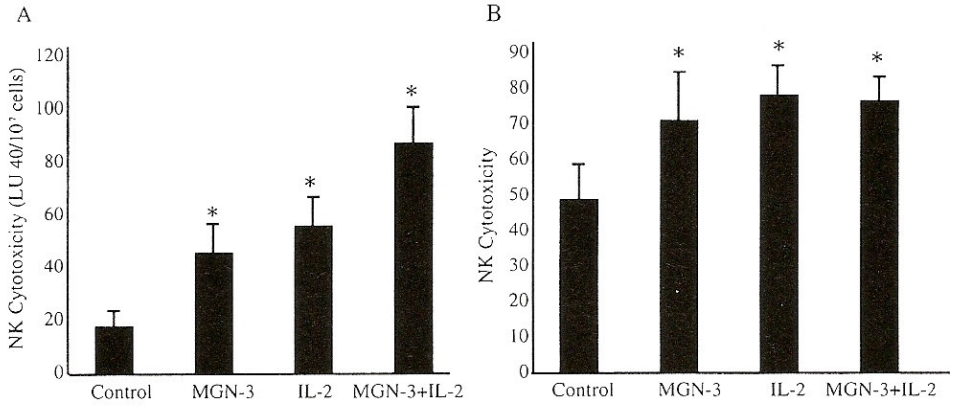

Figure 1

(A) Increased cytotoxicity mediated by peripheral blood lymphocytes (PBLs) after treatment with MGN-3 *in vitro* in the presence or absence of interleukin-2 (IL-2).

PBLs from five donors were incubated with MGN-3 (0.5 mg/m*l*) and interleukin-2 (IL-2; 500 U/m*l*) for 16 hours, after which they were examined for natural killer (NK) cell activity by ^{51}Cr-release assay. Activity expressed as lytic units at 40%. **P*<0.001.

(B) Action of MGN-3 on activity of purified NK cells.

Purified NK cells were incubated with MGN-3 (0.5 mg/m*l*) in the presence or absence of IL-2 (500 U/m*l*) overnight. Activity examined at effector: target (E: T) ratio of 12: 1 M±standard deviation of five individuals. **P*<0.01.

of a wide range of BioBran/MGN-3 concentrations (1-1,000 μg/m*l*) on TNF-α production. **Figure 2A** demonstrates that BioBran/MGN-3 is a potent TNF inducer. The effect was dose-dependent. The level of TNF-α did not change at 1 to 10 μg/m*l* but increased at a concentration of 0.1 mg/m*l* and maximized at concentration of 1 mg/m*l*. IL-2 alone had no effect on TNF-α production; however, a synergistic effect of BioBran/MGN-3 and IL-2 was observed.

2) Production of TNF-α by PBLs

Subsequently, two concentrations of BioBran/MGN-3 (1 and 0.1 mg/m*l*) were selected and applied to 25 donors. **Figure 2B** shows that addition of BioBran/MGN-3 to PBLs significantly increased TNF-α secretion in all subjects. The variation among subjects in response toward BioBran/MGN-3 is clear. Compared to control, untreated samples, subjects treated with 1 mg/m*l*, could be divided into three groups: G1 (40%; 10 of 25) showed an increase in their TNF-α level up to less than 20-fold; G2 (24%; 6 of 25) showed an increase in TNF-α level between 20-and 100-fold; and G3 (36%; 9 of 25) were highly responsive, with an increase in TNF-α level greater than 100-fold. Further, the data in **Figure 2B** show that the increase in TNF-α secretion was augmented synergistically in the presence of IL-2 (P<0.001).

3. Production of TNF-α by Purified NK Cells

Figure 2C depicts data obtained when NK cells were purified and cultured with BioBran/MGN-3 in the presence or absence of IL-2. Data show a ninefold increase in TNF-α production after treatment with BioBran/MGN-3 alone at a concentration of 1 mg/m*l*. IL-2 treatment demonstrated no change in the TNF-α level as compared to controls. TNF-α production did not increase further after combined treatment of

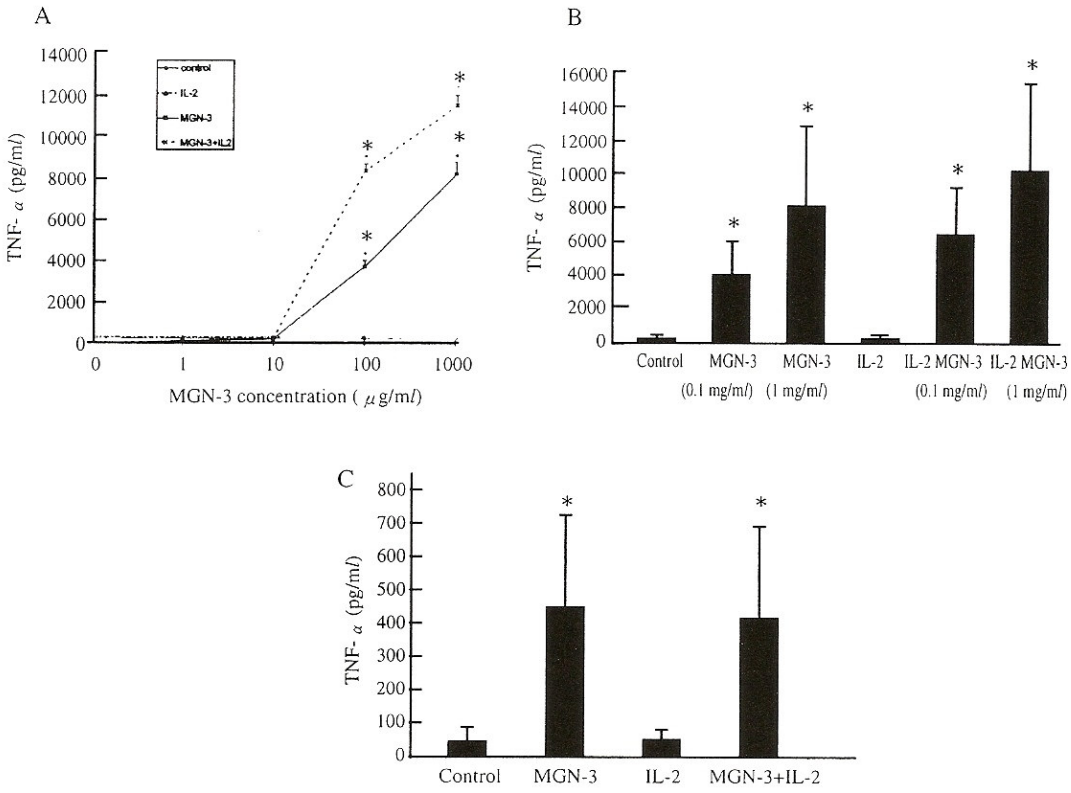

Figure 2

(A) Titration experiments for tumor necrosis factor- α (TNF- α) production by peripheral blood lymphocytes (PBLs).
PBLs were incubated with a wide range of MGN-3 concentrations (1-1,000 μg/ml) in the presence or absence of interleukin-2 (IL-2; 500 U/ml) for 16 hours.
Supernatant was harvested and subjected to enzyme-linked immunosorbent assay (ELISA). *P<0.001.
(B) Triggering of tumor necrosis factor- α (TNF- α) secretion by PBLs treated with MGN-3.
PBLs (5×10^6) were treated with 0.1 mg/ml and 1 mg/ml MGN-3, 500 U/ml IL-2, and the combination of IL-2 and MGN-3.
After 16 to 18 hours of incubation, the supernatants were harvested and subjected to TNF- α ELISA.
Data of 25 individual donors. *P <0.001.
(C) Triggering of TNF- α secretion by purified natural killer (NK) cells treated with MGN-3.
NK cells were treated with MGN-3 (1 mg/ml) in the presence or absence of IL-2 (500 U/ml) for 16 hours.
Supernatants were harvested and subjected to TNF- α ELISA.
Data of three subjects, each tested in triplicate. *P<0.01.

BioBran/MGN-3 and IL-2, as compared to use of either agent alone.

4. Increase in IFN- γ Secretion

1) Titration Studies

With respect to IFN- γ secretions, titration experiments were conducted also to examine the effect of a wide range of BioBran/MGN-3 concentrations (1-1,000 μg/ml) in the presence and absence of IL-2 on

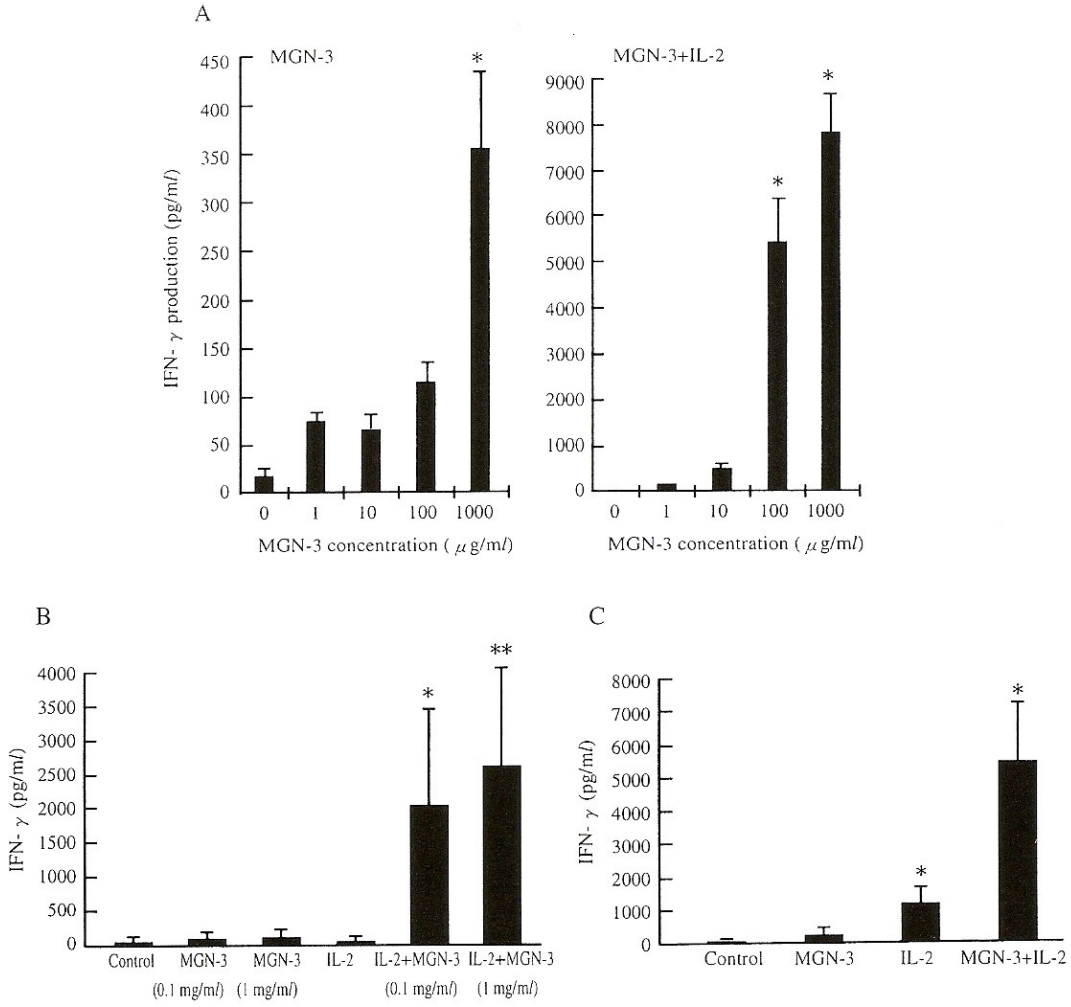

Figure 3

(A) Titration experiment for interferon-γ (IFN-γ) production by peripheral blood lymphocytes (PBLs).

PBLs were incubated with a wide range of MGN-3 (1-1,000 μg/ml) alone or in the presence of interleukin-2 (IL-2; 500 U/ml) for 16 hours. Supernatants were harvested and subjected to enzyme-linked immunosorbent assay (ELISA). *P<0.001.

(B) MGN-3-mediated induction of IFN-γ secretion by PBLs.

The treatments were conducted as described.

After 16 to 18 hours of incubation, supernatants were removed and subjected to IFN-γ ELISA assay.

Data from 14 individual donors. *P<0.03; **P<0.02.

(C) Induction of IFN-γ secretion by purified natural killer cells treated with IL-2 and MGN-3.

Natural killer cells were treated with MGN-3 at concentrations of 1 mg/ml in the presence or absence of IL-2, as described.

The supernatants were harvested and subjected to IFN-γ ELISA.

Data of three subjects, each tested in triplicate. *P<0.01.

IFN-γ production (**Figure 3A**). BioBran/MGN-3 proved to be an IFN-γ inducer in a dose-dependent manner. IFN-γ secretion was noticeable at 1 to 10 μg/m*l*, was increased further at 0.1 mg/m*l*, and was maximized at BioBran/MGN-3 concentration of 1 mg/m*l*, whereas IL-2 treatment showed no change in IFN-γ level. Furthermore, a synergistic effect was observed when BioBran/MGN-3 was coupled with IL-2, resulting in a dramatic several-fold increase in IFN-γ production, as compared to use of either agent alone.

5. Production of IFN-γ by PBLs

Consequently, two concentrations of BioBran/MGN-3 (0.1 and 1 mg/m*l*) were applied to PBLs from 14 donors. **Figure 3B** shows that BioBran/MGN-3 treatment increased IFN-γ secretion with individual variation among subjects. Addition of BioBran/MGN-3 to PBL samples in the presence of IL-2 resulted in a synergistic increase in IFN-γ secretion in all subjects. The synergistic effect was BioBran/MGN-3-dose-dependent : 52-fold induction at BioBran/MGN-3 concentration of 0.1 mg/m*l* (P<0.03) and 66-fold at BioBran /MGN-3 concentration of 1 mg/m*l* (P<0.02).

1) Production of IFN-γ by Purified NK Cells

Figure 3C shows an increase in IFN-γ secretion after culture of NK cells with BioBran/MGN-3 at a concentration of 1 mg/m*l*. Treatment with IL-2 significantly increased IFN-γ level (P<0.01), and again, a synergistic effect of BioBran/MGN-3 in the presence of IL-2 was observed clearly (P<0.01).

6. Induction of Key Cell Surface Recepors on NK Cells by BioBran/MGN-3 and IL-2

1) Upregulation of CD69 Early Activation Antigen

The mechanism by which BioBran/MGN-3 activated both the cytotoxic and secretory pathways of NK cells was examined. Addition of BioBran/MGN-3 to PBLs induced significant upregulation of CD69, an early activation marker on NK cells. The induction of CD69 cell surface receptor expression by BioBran /MGN-3 was higher than those observed by the IL-2 treatment of PBLs (**Figure 4A**).

2) Upregulation of IL-2 Receptor Alpha Chain (CD25)

Figure 4B shows significant upregulation of the IL-2 receptor α chain (CD25) expression after treatment of PBLs with BioBran/MGN-3. The expression of CD25 on BioBran/MGN-3-treated NK cells was higher than in those induced by IL-2 treatment alone.

3) Upregulation of CD54 Adhesion Molecule

The addition of BioBran/MGN-3 to NK cells resulted in the upregulation of CD54, the adhesion molecule. Treatment of NK cells with the combination of IL-2 and BioBran/MGN-3 augmented the intensity of CD54 expression on NK cells (see **Figure 4C**).

An upregulation of CD69, CD25 and CD54 also was observed on lymphocytes other than NK cells after BioBran/MGN-3 treatment but to a lesser extent. Indeed, the levels of CD69 expression on $CD4^{++}$ T cells were significantly lower than those observed on NK cells, both at the percentage and the mean intensity levels (see **Figure 4D**).

Discussion

Recently, we tested the ability of BioBran/MGN-3 to enhance human NK cell activity in both *in vivo* and *in vitro* culture conditions[1,14,15]. BioBran/MGN-3 is composed of denatured hemicellulose, which is

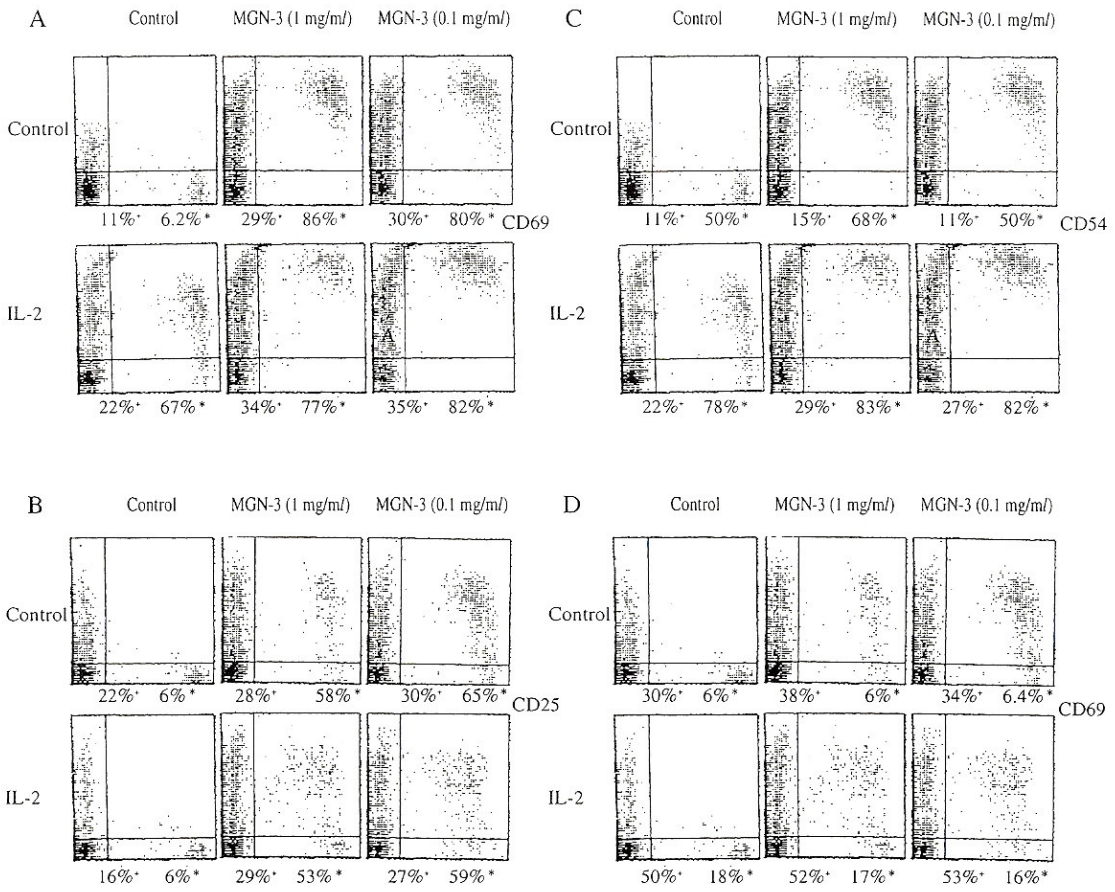

Figure 4

Upregulation of CD69, CD25, and CD54 surface receptor expression on natural killer (NK) and T cells by MGN-3 and interleukin-2 (IL-2) treatments.

Peripheral blood lymphocytes (PBLs) were treated with MGN-3 (0.1 and 1 mg/ml) and IL-2 (500 U/ml) for 16 to 18 hours.

(A) The treated samples were washed and stained with the FITC-conjugated CD16 and PE-conjugated CD69 monoclonal antibodies. The number on the bottom represents the percentage of cells positive for CD69 expression.

*Percentage of cells positive for CD69 expression within CD16-positive natural killer cells; ⁺percentage of cells positive for CD69 expression within CD16-nagative fraction of peripheral blood mononuclear cells (PBMCs).

(B) FITC-conjugated CD16 and PE-conjugated CD25 were used for the staining of the lymphocyte.

The numbers on the bottom represent the percentage of positive cells for CD25 expression.

*Percentage of cells positive for CD25 expression within CD16-positive NK cells; +percentage of cells positive for CD25 expression within CD16-nagative fraction of PBMCs.

(C) Staining with FITC-conjugated CD16 and PE-conjugated CD54.

*Percentage of cells positive for CD54 surface expression within CD16-positive NK cells; +percentage of cells positive for CD54 surface expression within CD16-nagative fraction of PBMCs.

(D) FITC-conjugated CD4 and PE-conjugated CD69.

*Percentage of cells positive for CD69 surface expression within $CD4^+$ fraction of PBMCs; +percentage of cells positive for CD69 surface expression within $CD4^-$ fraction of PBMCs.

obtained by reacting rice bran hemicellulose with multiple carbohydrate-hydrolyzing enzymes from shiitake mushrooms. 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[1-13]. The ability of BioBran/MGN-3 to activate highly purified NK cells from peripheral blood indicates that the activity is attributed only to the NK cell fraction.

In this study, we analyzed *in vitro* the possible mechanisms by which BioBran/MGN-3 enhances NK cell activity ; we evaluated cytotoxic and secretory functions of NK cells in the presence of BioBran/MGN-3. Increase in NK cell cytotoxic activity after treatment with BioBran/MGN-3 was paralleled with an increase in cytokine secretion. Significant levels of TNF-α secretion were triggered by the presence of BioBran /MGN-3 in a dose-dependent manner. Researchers have established that TNF-α induces the expression of IL-2Rα[20-22]. Moreover, addition of the anti-TNF-α antibody to IL-2-treated NK cells inhibited IL-2Rα upregulation significantly[20]. The blocking of TNF receptor function by antibodies to p75 and p55 TNF receptors also was shown to inhibit the generation of lymphokine-activated killer cells[23]. Therefore, the induction of TNF-α secretion by BioBran/MGN-3 might represent the first step in NK cell activation and might be responsible for the observed increases in IL-2Rα (CD25) expression. The increase in IL-2Rα expression by BioBran/MGN-3 and its association with ths IL-2Rβ/γ chain forms the high-affinity binding site for IL-2 (kd = 10pM)[24], which results in the observed increase in cellular response to IL-2 binding.

IL-2, in turn, has been shown to upregulate TNF-receptor chains, thus establishing a feedback loop whereby IL-2 will increase TNF-α responsiveness and TNF will augment IL-2 responsiveness[20]. As a result of such interaction, a synergistic response will be obtained when both cytokines are present in the cell cultures. Therefore, observation of a synergistic function in the presence of IL-2 and BioBran/MGN-3 is expected, because BioBran/MGN-3 can trigger significant TNF-α secretion. Addition of IL-2 to BioBran/MGN-3 at concentrations of 0.1 and 1 mg/m*l* elevated TNF-α production by 37.5- and 59.3-fold, respectively. *In vitro* culture of PBLs and highly purified NK cells with either agent alone resulted in a significant increase in NK cell activity. In addition, the synergistic effect of both agents was detected against PBLs but not against highly purified NK cells, because IL-2 treatment alone maximized NK activity.

INF-γ secretion has been shown to upregulate TNF receptor II (p75) expression, whereas TNF-α exerts its function through both the TNFRII and TNFRI[22]. Induction of TNFRII expression and signaling is important for cellular proliferation and induction of the NF-κB transcription factor[25]. Thus, an intimate relationship among TNF-α, IFN-γ, and IL-2 determines the fate of NK cell activation in BioBran/MGN-3-treated cells. BioBran/MGN-3-treated NK cells have increased expression of CD69, an early activation antigen, which has been correlated with increased cytokine secretion by NK cells[20]. TNF-α also plays an important role in the induction of CD69 and CD54 cell surface receptors. CD69 is a 28- to 32-kDa surface homodimer[26], which is induced rapidly and is phosphorylated in IL-2-activated NK cells[27]. Furthermore, the expression of this antigen correlated well with the levels of cytotoxicity observed in IL-2-stimulated free cells[20]. Moreover, anti-TNF-antibody treatment of IL-2-activated cells resulted in inhibition of CD69 cell surface expression. Thus, TNF-α triggered by BioBran/MGN-3 might be responsible also for upregulation of CD69 surface expression and for the increase in both cytotoxicity and cytokine secretion. Therefore, activation of TNF-α secretion by BioBran/MGN-3 plays a central role in the regulation of NK cell activity and function.

Parallel to CD25 and CD69 induction, an upregulation of CD54 (ICAM-1) was observed by BioBran /MGN-3-treated NK cells. Several lines of evidence suggest a possible role for CD54 in adhesion and

cytotoxicity by NK cells[6, 28, 29]. Indeed, increases in CD54 expression were observed in IL-2-activated PBLs[30]. Thus, increased NK cell cytotoxic function by BioBran/MGN-3 could be due partly to the ability of BioBran/MGN-3 to augment the expression of ICAM-1. Treatment of the PBLs with BioBran/MGN-3 *in vivo* resulted in a significant increase in effector cell binding capacity to their tumor cell targets[1].

Though demonstrating a significant increase in TNF-α and IFN-γ production after treatment of PBLs with BioBran/MGN-3, lower levels of these cytokines were observed when purified NK cells were tested. Although increased activation of $CD4^+$ T cells was observed in the presence of BioBran/MGN-3, levels of such activation were lower than those obtained by BioBran/MGN-3-treated NK cells, suggesting that BioBran/MGN-3 primarily targeted the NK cells, whereas other cells serve as accessory cells for NK activation.

In conclusion, we have presented evidence for the role of BioBran/MGN-3 in activation of NK cytotoxicity, induction of cytokine production, and upregulation of key NK cellular receptors. Identification and purification of the active fraction in BioBran/MGN-3, which is responsible for triggering functional activation and upregulation of key surface receptors in NK cells, awaits further investigation.

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Effect of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on NK Cell Activity of Human Peripheral Blood Lymphocytes

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Introduction

Every day, genetic damage is caused by a variety of environmental factors, such as air pollution due to exhaust gases, chemical carcinogens including pesticides, and lifestyle habits such as unbalanced meals and cigarette smoking. Cells with damaged genes cannot behave normally and some become proliferative tumour cells. These abnormal cells are eliminated by white blood cells and natural killer (NK) cells, which carry out immune functions at an early stage before the tumour cells become fully malignant and cause widespread disease. Tumour cells that fail to be eliminated by early immunity can be removed by a more powerful immune mechanism. The cells involved include macrophages, dendritic cells, and lymphocytes such as helper T cells and killer T cells. These carry out acquired immunity, and the anti-cancer immuno-modulatory substances (cytokines) produced play important roles in this process. Typical cytokines involved include interleukin 2 (IL-2), interferon γ (IFN-γ), interleukin 12 (IL-12), interleukin 18 (IL-18), and tumour necrosis factor (TNF-α). The reactions mediated by cytotoxic cells such as NK cells and killer T cells are referred to as cellular immunity. C ancer patients are known to show a decrease in cellular

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immune activity across a range of functions.[1]

Plant polysaccharides derived from mushrooms and herbs counteract the decline in immune function by enhancing lymphocyte cytotoxicity and their ability to produce cytokines, and thus exert an anti-cancer effect. Mushrooms which have been shown to exert anti-tumour or immunomodulatory effects include agaricus, shiitake (lentinan), reishi, enokidake, maitake (maitake D-fraction), kawaratake (krestin), and suehirotake (sonifilan). Some of these are used in dietary supplements. β-1,3-glucan polysaccharides have been shown to be responsible for the lymphocyte stimulating effect of these mushrooms.[2] However, β-1,3-glucans are a polymeric dietary fibre, which passes through the intestinal tract without being absorbed. To exert an immunomodulatory action, the active ingredient must be absorbed into the bloodstream and make contact with effector cells such as lymphocytes and macrophages. It is thought that some of the polysaccharides degraded by enteric bacteria are absorbed and exert such an effect, while most pass unused through the intestine.

In this study, we investigated the physiologically active ingredient that activates cellular immunity in BioBran/MGN-3 with a relatively low molecular weight. BioBran/MGN-3 mainly contains modified hemicellulose, produced by partially degrading a soluble hemicellulose fraction from rice bran with a carbohydrate-hydrolyzing enzyme complex from shiitake mushroom culture filtrate. This product is already on the market and has been reported to be effective orally against various diseases including cancer[3] and HIV infection.[4] Studies have also been made into its ability to scavenge reactive oxygen species and to activate macrophages, and its therapeutic effect in the treatment of diabetes mellitus.

The main physiological effect of this product is its action on biological defences in which NK cells play a major role. Thus, it has been demonstrated that culture of peripheral blood lymphocytes with this product increases cytotoxicity of NK cells.[5]

NK cells are lymphocytes that are able to kill certain target cells without becoming sensitised to antigen; they are neither T cells nor B cells and account for about 5%-10% of human peripheral blood lymphocytes.[6,7] When killer T cells, which differentiate from T cells after stimulation by virally infected cells or tumour antigens, find and kill target cells, receptors on the T cell surface recognise antigen-derived peptides and MHC (major histocompatibility complex) proteins at the same time and attack the cells which carry them. However, in order for NK cells to mount an attack, an MHC antigen should not exist, and the antigen need not to be presented by an MHC molecule. In other words, the cytotoxicity of NK cells is not restricted by the MHC, meaning that they can attack different cell types including syngeneic, homogeneous, and heterogeneous cells.[7-9] NK cells have attracted attention for their particular properties, including immunosurveillance of tumours and an ability to prevent viral infection. It has also been suggested that NK cells are closely involved with the differentiation and growth of haematopoietic stem cells, organ transplantation, and the development of certain haematological diseases, collagen disease, diabetes melli-tus, and gastrointestinal disorders, through their production of cytokines and killer activity.[10] As mentioned above, NK cells play an important role at the forefront of biological defence. Enhanced cytotoxicity resulting from activation of NK cells may therefore promote elimination of abnormal cells and activation of the immune system overall by increased cytokine production.

The mechanism of NK cell activation by BioBran/MGN-3 and the identity of the active constituent are unknown. In this study, we performed an investigation with the aim of defining the active ingredient and elucidate the mechanism by which it activates NK cells.

Materials and Reagents

BioBran/MGN-3, which is commercially available, was provided by Daiwa Pharmaceutical.

The blood cell fraction was provided by Funabashi Red Cross Blood Center. Blood was collected in a 200 m*l*-bag containing as anti-coagulant acid citrate dextrose (ACD), and 200 m*l* was reduced to about 40 m*l* by removal of plasma. The blood cell fraction therefore contained a small amount of ACD from the original blood.

Human chronic myelogenous leukaemia cells (K-562) were from Dainippon Pharmaceutical Co., Ltd. IL-2 and INF-γ were from Pepro Tech. Unless specified, other reagents are commercially available products of special grade.

Methods

(1) Cell culture

The culture medium for K-562 cells was RPMI 1640 supplemented with 10% foetal calf serum(FCS) and 0.2 mg/m*l* kanamycin. Cells were cultured at 37℃, with 5% carbon dioxide, and 100% relative humidity.

(2) Preparation of BioBran/MGN-3 fractions

BioBran/MGN-3 was dissolved at 0.2 g/m*l* in distilled water(dw), the solution was centrifuged at 5400× g for 30 minutes, and the supernatant collected. This was applied to a Sephadex G-25 column (5.0×24cm) equilibrated with dw and also eluted with dw. Blue dextran and dinitrophenylalanine were used as molecular weight markers. Collected fractions were freeze-dried, redissolved at 0.2 g/m*l* in dw, and centrifuged at 5400×g for 30 minutes. The supernatant was applied to a Sephadex G-75 column (2.1×42cm) equilibrated and eluted as before. Using blue dextran and cytochrome C as mw markers, fractions were collected and freeze-dried.

(3) Cell preparations

(A) Separation of peripheral blood lymphocytes (PBMC)

Lymphocytes were isolated from the blood cell fraction using Ficoll-Paque Plus.[1] Ten m*l* of the blood cell fraction was diluted with 20 m*l* of PBS (phosphate buffered saline) and overlaid onto 15 m*l* of Ficoll-Paque. This was centrifuged at 18-20℃ and 400×g for 30 minutes and the lymphocyte fraction collected. The lymphocytes were resuspended in at least three times the volume of PBS, and the suspension was centrifuged three times at 18-20℃ and 100×g for 10 minutes. Finally, the cells were suspended in 10% FCS-RPMI 1640 at 5×10^6/m*l*.

(B) Stimulation of PBMC by different fractions

The above cell suspension was used as the negative control and addition of 100 U/m*l*-IL-2 was used as the positive control. Each fraction was added to the PBMC suspension to a final concentration of 100 mg/m*l* and cultured at 37℃ in a CO_2 incubator for 3-5 days. Changes in NK activity after culture were measured.

(C) Preparation of target cells

Culture of K-562 cells at 1×10^5 cells/m*l* was started 2 days before measurement, an equal volume of 10%

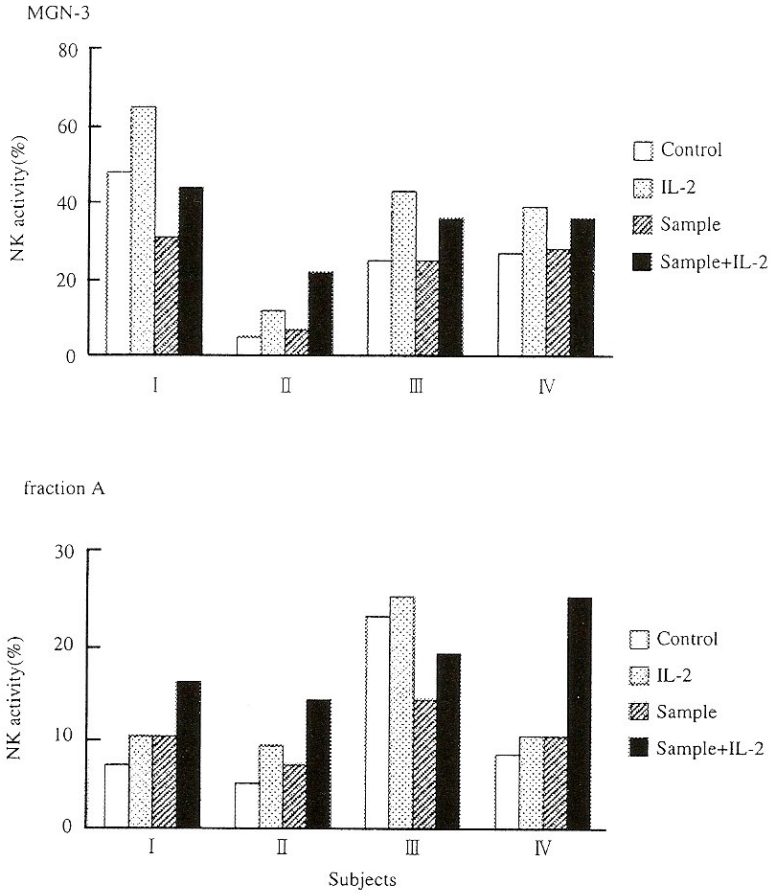

Fig.1 NK activity of MGN-3 or fraction A

FCS-RPMI 1640 was added one day before measurement, and K-562 cells (2-5×10^5 cells/m*l*) in logarithmic phase were used in experiments.

(D) Measurement of NK cell activity

NK cell activity was measured using an established fluorescence method.[12]

Results and Discussion

As blood two days after collection was used in this study, the effectiveness of the separated PBMC for assay of NK activity and toxicity of BioBran/MGN-3 for PBMC were studied first. Viable and dead cells were counted using the trypan-blue elimination method[11] after culture for 1,3, and 5 days. Viability was 92% immediately after separation and 80% after 5 days in culture. No significant differences in viability were observed in PBMC treated with BioBran/MGN-3. From this result, it was judged that BioBran is non-toxic for PBMC.

After partial purification of BioBran/MGN-3, the fraction obtained by Sephadex G-25 chromatography

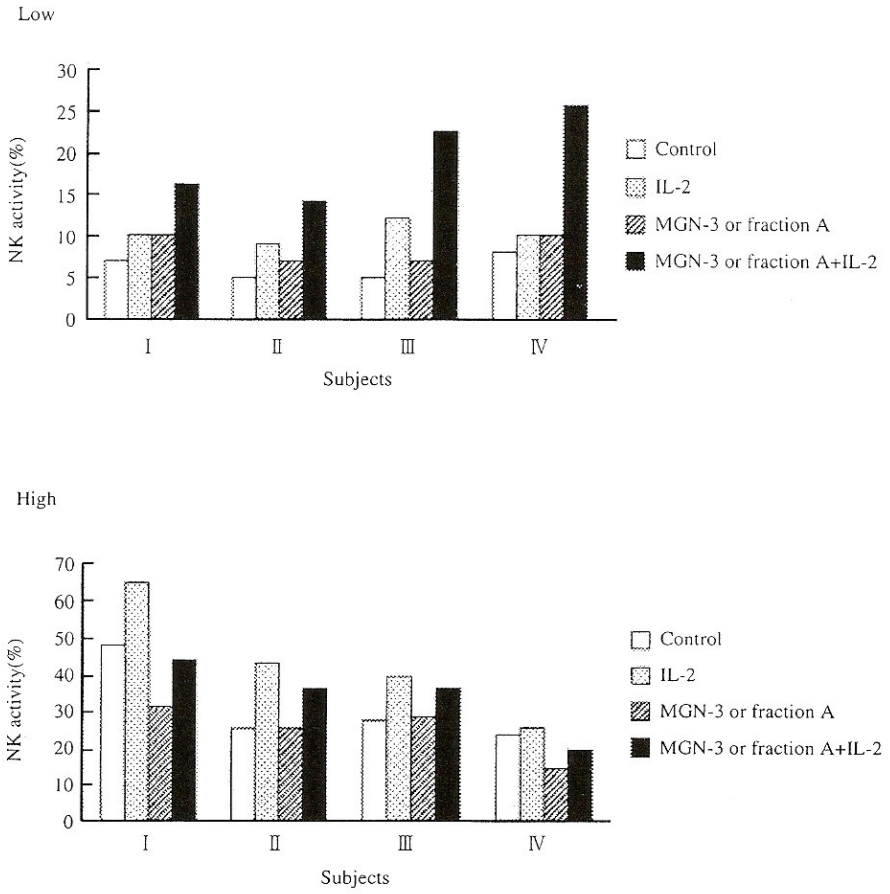

Fig.2 Effects for the low or high NK activity subjects

was designated A. Of the fractions obtained by Sephadex G-75 chromatography, that corresponding in molecular weight to cytochrome C was designated B, and that between blue dextran and cytochrome C was designated C. NK activity increased in the presence of all three fractions, but the effect depended on the cells used. Concomitant treatment with IL-2 tended to increase NK activity more than treatment with any of the fractions alone. However, the effect varied greatly depending on the cells used, and the activity did not increase in a similar way. As shown in **Fig. 1**, the degree of increase in NK activity also varied in the positive control containing IL-2. Treatment with BioBran/MGN-3 and fraction A increased NK activity in some cases but left the activity unchanged or reduced in others. Depending on the cells used, combined treatment with BioBran/MGN-3 and IL-2 produced conflicting results, increasing activity in some cases but decreasing it in other cases relative to treatment with IL-2 alone.

Analysis of the results from all blood cells showed that the effect on samples with a high control NK activity was different from that on samples with low activity. The results are summarised in **Fig. 2**. In blood cells with low initial NK activity, treatment with either BioBran/MGN-3 or IL-2 led to increased activity, which was higher after combined treatment with both than after either one alone. In blood cells

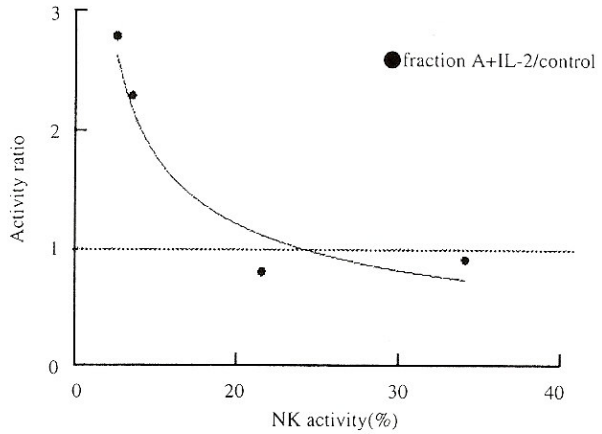

Fig.3 Correlation curves between fraction A with IL-2 and control

with high initial NK activity, however, IL-2 increased the activity but BioBran/MGN-3 caused almost no increase, or in fact decreased it in some cases. During combined treatment with IL-2 and BioBran/MGN-3, BioBran/MGN-3 seemed to inhibit the NK activity increased by IL-2. To investigate these seemingly contradictory results, a graph (**Fig. 3**) was plotted to show the value of NK activity after combined treatment with IL-2 and BioBran/MGN-3 divided by control NK activity (the degree of activation) against the control value (baseline activity). This clearly indicates that BioBran/MGN-3 tended to inhibit the increase in NK activity when the baseline NK activity of blood cells was above a certain value.

In this study, molecular-weight fractionation was also performed using an ultrafiltration membrane on the fraction obtained by affinity chromatography. A molecular species with the effect of increasing NK activity was found in the 12-15 kDa fraction after gel filtration chromatography and in the 10-30 kDa fraction after ultrafiltration. The two separation procedures produced similar results, suggesting that the size of this entity is within these ranges. However, as the estimates for molecular weight for both methods are based on the assumption that the molecule is spherical, the actual size of this sugar compound may be lower.

The results for the active fraction separated by ultrafiltration showed that the degree of activation of NK cells was dose-dependent. The fraction was shown to activate NK cells even at the low concentration of 10 mg/m*l*.

The compound produced results which appeared at first sight to be inconsistent. However, it is unlikely that two different substances are responsible independently for the increase and decrease of NK activity. It is unknown how BioBran/MGN-3 regulates NK activity at an appropriate physiological level *in vivo*.

There are many reports of molecules with immunomodulatory effects, mainly enhancement of NK activity, but BioBran/MGN-3 is particularly interesting as it increased NK activity in PBMC with low initial NK activity but decreased activity in PBMC with high initial NK activity. NK cells play an important role, particularly in cellular immunity, where they are activated by cytokines. However, when the immune response is expressed as humoral immunity, it leads to the production of cytokines involved in antibody production and allergic reactions. Some of these cytokines inhibit tumour suppression by cellular immunity, resulting in an inhibition of NK activity.[9,13,14] Given this, it is thought that BioBran/MGN-3 may stimulate the production of cytokines determining cellular immunity or humoral immunity, resulting in an

increase or decrease of NK activity.

The immunomodulatory effect of mushrooms is mainly attributable to β-glucan, which activates macrophages through cell surface receptors.[15] This β-glucan receptor is less specific and also reacts with mannose and N-acetyl-D-glucosamine.[16] Macrophages recognise sugar chains on cell surfaces and exert a phagocytic action. This enables them to recognise lectins and many types of sugars. Activation of the immune system by BioBran/MGN-3 may be mediated by macrophages.

As the blood cell fraction used in this study contained all the lymphocytes from the original blood and not just NK cells, in order to verify the above hypothesis it will be necessary to separate different lymphocyte populations and analyse the activity of each one. No clear effect of BioBran/MGN-3 was demonstrated on KHYG-1, a cell line established from NK cells. IL-2 is required to culture KHYG-1, and thus cells of KHYG-1 always have a high NK activity. This is a possible reason why BioBran/MGN-3 had no clear effect on KHYG-1.

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II-1-6

Enhancement of Natural Killer Cell Activity of Aged Mice by Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Abstract

The present study is aimed to examine the possibility of enhancement of natural killer (NK) cell activity in aged C57BL/6 and C3H mice using BioBran/MGN-3, a modified arabinoxylan from rice bran. Intraperitoneal injection of BioBran/MGN-3 (10 $mgkg^{-1}$ per day) caused a remarkable increase in the peritoneal NK activity as early as 2 days (35.2 lytic units), and the level remained elevated through day 14. The control aged mice had a level of 5.8 lytic units. Enhancement in NK activity was associated with an increase in both the binding capacity of NK cells to tumour targets and in the granular content as measured by BLT-esterase activity. Treatment did not alter the percentage of peritoneal NK cells. Data showed that peritoneal macrophages inhibit NK activity. In conclusion, BioBran/MGN-3 enhances murine NK activity of aged mice and may be useful for enhancing NK function in aged humans.

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Introduction

Immunodeficiency of ageing is manifested in terms of increased infection, cancer and autoimmunity. Immunodeficiency is apparently a multifactorial problem, where the loss of immunologic vigour plays a major role in the above-mentioned diseases. For example, the number of white blood cell subpopulations does not change appreciably with age, however qualitative changes in the leukocytes do appear to occur with age (Itoh et al. 1982; Albright & Albright 1983; Ghoneum et al. 1991a, b; Miller 1996; Pawelec et al. 1997, 1998; Greeley et al 2001; Rafi et al. 2003). Several studies have shown that natural killer (NK) cells, the activity of which resides in large granular lymphocytes, are important in the natural resistance against tumour (Lotzova 1984; Moretta et al. 2002), viral (Leong et al. 1998; Biron et al. 1999) and bacterial infection (Ghoneum et al. 2003). The functional significance of these cells has attracted considerable interest for its possible role in host defence against cancer (Herberman 1983, 2002; Wu & Lanier 2003).

Our earlier studies (Ghoneum et al. 1987, 1989, 1991a, b) and those of others (Itoh et al. 1982; Albright & Albright 1983) clearly demonstrated an age-association defect in NK activity. Therefore, several attempts have been made to augment NK activity during ageing. Whithin the last two decades, extraordinary emphasis has been placed on biological response modifiers (BRMs) as anticancer agents: interleukin-2 (Konjevic et al. 2003), interferon-γ (Appasamy et al. 1994; Allavena et al. 1998), killed streptococcal preparations (OK432) (Kurosawa et al. 1996), *Corynebacterium parvum* (Ghoneum et al. 1987), and bacilli Calmette-Guerin (Mizutani & Yoshida 1994). However, the clinical use of these BRMs has been severely limited because of their *in vivo* cytotoxicity. In this study, we examined the effect of a food supplement BioBran/MGN-3, for possible enhancement of NK cell activity in aged mice BioBran/MGN-3 is an arabinoxylan from rice bran that has been modified by carbohydrate hydrolysing enzymes from shiitake mushrooms (Ghoneum 1998a). We have previously reported that BioBran/MGN-3 enhances NK cell activity in healthy humans (Ghoneum 1998b), and increases the production of TNF-α by human peripheral blood lymphocytes (Ghoneum & Jewett 2000). It also sensitizes human leukaemia cells to death recepter (CD95) induced apoptosis (Ghoneum & Gollapudi 2003). In a double-blind study, Tazawa et al. (2003) found a prophylactic effect with the use of BioBran/MGN-3 against the common cold syndrome. In addition, BioBran/MGN-3 accelerated the protection against severe loss of bodyweight of mice due to treatment with the chemotherapeutic agent cisplatin (Jacoby et al. 2001; Endo & Kambayashi 2003). In this study, we tested the ability of BioBran/MGN-3 to enhance NK cell activity in aged mice, and to elucidate the possible mechanism that underlies the BioBran/MGN-3 effect.

Materials and Methods

1. Animals and materials

The mice used in this study were the inbred strain of aged C57BL/6 and C3H female mice (18 months old) and were purchased from the Animal Laboratory, University of California, Berkeley, CA. Mice were housed five per cage and were permitted free access to water and food; they were accommodated for 1 week before experiments.

Complete medium (CM) consisted of RPMI-1640 supplemented with 10% fetal calf serum. 100 units penicillin and 100 μ g streptomycin.

Target cells were the YAC-1 cell line, a Moloney leukaemia virus-induced mouse T-cell lymphoma of A/Sn mice origin, which was maintained in CM at a starting density of 3×10^5 cells mL^{-1}.

BioBran/MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushrooms; it contains polysaccharides (β1, 3-glucan and activated hemicellulose). BioBran /MGN-3 was provided by Daiwa Pharmaceuticals Co., Ltd, Tokyo, Japan.

2. *In vivo* treatment protocol

BioBran/MGN-3 was orally administered to two groups of mice daily in a volume of 0.1 mL at a concentration of 10 $mgmL^{-1}$ of BioBran/MGN-3 (dry weight) in water or by intraperitoneal injection in a volume of 0.1 mL at the same concentration of BioBran/MGN-3 in phosphate-buffered saline. The does of 1 mg of BioBran/MGN-3 per mouse was calculated from that recommended for humans (50 $mgkg^{-1}$). Control mice were given saline solution alone. At 2, 5 and 14 days after treatment, the peritoneal and splenic NK activity, as well as the cellularity of these tissues, were examined.

3. Preparation of peritoneal exudate cells (PECs), splenic NK cells and bone marrow NK cells

At 2, 5 and 14 days after treatment with BioBran/MGN-3, mice were killed by cervical dislocation, and PECs were isolated as follows: 5 mL of HBSS was injected intraperitoneally, the abdomen was massaged and 90% of the injected volume was recovered. Cell viability was 95%, as determined by the trypan-blue exclusion test. Spleens were removed, teased in CM, and contaminating erythrocytes were lysed with distilled water. Splenic lymphocytes were pelleted, and washed three times with HBSS. Bone marrow cells were flushed out from femur and tibia and washed in HBSS.

4. Measuring NK activity by the ^{51}Cr-released assay

The ^{51}Cr-released assay was used to determine NK cell activity in the peritoneum, spleen and bone marrow. In brief, 5×10^6 YAC-1 cells were labelled with 100 μCI $^{51}CrO_4$ solution (New England Nuclear, Boston, MA) for 1h at 27℃. Cells were washed four times with HBSS and resuspended in CM at 1×10^5 cells mL^{-1}. YAC-1 cells (1×10^4) were then pipetted into each well of 96-well round-bottomed Linbro plates. Effector cells were pipetted to quadruplicate wells to give effector/target cells ratios of 25 : 1, 50 : 1 and 100 : 1. The plates were incubated at 37℃ for 4h, then centrifuged at 1000 g for 5 min, and 0.1 mL of supernatant from each well was collected and counted in a gamma counter. The percentage of isotope released was calculated by the following formula:

$$\text{Lysis (\%)} = \frac{\text{(experimental release} - \text{spontaneous release)}}{\text{(total release} - \text{spontaneous release)}} \times 100$$

where spontaneous release is cpm from YAC-1 incubated in CM without effector cells and total release is cpm from YAC-1 incubated in Triton X-100. Results were expressed in lytic units (LU)/10^7, with 1LU being the number of effector cells required to lyse 5% and 15% of YAC-1 targets by peritoneal NK (P-NK) and splenic NK cells, respectively.

5. Flow cytometry

PECs were prepared from saline-treated and BioBran/MGN-3-treated mice. The percentage of peritoneal NK (P-NK) cells was demonstrated by their expression of NK 1.1 marker, and determined by flow cytom-

etry. Cells were first gated with forward and side scatter characteristics, and then the percentage of surface marker positive cells was measured. The monoclonal antibodies used were anti-NK 1.1 (Pharmingen, San Diego, CA).

6. BLT-esterase release assay

N-a-CBZ-L-Lysine thiobenzyl ester (BLT)-esterase activity was examined according to the procedure of Green & Shaw (1979) with little modification. In brief, PECs (3×10^6 cells mL^{-1}, 100 μL/well), from BioBran/MGN-3- and saline-treated aged mice were incubated with YAC-1 target cells (9×10^6 cells mL^{-1}, 100 μL/well) for 4h at 37℃ in RPMI 1640, in V-bottomed 96-microwell plates. The relative amount of secreted BLT-esterase activity was determined by incubating triplicate samples of supernatant (100 μL) and reaction buffer (100 μL) for 30 min at 37℃ in 96-well ELISA plates. The reaction buffer contained 0.1M Tris-HCl, pH 8.1, 0.2 mM DTNB, and 0.2 mM BLT (Sigma, St Louis, MO). The colour was allowed to develop for 40 min. Optical density readings were taken at 405 nm using an ELISA plate reader.

7. Morphological assessment of NK granularity

The effect of BioBran/MGN-3 on the level of granularity of P-NK cells was examined in cytospin preparations as previously described (Itoh et al. 1982). P-NK cells were purified as follows: macrophages and B-cells were removed by sequential incubation in CM in 150-cm^2 flasks for 1h at 37℃ and on nylon wool columns. Non-adherent mononuclear cells from nylon wool columns were fractionated by overlaying onto Percoll discontinuous gradients. For morphological assessment of NK granularity, P-NK cells (10^5 mL^{-1}) from BioBran/MGN-3- and saline-treated aged mice were centrifuged on slides at 200 g for 5 min using a cytospin cytocentrifuge (Shandon Southern Inst., Sewickley, PA, USA). Slides were air dried, fixed in 100% MeOH for 5 min, and then stained with 4% Giemsa solution for 15 min.

8. Percentage conjugate formation

The percentage of conjugate formation between P-NK cells and YAC-1 cells was examined in cytospin preparations (Itoh et al. 1982). In brief, peritoneal non-adherent cells (10^5 mL^{-1}) from BioBran/MGN-3 and saline-treated aged mice were mixed with YAC-1 cells (10^6 cells mL^{-1}) at 4℃ for 1h. The cells were centrifuged on slides at 200 g for 5 min using a cytospin cytocentrifuge and stained with Giemsa as previously described. The percentage of NK cells binding to YAC-1 cells within 200 cells was calculated in triplicate samples.

9. Suppressive effect of peritoneal macrophage (P-Mϕ) on P-NK cell activity

A set of experiments was carried out to examine the suppressive effect of P-Mϕ on P-NK cell activity. C57BL/6 mice were injected intraperitoneally with BioBran/MGN-3 for 5 days and the natural cytotoxicity by P-Mϕ, PECs, and PECs depleted of P-Mϕ (non-adherent cells) was examined separately by ^{51}Cr-released assay.

10. Admixture of P-Mϕ added to the non-adherent cells

To further confirm the suppressive effect of P-Mϕ, an admixture of equal numbers of P-Mϕ and non-adherent cells was examined for NK activity. The admixture contained cells at an effector/target ratio of 50 : 1. Results were compared with non-adherent cells alone.

Table 1 *In vivo* **affect of MGN-3 on tissue cellularity**

Strain of mice	Time after MGN-3 treatment (days)	Spleci cells ($\times 10^6$)		Peritoneal cells ($\times 10^6$)	
		MGN-3	Control	MGN-3	Control
C57BL/6	2	60±1** (150%)	40±2.3	9.4±1* (470%)	2.0±0.08
	5	61±0.08** (145.2%)	42±0.08	9.7±0.08 (461.9%)	2.1±0.08
	14	85.5±1.75** (192%)	44.5±2.1	8.5±1.05* (404.7%)	2.1±0.5
C3H	5	70±2.5** (166.7%)	42±3.7	6.8±0.4* (453.3%)	1.5±0.3

The percentage induction is given in parentheses.
Data represent the mean percentage of total peritoneal and splenic cells from C57BL/6 and C3H mice after intraperitoneal treatment with MGN-3.
Means±s.e.m of six mice at each time point were examined separately.
*P<0.01. **P<0.025, significantly different compared with control saline-treated aged mice.

11. BioBran/MGN-3 treatment by oesophageal tubing

Another set of experiments was carried out to examine splenic NK activity C57BL/6 mice. The mice were given BioBran/MGN-3 through oesophageal tubing at a concentration of 10 mg in 100 μL distilled water/mouse. After 14 days, the mice were killed and examined for splenic NK activity.

12. *In vitro* effect of BioBran/MGN-3 on NK activity

Splenic cells (1×10^6 cells mL^{-1}) from aged C57BL/6 mice were cultured with BioBran/MGN-3 at concentrations of 25 and 100 $\mu g mL^{-1}$. At 16h after treatment, cells were washed twice in CM and were examined for NK activity.

13. Statistical analysis

A two-tailed Student's t-test was used to determine the degree of significance between NK cell activity of control and BioBran/MGN-3-treated mice.

Results

1. Peritoneal and splenic cellularity

Results of the effect of intraperitoneal treatment with BioBran/MGN-3 on peritoneal and splenic cellularity are shown in **Table 1**. BioBran/MGN-3 significantly increased peritoneal cellularity (404-470% of control) in C57BL/6 and C3H mice. The effect was noted as early as 2 days, and remained elevated through day 14. An increase in splenic cellularity in both strains of mice was also noted but to a lesser extent (145-192% of control).

2. NK activity after intraperitoneal injection

Results in **Table 2** show that intraperitoneal injection with BioBran/MGN-3 resulted in enhancement in P-NK activity. This was detected as early as 2 days (35.2 LU), and the level remained elevated through Day 14 as compared with saline-treated aged mice. A similar pattern of increase was observed with the P-

Table 2 Effect of intraperitoneal injection of MGN-3 on natural killer (NK) cell activity in the peritoneal cavity (PC) and spleen

Strain of mice	Treatment	Day of assay	NK activity							
			PC				Spleen			
			25: 1	50: 1	100: 1	LU 5%	25: 1	50: 1	100: 1	LU 15%
C57BL/6	Saline	2	1.1	1.5	2.5	5.8	4.1	9.3	14.3	9.6
	MGN-3	2	4.0	7.1	9.2	35.2*	2.5	8.2	11.9	9.0
	Saline	5	0.8	1.7	2.4	6.2	5.1	8.0	10.3	7.7
	MGN-3	5	3.9	8.1	11.2	37.0*	6.1	9.2	14.0	9.2
	Saline	14	1.3	2.2	3.0	6.6	5.3	8.3	14.4	9.1
	MGN-3	14	4.4	6.7	8.8	34.0*	5.5	7.8	12.5	8.5
C3H	Saline	5	0.1	0.3	1.0	5.0	3.8	8.4	13.0	9.1
	MGN-3	5	1.2	2.4	7.5	24.1*	4.5	10.5	14.5	10.0

MGN-3 was injected into C57BL/6 and C3H mice.
Pooled lymphoid cells were prepared from the peritoneal cavity or spleens of saline-or MGN-3-treated mice.
Cells were harvested at 2, 5 and 14 days after treatment.
NK activity was assayed in a 4-h Cr-release assay against YAC-1 target cells at effector/target cell ratios of 25 : 1, 50 : 1 and 100 : 1 and expressed as the number of lytic units (LU).
*P<0.01, significantly different compared with saline-treated mice.

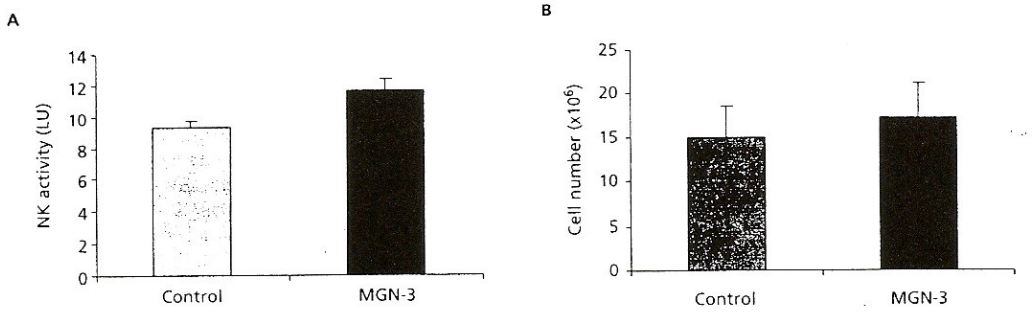

Figure 1 *In vivo* action of MGN-3 on bone marrow natural killer (NK) cell activity.
A : NK activity in C57BL/6 mice was examined 14 days after intraperitoneal treatment with MGN-3.
Activity of NK cells was measured by 4-h Cr-release assay at an effector/target ratio of 100 : 1.
B : The cell number of bone marrow was also examined.
Data are mean ± s.e.m of four mice examined separately.

NK activity of C3H mice after injection with BioBran/MGN-3. However, intraperitoneal treatment with BioBran/MGN-3 did not increase splenic NK cell activity at 2, 5 or 14 days after treatment in either strain of mice (**Table 2**).

3. Bone marrow NK activity

Bone marrow NK activity was examined in C57BL/6 mice at 14 days after intraperitoneal injection with

Table 3 Effect of oral treatment of MGN-3 on natural killer (NK) cell activity in the peritoneal (PC) cavity and spleen

Treatment	Day of assay	NK activity							
		PC				Spleen			
		25: 1	50: 1	100: 1	LU 5%	25: 1	50: 1	100: 1	LU 15%
Saline	14	0.3	1.1	2.0	5.5	4.1	8.0	10.6	8.4
MGN-3	14	0.6	2.1	3.3	6.3	8.1	16.2	19.9	16.7*

MGN-3 was injected into C57BL/6 mice.
Pooled lymphoid cells were prepared from the peritoneal cavity or spleens of saline- or MGN-3-treated mice.
Cells were harvested 14 days after treatment.
NK activity was assayed in a 4-h Cr-release assay against YAC-1 target cells at effector/target cell ratios of 25 : 1, 50 : 1 and 100 : 1 and expressed as number of lytic units (LU).
*P<0.01, significantly different compared with saline-treated mice.

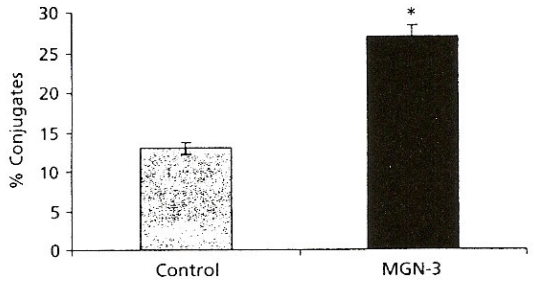

Figure 2 Percentage conjugates between natural killer (NK) cells and YAC-1 target cells from C57BL/6 mice examined at 5 days after intraperitoneal injection with MGN-3 and saline.
*P<0.01, compared with control saline-treated mice.

BioBran/MGN-3. As shown in **Figure 1**, BioBran/MGN-3 did not significantly increase either NK cell activity or the number of cells as compared with control mice.

4. NK activity after oral treatment

Oesophaged tubing of 10 mg in 100 μL/mouse resulted in a 200% increase in splenic NK cell activity in C57BL/6 mice at 14 days after treatment. On the other hand, P-NK cells showed no change in activity after treatment with BioBran/MGN-3 as compared with saline-treated mice (**Table 3**).

5. Percentage of P-NK cells

A surface marker for NK cells, NK1.1, was used to examine the frequency of P-NK cells. Results showed no significant differences in the percentaged of P-NK cells between the mice treated with BioBran/MGN-3 (16%) and those treated with saline (14%).

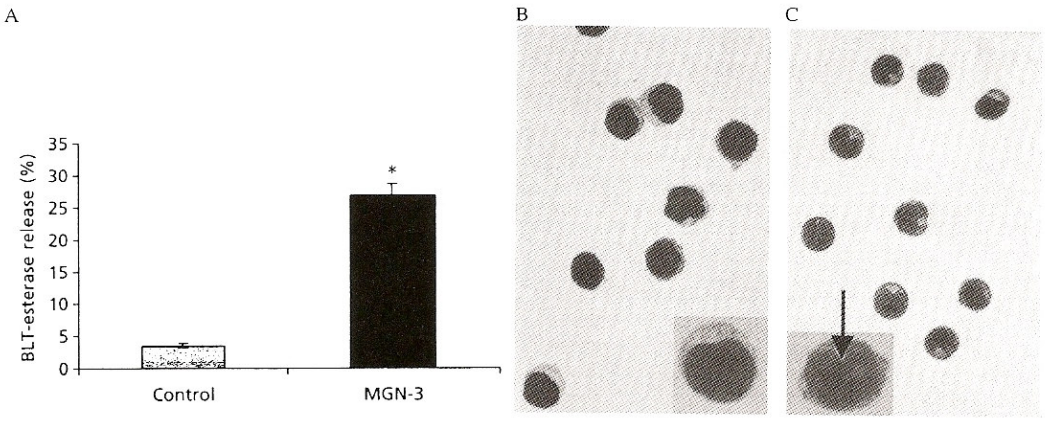

Figure 3

A : *In vivo* action of MGN-3 on BLT-esterase activity.
BLT-esterase activity was examined in C57BL/6 mice injected intraperitoneally with MGN-3 and saline.
Data are mean±s.e.m of three experiments.
*$P<0.01$, significantly different compared with control saline-treated aged mice.
B : Cytocentrifuge preparation of peritoneal NK (P-NK) cells of saline-treated mice.
P-NK cells were used in a purified from using Percoll discontinuous gradients.
Notice the high nuclear cytoplasmic ratio and absence of granules in control aged mice.
C : Preparation of NK cells after MGN-3 treatment.
Notice the high granularity of NK cells.
Notice one NK cell with an arrow pointing to the granules.
(Giemsa stain, ×740.)

6. Percentage conjugate formation

The effect of BioBran/MGN-3 on the binding capacity of P-NK cells to YAC-1 cells was examined in cytospin preparations. As shown in **Figure 2**, BioBran/MGN-3-treated P-NK cells demonstrated 26% conjugate formation as compared with saline-treated P-NK cells (13%); this represents a 2-fold increase.

7. Granularity of P-NK cells

BioBran/MGN-3-treated P-NK cells demonstrated a remarkable increase in the BLT-esterase activity. **Figure 3** shows an increase in the BLT-esterase activity at 5 days as compared with saline-treated mice. This observation was further confirmed in cytospin preparations. **Figure 3B, C** shows NK cells from aged mice with a low or absent granular content, yet BioBran/MGN-3-treated mice at 5 days demonstrated an increase in NK cell granular content.

8. Suppressive effect of P-Mϕ on P-NK cell activity

The suppressive effect of P-Mϕ on P-NK cell activity after treatment with BioBran/MGN-3 was examined. **Figure 4A** shows that P-Mϕ alone sowed an undetectable level of antitumour activity. On the other hand, natural cytotoxicity by BioBran/MGN-3-treated PEC showed 7%, while PEC depleted of P-Mϕ (the non-adherent cells) demonstrated a further increase to 16%. An admixture of P-Mϕ and non-adherent cells resulted in a significant inhibition of NK activity exhibited by non-adherent cells (33%) as compared with non-adherent cells alone (**Figure 4B**).

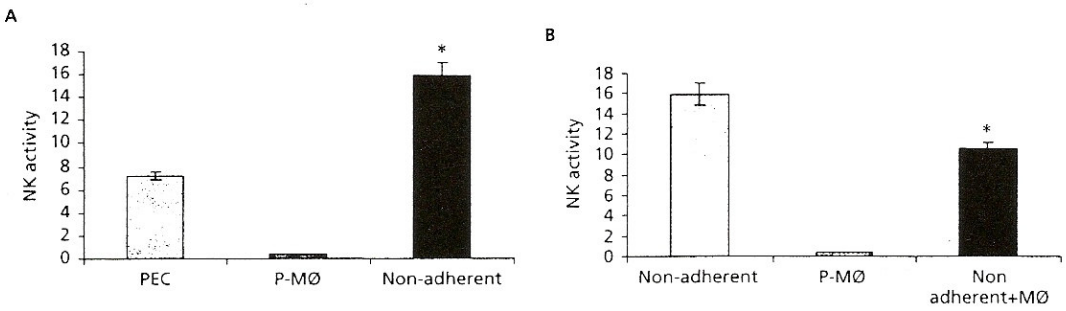

Figure 4 ***In vivo*** **effect of MGN-3 on peritoneal exudate cells (PECs), macrophages (P-Mϕ), and non-adherent cells.**

A : Aged C57BL/6 mice were injected intraperitoneally with MGN-3 for 5days and natural killer (NK) activity was examined at an effector/target ratio of 50 : 1 (A)

*P<0.025, compared with PECs.

B : Admixture of P-Mϕ to non-adherent cells from MGN-3-treated aged mice.

NK activuty was examined at an effector/target ratio of 50 : 1.

*P<0.025, significantly different compared with non-adherent cells alone.

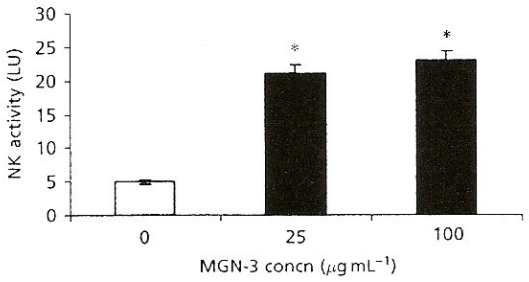

Figure 5 ***In vitro*** **action of MGN-3 on natural killer (NK) cell activity.**

Splenic lymphcytes from aged C57BL/6 mice were cultured for 16h in the presence or absence of MGN-3.

Activity was measured by 4-h Cr-release assay and expressed as number of lytic units (LU).

Data are mean ± s.e.m of three experiments.

*P<0.01, significantly different compared with control untreated cells.

9. *In vitro* action of BioBran/MGN-3 on NK activity

Culture of lymphocytes from aged C57BL/6 mice with BioBran/MGN-3 for 16h resulted in a significant induction of NK activity as follows: 21 and 23 LU at a concentration of 25 and 100 μgmL^{-1}, respectively, as compared with control untreated cells (**Figure 5**).

Discussion

In the present study, we demonstrated that BioBran/MGN-3 enhances murine NK activity in aged mice both *in vivo* and *in vitro*. The increase in NK activity was not due to simple intercompartmental redistribution of NK cells, but rather to an actual increase in the activity of the effector cells. This was evidenced

by examining the NK activity and the cell number of other lymphoid organs. Intraperitoneal injection with BioBran/MGN-3 did not significantly alter NK activity in the spleen or bone marrow, despite an increase in cellularity.

The mechanism by which BioBran/MGN-3 enhances murine NK activity may involve enhancing both the binding and the lethal hit. The study showed an increase in the percentage of conjugates of NK cells to their tumour targets after treatment with BioBran/MGN-3, which may be attributed to an increase in the expression of adhesion molecule (ICAM-1) on NK cells (Ghoneum & Jewett 2000) or other cell surface markers, for example LFA-1. The increase in conjugation after treatment with BioBran/MGN-3 may also suggest involvement of other effector cytotoxic cells such as CTL killing YAC-1 targets, since the percentage of NK cells did not alter with BioBran/MGN-3. The study also indicated an increase in the activity of the granule lytic BLT-esterase. It has been reported that aging is associated with a decline in the percentage of murine NK cells that carry granules (Ghoneum et al. 1989), and in the perforin expression in aged human NK cells (Rukavina et al. 1998).

Data demonstrated that P-Mϕ exhibit an inhibitory effect on NK cells. This was indicated by the ability of BioBran/MGN-3 to significantly increase the activity of P-NK cells that had been depleted of Mϕ. Earlier studies by Irimajiri et al. (1985) suggested that the active adherent suppressor cells, possibly macrophages, are responsible for the suppression of aged murine NK cell activity. We do not know the mechanism by which P-Mϕ suppress P-NK activity after treatment with BioBran/MGN-3, but it may involve increased production of H_2O_2 (Kono et al. 1996).

The increase in P-NK activity and in PECs is due to the direct effect of BioBran/MGN-3; the possibility that repeated injections of BioBran/MGN-3 caused any persistent peritoneal inflammation was excluded since control mice were repeatedly subjected to injections of saline solutions without the increase in P-NK activity or in PECs. The immunomodulatory effect of BioBran/MGN-3 was further confirmed by *in vitro* studies of a co-culture of BioBran/MGN-3 with NK cells for 16h. The increase in both splenic and peritoneal cellularity was a consistent finding in both strains of mice. The reason for this finding is not clear but it could be attributed to cell proliferation in these compartments. We have previously reported a significant increase in human T- and B-cell proliferation after ingestion of BioBran/MGN-3 (Ghoneum 1998a). Further studies are needed to clarify this point.

The inability of BioBran/MGN-3 to enhance splenic NK activity *in vivo* is not fully understood, but it could be attributed to the dilution of NK cells in the spleen due to an expansion of other cell populations. As shown in **Table 1**, splenic cellularity in BioBran/MGN-3-treated mice was increased. It has been reported that NK cells in different tissues display a differential response towards the enhancing effect of other BRMs. For example, treatment with *Candida albicans* remarkably inceased P-NK activity without an effect on splenic NK activity (Marconi et al. 1985). On the other hand, introducing BioBran/MGN-3 into the mice by oesophageal tubing increased splenic NK activity (200% as compared with control mice). This suggests that the enhancement of splenic NK cell activity depends on how this agent is introduced into the body.

Conclusions

The results of the present study show that intraperitoneal injection and oral treatment with BioBran/MGN-3 resulted in a significant enhancement of murine peritoneal and splenic NK cell activity, respectively.

The increase in NK activity was associated with an increased level of NK granularity. We conclude that BioBran/MGN-3 has the ability to enhance NK activity in aged mice and may be useful for enhancing the NK function in ageing humans.

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II-2-1

Anti-HIV Activity *In Vitro* of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Abstract

BioBran/MGN-3, an arabinoxylan from rice bran that has been enzymatically modified with extract from *Hyphomycetes mycelia*, was tested for anti-HIV activity *in vitro*. BioBran/MGN-3 activity against HIV-1 (SF strain) was examined in primary cultures of peripheral blood mononuclear cells. BioBran/MGN-3 inhibited HIV-I replication by: (1) inhibition of HIV-1 p24 antigen production in a dose dependent manner —BioBran/MGN-3 at concentrations of 12.5, 25, 50, and 100 μg/m*l* showed 18.3, 42.8, 59, and 75% reduction in p24 antigen, respectively; and (2) inhibition of syncytia formation maximized (75%) at concentrations of 100 μg/m*l*. Further studies showed that ingestion of BioBran/MGN-3 at concentration of 15 mg/kg/day resulted in a significant increase in T and B cell mitogen response at 2 months after treatment: 146% for PHA, 140% for Con A, and 136.6% for PWM mitogen. We conclude that BioBran/MGN-3 possesses potent anti-HIV activity and in the absence of any notable side effects, BioBran/MGN-3 shows promise as an agent for treating patients with AIDS.

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II-2-1 Anti-HIV Activity *In Vitro* of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

Purpose

Human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS). HIV is one of the principal threats to human life worldwide. According to several sources (Center for Disease Control, American Red Cross, and other public health agencies) there are approximately 1.5 million HIV infected people in the United States and as many as 50 million worldwide. It has been estimated that by the year 2000, 110 million will be infected with HIV (2% of the world population). Drugs such as AZT and other nucleoside analogues pose as major problems in slowing the progression of the disease. At the last AIDS International Conference held in Vancouver, Canada, there was very little promise of a vaccine. The lack of vaccination or effective treatment send alarming singnals. Therefore, there is great interest and need to identify anti-HIV agents that are not only active against the virus, but also can potentiate the host immune system without having deleterious side effects. Recently we demonstrated that BioBran/MGN-3, a modified arabinoxylan from rice bran, is a potent biological response modifier (BRM) that is able to enhance natural killer (NK) cell activity in cancer patients[1,2]. In this study we showed that BioBran/MGN-3 inhibited HIV replication in patients' peripheral blood mononuclear cells (MNC) as well as syncytia formation. BioBran/MGN-3 also increases T and B cell mitogen response upon ingestion. These studies demonstrated BioBran/MGN-3 has strong anti-HIV activity and may be of value in combination therapy in the treatment of HIV-1 infected patients.

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 Basidiomycetes mycelia. It is a polysaccharide that contains β-1, 4 xylopyronase hemicellulose (**Fig.1**). MGN-3 is commercially known as Biobran (Daiwa Pharm., Co., Ltd., Tokyo, Japan).

2. Complete medium (CM)

RPMI-1640 (Sigma) was supplemented with 1% antibiotics (v/v) and 20% (v/v) fetal bovine serum and recombinant IL-2.

3. Production of HIV-1 p24 antigen

MNCs from 3 healthy individuals were incubated (37℃) with PHA (5 μg/m*l*) for 3days and then washed before incubation (37℃, 1 hr) with HIV-1 SF strain (HIV-1 p24 of 3000 pg/10^6 cells). MNCs were then washed 3× with PBS to remove unbound virus. Infected cells were incubated (37℃, 7 days) either with or without BioBran/MGN-3 at various concentrations (0-100 μg/m*l*), in CM. Half of the medium was changed twice per week with corresponding BioBran/MGN-3 concentrations. At the end of the incubation period, culture supernatants of HIV-1 infected cells were collected and analyzed for viral production. HIV-1 p24 was measured by antigen capturing ELISA using a commercially available kit (DuPont NEN, Boston, MA) according to the protocol provided by the manufacturer.

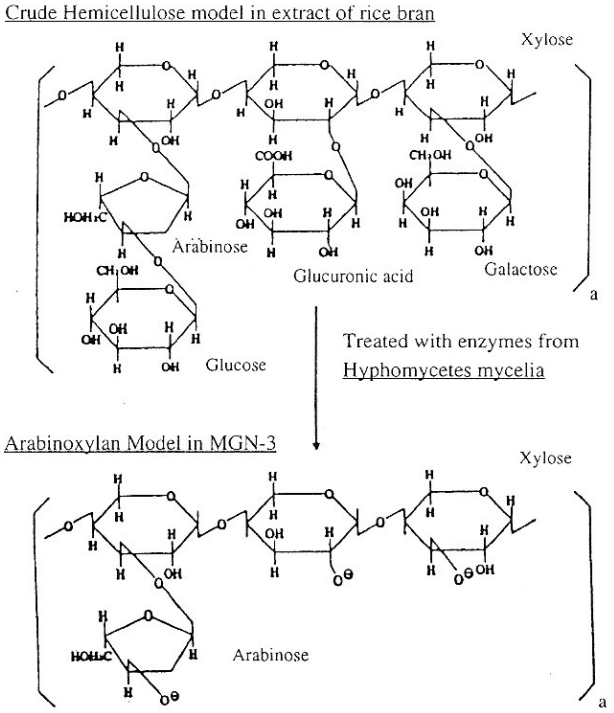

Fig. 1 Crude hemicellulose model in extract of rice bran treated enzymatically by glycosidases from *Hyphomycetes mycelia*

Main chemical structure of MGN-3.

It is an arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain.

4. Syncytia formation

A slight modification of Johnson and Walker[3] cell fusion assay was used. Briefly, MNC from 5 AIDS patients were cultured with PHA and in the presence or absence of BioBran/MGN-3 at various concentrations (0-100 μg/m*l*). HIV-infected MNC cells were incubated (37℃) in flat bottom 96-well plates (2×10^4 /well). Cultures were then examined after 7 days. The total number of syncytia were counted per well and are reported as number of syncytia/well.

5. *In vivo* T and B lymphocyte proliferation

We investigated the *in vivo* effects of BioBran/MGN-3 on T and B cell proliferation using ^{3}H-thymidine uptake. Five healthy control subjects were given BioBran/MGN-3 at concentrations of 15 mg/kg/d orally for two months. MNCs were prepared from peripheral blood of these individuals before treatment (base line) and at two months after treatment. MNCs were incubated with or without 10mg/m*l* of phytohemagglutinin (PHA), Concavalin A (Con A), or pokeweed mitogen (PWM) for three days. One mCi of ^{3}H-thymidine was added to the cell cultures for the last 8 hours. DNA was harvested and ^{3}H-thymidine uptake was determined by scintillation counter.

Table 1 Dose deependent inhibition of HIV-1 replication by MGN-3

MGN-3 dosage (μg/ml)	HIV-1 p24, pg/ml		
	Subject I	Subject II	Subject III
0	565 (100%)	720 (100%)	433 (100%)
12.5	534 (94.5%)	480 (66.7%)	364 (84.1%)
25	325 (57.5%)	333 (46.3%)	294 (67.9%)
50	263 (46.5%)	139 (19.3%)	248 (57.3%)
100	132 (23.4%)	77 (10.7%)	178 (41.1%)

Note. Date from three different subjects examined at 7 days.

6. Cell viability

Viability was measured by calorimetric method using the tetrazolium salt MTT assay. A mitochondrial dehydrogenase catalyzes the formation of blue formazan crystals from tetrazolium salt. The amount of formazan produced is proportional to the number of living cells. Briefly, HIV-1 infected cells at 4, 7 and 11 days post-infection were dispensed in triplicate into 96 well round bottom tissue culture plates. MTT (50 μg) was added to each well and the plates were incubated for 4 hrs at 37℃. The formazan crystals were solubilized with 40 mM HCl/isopropanol and the optical density at 590 nm was measured using an ELISA plate reader (Molecular Devices, Menlo Park, CA).

7. Statistical analysis

A student's *t*-test was used to examine the significance of the differences between control and BioBran /MGN-3 treated cells *in vitro* as well as differences in T and B cell mitogen response before and after treatment *in vivo*.

Results

1. Production of HIV-1 p24 antigen

BioBran/MGN-3 inhibited HIV-1 replication in MNC in dose dependent manner. As shown in **Table 1**, BioBran/MGN-3 caused inhibition in HIV p24 antigen production in all subjects, however, there was a clear differential response among different individuals towards BioBran/MGN-3, inhibitory effect by BioBran/MGN-3. The effect of BioBran/MGN-3 at low concentration (12.5 μg/ml) on subject I was minimal (5.5%) while the same dose caused 34% antigen production in subject II. Similarly, at high concentrations (100 μg/ml) of BioBran/MGN-3, the percentage of p24 antigen inhibition varied greatly among the three subjects (59%-90%). Data in **Fig.2** summarizes the mean and SD of the results depicted in **Table 1**. At concentrations of 25, 50, and 100 μg/ml, BioBran/MGN-3 demonstrated 18.3, 42.8, 59 and 75% inhibition in the production of HIV-1 p24 antigen, respectively.

2. Effect on syncytia formation

We conducted studies on the effect of BioBran/MGN-3 on HIV induced syncytia formation *in vitro*. Results in **Table 2** showed that BioBran/MGN-3 siginificantly inhibited syncytia formation. The effect

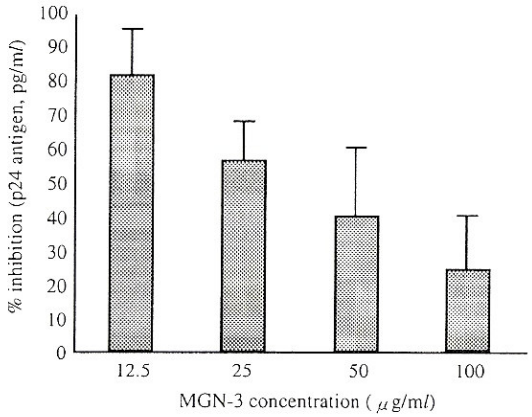

Fig. 2 Effect of MGN-3 on production of HIV-1 p24 antigen

Data represent mean ±S.D. of three different individuals from Table 1.

Table 2 Inhibition of syncytia formation by MGN-3

MGN-3 dosage (μg/ml)	Syncytia formation (SF)	
	No. of SF	% Inhibition
0	42±8	0
12.5	25.8±7	38.5
25	21.5±5	50
50	15.8±4	62.5
100	10.5±3	75

Note. Data represent means ±S.D. of five individuals examined at 7 days.

was dose dependent and maximum inhibition (75%) was observed at a concentration of 100 μg/ml.

3. *In vivo* effect of BioBran/MGN-3 on T and B cell proliferation

The *in vivo* effect of BioBran/MGN-3 on cell proliferation was studied using 3H uptake. MNC were prepared from peripheral blood of five healthy individuals who were given BioBran/MGN-3 at concentration of 15 mg/kg daily for two months. **Fig.3** showed that treatment with BioBran/MGN-3 resulted in significant changes in MNC proliferation. MNC is the presence of PHA (T cell mitogen) exhibited signicant increase in cell proliferation (146%) as compared with baseline value ($P<0.001$). Similar results were observed when Con A mitogen was used (140%, $P<0.001$). MNC showed 136.6% increase in their proliferative response to PWM, a B cell mitogen as compared to base line value ($P<0.05$).

4. Cell viability

The effect of BioBran/MGN-3 on the viability of HIV-1 infected cells was examined. MTT assay detected no significant differences between treated cells and controls examined at 4, 7, and 11 days post infection.

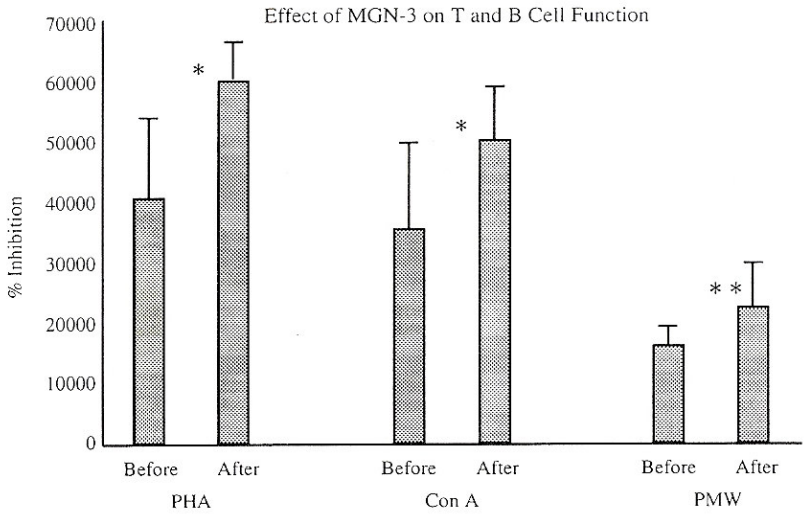

Fig. 3 ***In vivo*** **action of MGN-3 on T and B cell mitogen response at 2 months after treatment**
MNC were cultured for three days in the presence or absence of PHA, Con A and PWM.
^{3}H incorporation was examined.
Data represent mean±S.D. of five different individuals.
*P<0.001, **P<0.05.

Discussion

In this study we demonstrated that BioBran/MGN-3 possesses an inhibitory effect on HIV replication *in vitro* without cytotoxicity. BioBran/MGN-3 is composed of denatured hemicellulose that is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from Hyphomycetes, mycelia. 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 (**Fig.1**). BioBran/MGN-3 has proven to be a potent biological response modifier(BRM) that activates human natural killer(NK) cell activity *in vivo* and *in vitro*[1-2]. The results of this study also show that BioBran/MGN-3 acts as an anti-viral agent; it inhibited HIV-1 production in peripheral blood mononuclear (MNC) in vitro as manifested by: 1) inhibition of HIV-1 24 antigen production, and 2) inhibition of syncytia formation.

Side effects are one of the problems of using anti-HIV agents for treatment. The prolonged use of several drugs such as PI, azidothymidine, dideoxycytidine, dideoxyinosine and D4T are associated with severe toxicitity and development of drug resistance[4-6]. Therefore, many attempts have been made recently to develop new products that possess anti-HIV activity without the side effects. A number of plants belonging to the mint family (Labiatae) have been reported to have anti-viral activity against different viruses, including HIV[7-10]. Hyssop officialis contains several active ingredients that exhibit anti-HIV activity, for example, tannins[11], and polysaccharide (MAR-10) that inhibits production of HIV-1 antigen in HIV-1 infected MNC and in HUT78 T cell line[12]. Another polysaccharide from pine cones (Pinus parvifloria Sieb Zucc) has also been reported to inhibit HIV activity[13]. With respect to polysaccharide from rice bran.

II-2-1 Anti-HIV Activity *In Vitro* of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

Earlier studies demonstrated that extracted hemicellulose from rice bran fiber (RBF) has known unique biological effects; for example, a-glucan from rice bran show potent antitumor activity in mice[14], arabinose and xylose from RBF show defensive effects against bis (n-tributyltin) oxide (TBTO) induced thymic atrophy in rats[15]. Unprocessed RBF and cholestyramine have been observed to increase peripheral blood leukocyte in humans[16]. The polysaccharide used in this study acts as an interferon inducer[17] and has been tested as an anti-cancer agent in patients with different types of malignancy[2].

BioBran/MGN-3 was examined for toxicity using blood chemistry analysis for SMAC and liver enzymes (SGOT and SGPT). Five healthy subjects were given BioBran/MGN-3 orally at concentration of 45 mg /kg/d. After one month, no significant changes were detected in all parameters investigated. *In vivo* studies showed BioBran/MGN-3 has highly significant augmentory effects on lymphocyte proliferation as shown by mitogen response with PHA ($P<0.001$), Con A ($P<0.001$) and PWM ($P<0.05$). Moreover, cell viability was not affected in MNC up to 11 days post-treatment. Clearly BioBran/MGN-3 inhibits HIV-replication in a dose dependent manner and maximum effect was observed at a concentration of 100 μg/m*l*. The results also showed differential response among participants toward the inhibitory effect against HIV replication by BioBran/MGN-3. The mechanism by which BioBran/MGN-3 inhibits HIV replication is not fully understood. HIV infects CD4+ cells, primary T lymphocytes and macrophages by binding the CD4 receptors of the host cells. The inhibitory effect on HIV replication by BioBran/MGN-3 may be through the drug's interference with HIV replication post-entrance, alteration of chemokine receptors or chemokine production.

We conclude that the results generated in this investigation may represent the basis of future studies on clinical trials of BioBran/MGN-3 as an anti-HIV agent.

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II-3-1

A Study of a Cancer-Cell Growth Inhibiting Ingredient in Modified Arabinoxylan from Rice Bran (BioBran /MGN-3)

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Introduction

Various cells in the blood are derived from pluripotent haematopoietic cells in the bone marrow, which ultimately differentiate into erythrocytes, leukocytes, platelets, and lymphocytes. During the process of differentiation into leukocytes, haematopoietic cells change sequentially into myeloblasts, promyelocytes, myelocytes, and then metamyelocytes, finally developing into granulocytes including neutrophils, eosinophils, and basophils with their respective functions. Cells can repeat cycles of growth division a finite number of times at each stage before finally becoming terminally differentiated. Normal blood cells maintain a balance between growth and differentiation. However, if cells deviate from such a normal balance or if abnormal differentiation occurs at any stage and cells are unable to reach the terminally differentiated stage, they will become cancerous (leukaemia), where they will remain undifferentiated and continue to grow. If it were possible to discover a way to inhibit the growth of undifferentiated leukaemia cells and induce them to differentiate into terminally differentiated cells, this could provide a therapy for leukaemia.

In the early 1980s, Wang et al. in China reported that treatment of leukaemic cells from leukaemia patients

This article is a revision of a presentation given at the 2002 annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry.

with all-trans retinoic acid induced differentiation into normal cells. They used this in treatment of leukaemia although the number of patients was small. In 1988, it was reported that the differentiation inducer vitamin A compound (all-trans retinoic acid: ATRA) alone induced differentiation and allowed complete clinical remission[1]. These reports showed that differentiation-inducing therapy is feasible and highlighted studies of differentiation induction[2]. Complete remission implies that no leukaemic cells can be detected in peripheral blood and that the patient's physical condition returns to normal. This was tested in France[3], the U.S.[4] and Japan[5] again and the results were confirmed. However, acute promyelocytic leukaemia accounts only for 15% of all leukaemias. A differentiation inducer effective against all types of leukaemia as well as other types of cancer is needed.

The differentiation of leukaemic cells has been actively studied to understand the mechanism of leukaemia development and the nature of the cells and to develop therapies. Many types of leukaemic cell lines with different modes of differentiation have been established. Representative examples include HL60 (derived from acute promyelocytic leukaemia cells), K562 (from chronic myelocytic leukaemia cells), and U937 (from monocytic leukaemia cells). Growth potential and tumour-forming ability decrease depending on the level of differentiation and are completely lost in some cell lines. Differentiation inducers reported to date include vitamin A compounds, carcinogenesis promoters such as TPA (12-o-tetradecanoyl phorbol-13-acetate)[6], vitamins such as active vitamin D3 (1 α,25-dihydroxy vitamin D3)[7] and vitamin K2[8], polar compounds such as DMSO (dimethylsulfoxyde)[9], cytokines including rTNF-α (recombinant tumour necrosis factor-α)[10], camputothecin[11], daidzeinn[12], VP16 (4'-dimethylepipodophyllo-toxin ethyliden-β-D-glucoside)[13], bufalin[14], GGA(geranyl gerranyl acetone)[14], and actinomycin D[15].

Treatment with a growth/differentiation factor for normal haematopoietic stem cells causes normal stem cells to differentiate as they grow. This suggests that a coordinate regulatory mechanism is involved in the growth and differentiation of normal stem cells.[16] In leukaemic cells, however, the mechanisms of growth and differentiation are abnormal and uncoordinated, and growth becomes separated from differentiation in many cases[16-18]. The aforementioned differentiation inducers, such as TPA and active vitamin D3, are considered to cause differentiation of leukaemia cells through their particular differentiation-inducing functions rather than direct inhibition of growth. On the other hand, it has been reported that the addition of GM3 ganglioside to the HL 60 cell line caused inhibition of cell growth and differentiation into monocytes and macrophages[19]. These differentiation inducers are important compounds for differentiation-inducing therapy.

Compounds used in cancer treatment are designed to injure and kill cancer cells and many conventional chemotherapeutic agents are reported to induce apoptosis in cancer cells. Apoptotic cells were detected in cancer tissue of patients administered cisplatin, VP16, or retinoic acid[20,21]. Apoptosis is gene-programmed cell death that is not accidental and is reproducible. This cell death is physiologically meaningful and is frequently induced by a certain type of stimulation. Morphologically, the cell volume decreases without destruction of the cell membrane, accompanied by DNA fragmentation, nuclear chromatin aggregation, cytoplasmic swelling, and formation of apoptotic bodies. Subsequently, apoptotic cells are phagocytosed by macrophages and granulocytes and disappear without inducing inflammatory reactions[22]. A differentiation inducer that inhibited cancer cell growth by inducing differentiation and then caused apoptosis could be an ideal therapeutic agent.

In recent years, disorders due to immunodeficiency have increased and foods with biophylactic activity are now considered important for maintaining health. Dietary fibre not only has a mechanical action of

cleansing the gastrointestinal tract but also various physiological actions including an immunomodulatory effect, which is attracting attention. The present study was designed to search for a cell-growth inhibitor in BioBran/MGN-3, a commercially available complex product obtained by partial hydrolysis of a hemicellulose fraction from rice bran with a carbohydrase complex in a culture filtrate of shiitake fungi, a basidiomycete. In addition, the types of cancer cells on which the target compound exerts its effect were examined.

Materials and Reagents

BioBran/MGN-3 powder was provided by Daiwa Pharmaceutical. The human acute promyelocytic leukaemia cell line (HL60) was from Japan Health Sciences Foundation. The human chronic myelocytic leukaemia cell line (K562), liver cancer cell line (HepG2, HLE), pancreatic cancer cell line (PANC1), uterine cervical cancer cell line (HeLa), a colon cancer cell line (SW480), and a normal gingival fibroblast cell line (Gin1) were from Dainippon Pharmaceutical Co., Ltd. The cell culture medium for cell suspension, RPMI1640, was from Sigma and Dulbecco's Modified Eagle's Medium was obtained for adhesive cells. To these media were added foetal bovine serum (Intergen) at a final concentration of 10% and kanamycin at 0.2g/10m*l*. Culture conditions were temperature 37℃, carbon dioxide gas 5%, and humidity 100%.

Commercially available special-grade reagents were used for other reagents unless otherwise specified.

Methods

1. Preparation of BioBran/MGN-3 crude extract ingredient

BioBran/MGN-3 powder was suspended in distilled water at 0.2g/m*l* and stirred for about 30 mins. The suspension was centrifuged at 10,000×g for 20 mins and the supernatant was used as the crude extract. This was freeze-dried and used as the crude extract ingredient.

2. Partial purification of BioBran/MGN-3

BioBran/MGN-3 was suspended in 100% ethanol at 1g/m*l*, stirred for about 30 mins, and centrifuged at 10,000×g for 20 mins to obtain the precipitate, which was then dried and dissolved in distilled water at 0.2g/m*l*. The solution was centrifuged and the supernatant was applied to a Sephadex G-25 column for partial fractionation using distilled water as a separating solvent. The solution was separated into 3 fractions using blue dextran (mw about 2,000,000) and DNP-alanine (mw 269) as markers. The fraction in which blue dextran was eluted was Fraction A and that in which DNP-alanine was eluted was Fraction C. The intermediate fraction between Fractions A and C was Fraction B. These fractions were freeze-dried.

3. Evaluation of growth inhibiting effect

The initial cell density in the culture medium was set at 2.0×10^5 cells/m*l*. Each powdered fraction of BioBran/MGN-3 was dissolved in distilled water at 50mg/m*l* regardless of the crude extract fraction or partially purified fractions. The solution was added at 0-15% of the total volume of each cell culture medium and total volume was adjusted with phosphate buffered saline (PBS) to the same volume. The viable cell count at 3 days of culture was measured with a Cedex Analyze System (Innovatis). Taking as 100% the viable cell count for cell culture medium without addition of fractions of BioBran/MGN-3 (con-

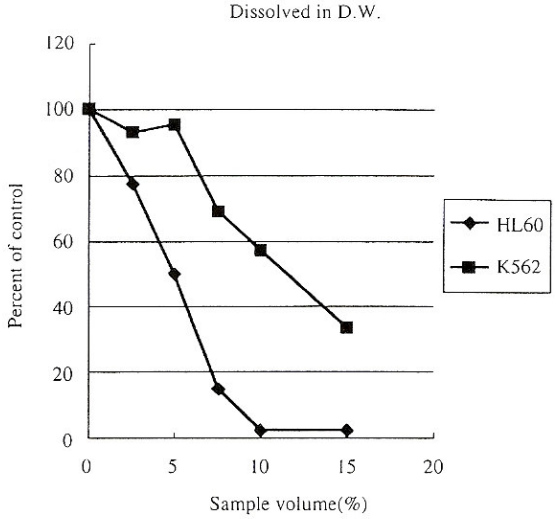

Fig.1 Effects of BioBran on the proliferation of HL60 and K562.

trol), the viable cell count for each added volume was expressed as the relative value and plotted as a graph.

Results and Discussion

After a solution other than culture medium and foetal bovine serum is added to cell cultures, the concentration of the culture medium decreases. The larger the volume of added solution, the lower the required concentration of culture medium. To confirm this, PBS was added to cell culture media and the effect of the additional volume on cell growth was studied. As a result, even if about 35% of the volume of culture medium was added, there was no effect on cell growth without a large difference in growing cell number (data not shown). This indicates that the addition of solutions at 0-15% of the culture medium volume had no effect on cell growth.

Figure 1 shows the results of adding BioBran/MGN-3 crude extract ingredient in distilled water to the HL60 and K562 cell lines in different volumes. The HL60 cell line showed a growth inhibition of about 85% after addition of 7.5% of the total volume (final concentration: 3.75 mg/m*l*), while the K562 cell line displayed growth inhibition of about 60% after addition of 15% of the total volume (final concentration: 7.5 mg/m*l*). Although both cell lines were derived from the same blood cells, the effect of growth inhibition on each was different. This may have been due to the type of cell line, i.e. the original cancer cells.

Next, a fraction obtained by treating BioBran/MGN-3 powder with ethanol was partially purified using Sephadex G-25 column chromatography and the fractions obtained at different molecular weights were investigated for growth inhibitory activity. The yield from 1 g of powder obtained after ethanol treatment was 75 mg for Fraction A, 513 mg for Fraction B, and 138 mg for Fraction C. The cell lines tested were HL60 and K562 and the volumes added were 5% of the total culture (final concentration: 2.5 mg/m*l*) and 10% (final concentration: 5.0 mg/m*l*). **Figure 2** shows the results obtained with the HL60 cell line and

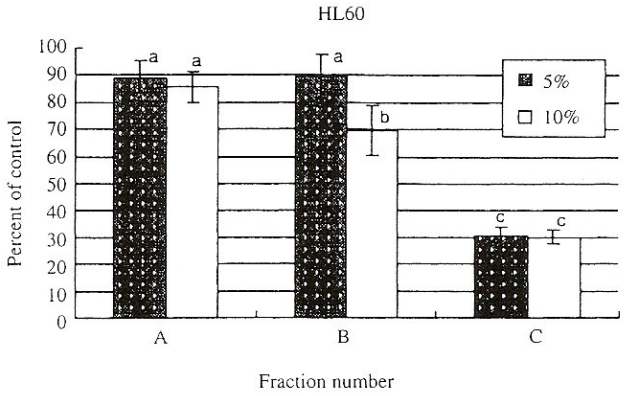

Fig.2 Effects of fractions from Sephadex G25 column chromatography on the proliferation of HL60.

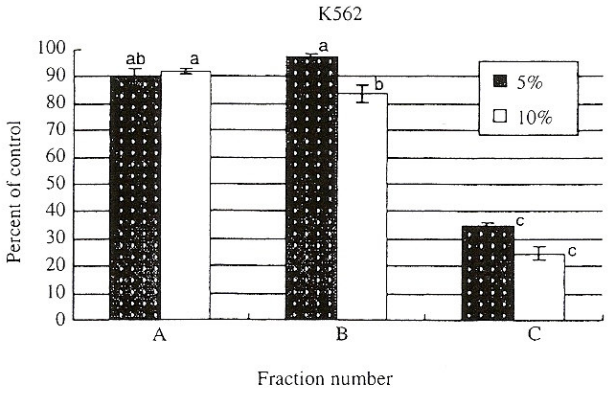

Fig.3 Effects of fractions from Sephadex G25 column chromatography on the proliferation of K562.

Figure 3 those with K562 cells. Both clearly show that Fraction C has a growth inhibitory activity. They also show that other fractions (Fractions A and B) have weak growth-inhibitory action on one cell line. However, the activities of Fractions A and B are very weak compared with that of Fraction C and they have almost no effect on the K562 cell line. The results for the other cancer cell lines, HepG2, HLE, PANC1, HeLa, and SW480, were almost the same although the magnitude of effect varied. Based on these results, Fraction C was used in subsequent evaluations of the cell-growth inhibitory component.

The cell-growth inhibitory effect of Fraction C was studied at concentrations of 0-15% with the above cancer cell lines and normal gingival fibroblast cell line, Gin1. The results in **Figure 4** show that growth inhibition is low in the normal cell line Gin1. This result could be interpreted in two ways: one possibility is that although the effect was weak, Fraction C inhibited the division of normal cells, while the other is

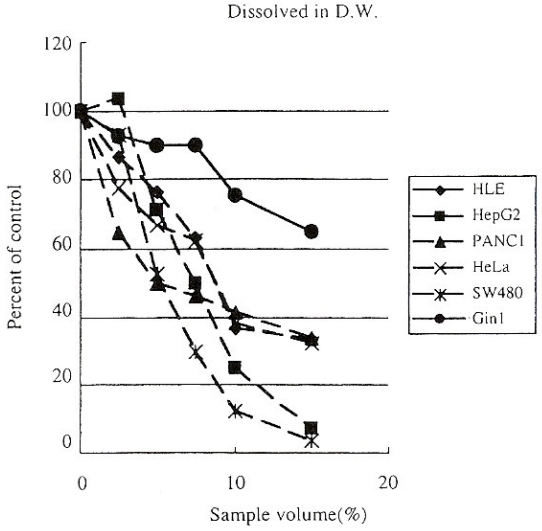

Fig.4 Effects of BioBran on the proliferation of HLE, HepG2, PANC1, HeLa, SW480 and Gin1.

that this normal cell line that exhibits division growth has some characteristics of cancer cells. Lymphocytes from human blood have been cultured with Fraction C but no adverse effects were observed. It cannot be ascertained whether Fraction C inhibits the growth of normal cells from this result, because although lymphocytes are normal cells, they were non-proliferative. To elucidate this point requires evaluation of the effect on normal proliferating cells. Fraction C had an inhibitory effect on all cancer cell lines studied, although the magnitude varied.

The growth-inhibitory component in Fraction C remains to be studied and identified. However, the results of the present study, together with fragmentary results obtained previously, show that the growth-inhibitory material mainly contains sugars or is a low-molecular weight oligosaccharide. The ingredient is likely to have the ability to induce partial differentiation of cancer cells. It is clear that its presence in culture medium inhibits cancer cell growth but it is unknown what effect it may have on the growth of normal cells. When cells with inhibited growth were separated from the culture, washed and recultured in medium without this ingredient, the cells grew at the usual rate.

Even if the substance has only the effects mentioned above, the fact that it can inhibit cancer cell growth and activate immune cells during the inhibition suggests a poteintial use in cancer therapy.

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II-4-1

Scavenging Activity of Modified Arabinoxylane from Rice Bran (BioBran/MGN-3) with Natural Killer Cell Activity on Free Radicals

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Key words: Biobran, Arabinoxylane, MGN-3, Free radical

Summary

BioBran/MGN-3, which produces a remarkable increase in natural killer cell activity, showed a high scavenging rate on hypoxanthine-xanthine oxidase-generated superoxide anion radicals, and on ferrous sulfate-hydrogen peroxide and UV light reaction system generated hydroxyl radicals. The S-group of BioBran/MGN-3 fractions (L>10,000 molecular, 10,000>M>3,000 molecular and 3,000 molecular>S) showed the highest scavenging rate on superoxide anion radicals and the UV light reaction system. There was no difference in the scavenging rate for hydroxyl radicals by the Fenton reaction.

Introduction

BioBran/MGN-3 is a modified arabinoxylan from rice bran, which consists of modified hemicellulose

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produced by treating the water-soluble hemicellulose fraction with enzymes (glycosidases) from shiitake fungi culture filtrate. BioBran/MGN-3 is an arabinoxylan with a xylose in its main chain and an arabinose polymer in the side chain (Fig. 1). It has been reported to increase NK cell activity in cancer patients with decreased immunity[1]. This compound and its fractions, separated on a Sephadex G-25 column, were assessed for their active-oxygen ($\cdot O_2^-$, ·OH) scavenging activities using electron spin resonance (ESR: Japan Electronics JES-FR-30).

Materials and Methods

Eight g of material components of BioBran/MGN-3 was dissolved in 400 ml of ethanol and the supernatant was condensed by an evaporator. The subsequent component was eluted and fractionated on a Sephadex G-25 column and each component was named in descending order as L (L > 10,000 molecule), M (10,000 > M > 3,000 molecule), and S (3,000 molecule > S), and used for evaluation. Ultrapure water was added to these components to 20 mg/ml, subjected to shaking extraction for 10 minutes, and centrifuged (3,000 rpm, 5 min) to obtain the supernatant. The supernatant was further diluted to 2.0 mg/ml and 0.2 mg/ml sample solutions, and used for measurement.

Active enzyme scavenging activity was evaluated by measuring the superoxide anion radical ($\cdot O_2^-$) and hydroxyradical (·OH) scavenging activities of the Fenton reaction, and the scavenging activity of the hydroxyradical generated from ultraviolet irradiation.

·O2 scavenging activity was measured by the Spin Trap method using the HPX-XOD reaction. In detail, 50 μl of 2 mM hypoxanthine solution (HPX), 35 μl of 5.5 mM DETA-PAC solution, 15 μl of 9.2 M DMPO and 50 μl of sample solution were mixed, and timing was started concurrently with the addition of 50 μl of 0.4 U/ml xanthine oxidase solution (XOD). After stirring for 1 min, the spectrum of $\cdot O_2^-$ adduct generated inside the 120 min quartz cell was measured. The scavenging activity was calculated as a relative signal strength of $\cdot O_2^-$ adduct to the signal strength of the internal standard Mn. It was also calculated as the SOD concentration corresponding to the SOD activity of the sample, from the calibration curve of SOD at various concentrations.

·OH was measured using the Fenton reaction. Five ml of sample solution from each group was added to 75 μl of 1 mM $FeSO_4$ and mixed with 20 μl of 9.2 M 10-fold diluted DMPO. Then, 75 μl of 0.1 mM H_2O_2 was added, stirred for two minutes, placed in the cell, and sweeping was started 60 sec after H_2O_2 was added.

·OH was further measured using the generation system of the ultraviolet reaction. Two hundred fifty ml of sample solution and 40 μl of 9.2 M 10-fold diluted DMPO were mixed, 150 μl of 100 mM H_2O_2 was added, stirred, poured into a plastic container and after ultraviolet irradiation (365 nm, 4×10^3/J/m^2/min) for 5 min, it was placed in a cell and measured.

Spectral analysis using ESR (Japan Electronics JES-FR30) was performed under the following conditions:

Magnetic field sweep width: 335.6 mT
Magnetic field modulation: 0.1 mT
Gain: 125
Sweep time: 2 min
Response time: 0.1 sec
Temperature: room temperature

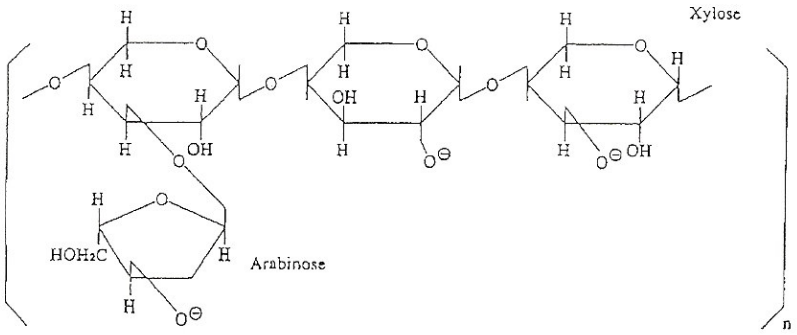

Fig. 1 Arabinoxylane model in BioBran

Results

The $\cdot O_2^-$ scavenging activity of BioBran/MGN-3 was dose-dependent: 64.6%, 23.0%, and 4.4% as scavenging rates at 20, 2.0, and 0.2 mg/ml, respectively. The SOD activities of samples for the calibration curve were 7.6, 0.9, and 0 U/ml, respectively. The ·OH scavenging activity, as determined by the Fenton reaction, was also dose-dependent: scavenging rates were 94.9%, 78.9%, and 3.3% at 20, 2.0, and 0.2 mg/ml, respectively. The UV-generated ·OH scavenging activity was 72.6%, 35.9%, and 11.5% as scavenging rates (**Table 1**).

The $\cdot O_2^-$ scavenging activity of each fraction of BioBran/MGN-3 increased with dose: scavenging rates were 39.9%, 10.4%, and 0% at 20, 2.0, and 0.2 mg/ml for the L fraction; 49.5%, 15.6%, and 0% for the M fraction; and 90.4%, 68.1%, and 26.4% for the S fraction. The S fraction had the highest scavenging activities. The SOD activities of the L, M, and S fractions were 5.0, 7.2, and 70.5 U/ml at 20 mg/ml; 0.8, 1.4, and 15.7 U/ml at 2.0 mg/ml; and 0, 0, and 2.6 U/ml at 0.2 mg/ml (**Table 1**).

The ·OH scavenging activities of each fraction of BioBran/MGN-3 at 20, 2.0, and 0.2 mg/ml were 97.2%, 34.4%, and 3.3% for the L fraction; 97.0%, 68.4%, and 8.7% for the M fraction; and 96.5%, 55.1%, and 4.2% for the S fraction in terms of scavenging rate. All the fractions had high activities. The UV-generated ·OH scavenging activities at 20, 2.0, and 0.2 mg/ml were 41.8%, 16.5%, and 1.0% for the L fraction; 45.4%, 9.9%, and 3.9% for the M fraction; and 71.0%, 54.9%, and 19.6% for the S fraction in terms of scavenging rates. The S fraction had the highest scavenging activities (**Table 1**).

Conclusion

The active oxygen-scavenging activity of BioBran/MGN-3, a plant polysaccharide processed food, was investigated in this study. We found that BioBran/MGN-3 had a high scavenging activity for $\cdot O_2^-$ and ·OH involved in ageing and diseases. The OH scavenging effect on the Fenton reaction was especially prominent.

The results of the measurements are shown in Table 1. The S fraction excelled all others in the inhibition of ·OH generation caused by $\cdot O_2^-$ and ultraviolet irradiation, and high scavenging activity was

observed in all fractions for the scavenging activity of ·OH generation in the Fenton reaction.

Table 1 Scavenging Activity of MGN-3 on Active Oxygen radical (·O_2^- and ·OH and UV light reaction ·OH)

Kind of Activie Oxygen and SOD activity	Scavenging ratio of Superoxyde anion radical (%)			SOD activity (U/ml)			Scavenging ratio of Hydroxyl radical by UV light reaction (%)		
	20	2.0 (mg/ml)	0.2	20	2.0 (mg/ml)	0.2	20	2.0 (mg/ml)	0.2
MGN-3	64.6	23.0	4.4	7.6	0.9	0	94.9 (72.6)	78.9 (35.9)	3.3 (11.5)
MGN-3-L	39.9	10.4	0	5.0	0.8	0	97.2 (41.8)	34.4 (16.5)	3.3 (1.0)
MGN-3-M	49.5	15.6	0	7.2	1.4	0	97.0 (45.4)	68.4 (9.9)	8.7 (3.9)
MGN-3-S	90.4	68.1	26.4	70.5	15.7	2.6	96.5 (71.0)	55.1 (54.9)	4.2 (19.6)

·O_2^-: HPX-XOD reaction, ·OH: Fenton reaction,
·OH by UV light reaction: 365 nm, $4 \times 10^3 J/m^2/min \times 5$

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A Basic Study of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on the Activation of Vital Defence

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Hideyuki KANNA, Kenji HIRAYAMA and Chiharu KUBO

Introduction

Modified arabinoxylan from rice bran (BioBran/MGN-3) is a plant polysaccharide processed food with a biophylactic action, which mainly contains polysaccharides such as hemicellulose, arabinoxylan and glycoprotein. BioBran/MGN-3 is known to have various biophylactic actions such as *in vitro* NK cell activity stimulation and an anti-HIV effect[1-2]. In the present study, the vital defence activating effect of BioBran/MGN-3 was studied in 3 animal experiments: Experiment 1 examined its effect on the survival in a lipopolysaccharide-induced lethal sepsis model, Experiment 2 studied the anti-stress effect on restraint stress, and Experiment 3 examined its effect on the survival of autoimmune disease-prone (NZB × NZW) F1 mice.

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Methods

1. Experimental procedures

Experiment 1

In the experiment, BALB/c mice (male, 5-7 weeks old) were used. 20 mg/kg and 200 mg/kg of BioBran/MGN-3 (provided by Daiwa Pharmaceutical Co., Ltd.) were dissolved into 0.5 ml of PBS, and via an oral zonde, administered every other day for 2 weeks, in total 7 times. Then 0.5 ml of PBS was administered orally over the same interval for the control group. 200 μg/mouse of LPS was administered intraperitoneally 12 hours after the final oral administration, and the conditions of the mice were observed over time. In another experiment, 100 μg/mouse of LPS was administered intraperitoneally in the BioBran/MGN-3 group and control group, the mice were euthanized 0, 2, 4, 8 hr after the LPS administrations, and peripheral blood was collected from the heart. Serum was separated, and IL-6 and TNF were measured. IL-6 activity was measured using the B9 cell line[3] and TNF activity was measured by bioassay in the WEHI164-13 cell line[4].

Experiment 2

BALB/c mice (male, 5 weeks old) were fed foods containing 0%, 0.25%, and 0.5% BioBran/MGN-3 and subjected to restraint stress in a metal cage for 12 hours, as previously reported[5]. The thymus and spleen were isolated before and after restraint to study the number of cells and lymphocyte subsets. The number of cells was counted using a hemocytometer and lymphocyte subsets were analyzed by flow cytometry (FACS caliber).

Experiment 3

Autoimmune disease-prone (NZB × NZW) F1 mice (female, 5 weeks old) were given the same foods as in Experiment 2 to observe body weight, proteinuria, and survival rate over time.

2. Statistical analysis

Measurements were expressed as means ± standard deviations. Survival was analyzed using the Kaplan-Meiyer method, and comparison between two groups was performed using Mantel-Cox tests. Others were analyzed by factorial ANOVA and then comparison was made between the two groups using unpaired *t* tests.

Results

1. Improvement of survival rates by BioBran/MGN-3 in a lipopolysaccharide-induced lethal sepsis model

As shown in **Fig. 1**, when 200 μg/mouse of LPS was administered, the survival rate significantly improved in groups where 20 mg/kg or 200 mg/kg of BioBran/MGN-3 was administered daily, compared with that in the control group (20 mg/kg BioBran/MGN-3 group vs. control group, $p = 0.0456$; 200 mg/kg BioBran/MGN-3 group vs. control group, $p = 0.0232$, by Mantel-Cox test). Also, when 100 μg/mouse of LPS was administered, all the mice survived in groups where 20 mg/kg or 200 mg/kg of BioBran/MGN-3 was administered daily, while 3 out of 10 mice died in the control group. Next, to establish the mechanism for improvement of survival rate by BioBran/MGN-3, blood concentrations of IL-6 and TNF were measured (**Fig. 2**). In the experimental group where BioBran/MGN-3 was administered, compared with

the control group, the circulating IL-6 level was significantly lower 2 hours after the administration of LPS (control group 702.9 ± 24.7 ng/ml, BioBran/MGN-3 group 403.1 ± 59.6* ng/ml; $p < 0.01$); however, 8 hr after the administration, it significantly increased (control group 88.5 ± 50.0 ng/ml, BioBran/MGN-3 group 441.0 ± 115.0* ng/ml; $p < 0.05$). Meanwhile, 4 hr after LPS administration, the blood TNF level significantly increased in the BioBran/MGN-3 group compared with that in the control group (control group 492 ± 187, BioBran/MGN-3 group 1816 ± 307* pg/ml; $p < 0.01$).

2. Study on anti-stress effect by BioBran/MGN-3

Subsequently, we studied the possible inhibitory effect of BioBran/MGN-3 in lymphocyte reduction in thymus and spleen caused by restraint stress, one of the non-inflammatory stresses. As shown in **Fig. 3**, in the 0.5% BioBran/MGN-3 group, the number of thymocytes decreased significantly with BioBran/MGN-3 intake alone. However, in either group, the number of cells in thymus and spleen significantly decreased by loading the restraint stress. Meanwhile, for the lymphocyte subset, although the characteristic fluctuation of subset accompanied by stress loading was observed[6], there was no difference between the BioBran/MGN-3 group and control group.

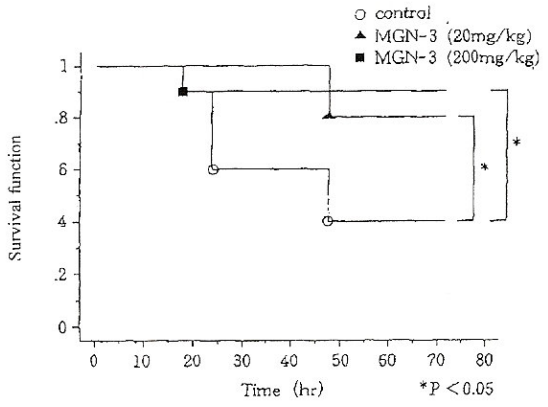

Fig. 1 Improvement of survival rates by MGN-3

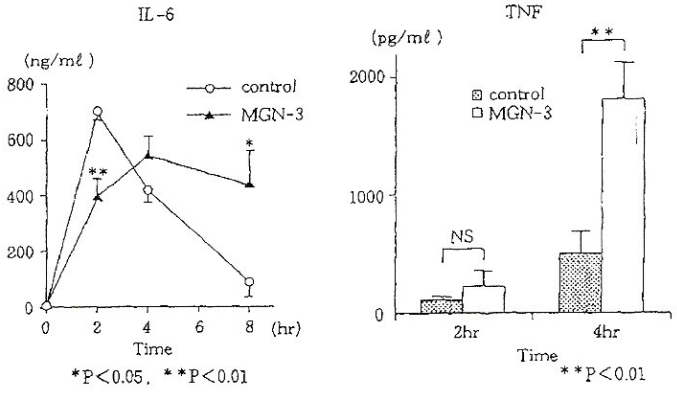

Fig. 2 Changes in blood levels of IL-6 and TNF

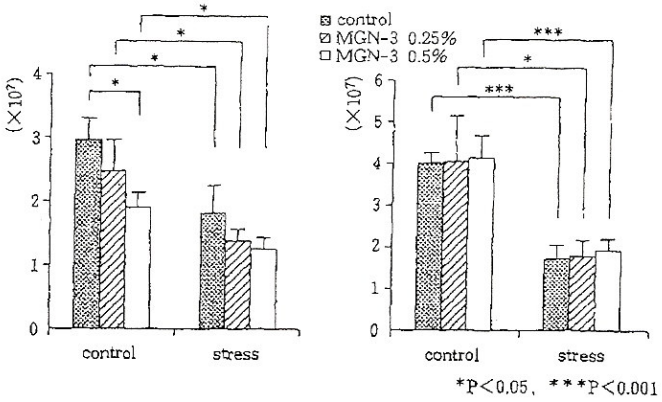

Fig. 3 Changes in numbers of cells in thymus and spleen

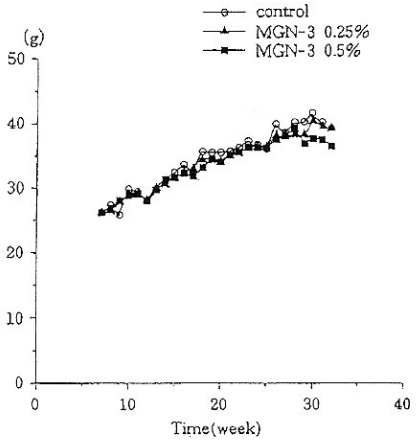

Fig. 4 Changes in body weight

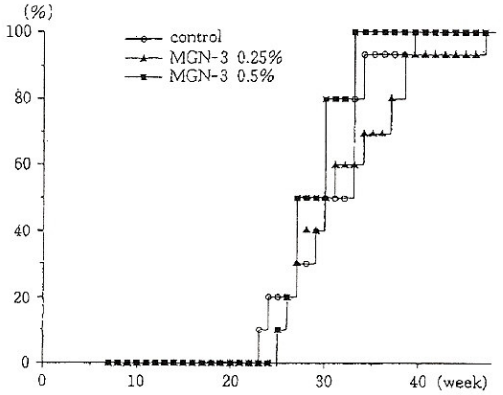

Fig. 5 Time-dependent changes in rate of animals with proteinuria

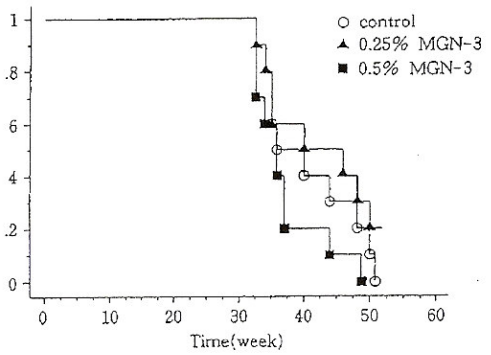

Fig. 6 Effect of MGN-3 on survival rate for (NZB × BZW) F1 mice

3. The effect of BioBran/MGN-3 on clinical signs in mice with collagen vascular diseases

As shown in **Fig. 4**, changes in body weight were similar for the BioBran/MGN-3 and control groups. Proteinuria occurred, in increasing order of frequency, in the 0.5% BioBran/MGN-3, control, and 0.25% BioBran/MGN-3 groups, and was positive in all mice at 46 weeks (**Fig. 5**). There was no significant difference in death rate between the 3 groups, but the 0.5 BioBran/MGN-3 group tended to have a higher death rate than the 0.25 BioBran/MGN-3 group (**Fig. 6**, 0.5% BioBran/MGN-3 vs 0.25% BioBran/MGN-3, $p=0.0769$).

Discussion

In the lipopolysaccharide-induced lethal sepsis model, a large amount of inflammatory cytokines (IL-1, IL-6, and TNF-α) released from the reticuloendothelial system of the whole body induces multiple organ failure, which leads to death of the animal. In this study, BioBran/MGN-3 significantly improved survival rate. One hypothetical explanation for this was that BioBran/MGN-3 may have inhibited the production of tissue-damaging cytokines by macrophages, and blocked reactions leading to damage to target cells. However, although the blood IL-6 level at 2 hours was decreased by BioBran/MGN-3 ingestion, the level of TNF that actually causes tissue damage was significantly increased in the BioBran/MGN-3 groups, suggesting that it is unlikely that BioBran/MGN-3 inhibited the production of inflammatory cytokines. TNF-α is a critical factor determining death or survival in this model. The *in vivo* effect of TNF-α is known to be controlled by competitive soluble TNF-receptors[7]. The mechanism of survival improvement by BioBran/MGN-3 is as yet unknown. Plausible theories for the mechanism are a contribution of cytokines with a competitive effect on TNF and the decreased sensitivity of target cells to TNF (for example, by down-regulation of receptors).

Non-inflammatory stress (restraint stress) decreases the numbers of cells in the thymus and spleen. As similar decreases in the numbers of cells were observed in the BioBran/MGN-3 groups subjected to restraint stress, it can be concluded that BioBran/MGN-3 had no anti-stress effect on the thymus or spleen. However, BioBran/MGN-3 decreased the number of cells in the thymus. This, together with its mechanism of action, remains to be studied.

SLE-prone (NZB × NZW) F1 mice will develop anti-DNA antibody in the blood and proteinuria with the

passage of time, and prematurely die of renal failure. In the present study, proteinuria appeared earlier and the death rate was increased in the 0.5% BioBran/MGN-3 group compared with the 0.25% BioBran/MGN-3 group, although the differences were not significant. Administration of BioBran/MGN-3 at large doses needs careful consideration. IFN-γ is known to exacerbate renal failure and increase the death rate in (NZB × NZW) F1 mice[8]. It has been reported that BioBran/MGN-3 promotes the *in vitro* production of IFN-γ by NK cells[1]. Thus, administration of BioBran/MGN-3 in large doses involves the risk of IFN-γ-induced renal damage. In conclusion, it was suggested that BioBran/MGN-3 may enhance Th1 type immune reactions.

Conclusion

The plant polysaccharide processed food arabinoxylan was studied for its effect on the vital defence activation in animals. The following results were obtained:

1) The food improved survival rates in an LPS-induced lethal sepsis model.
2) It had no anti-stress effect on restraint-decreased cells in the thymus and spleen.
3) It did not prolong the survival in a mouse model of autoimmune disease.

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Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) Beneficial for Weight Loss of Mice as a Major and Acute Adverse Effect of Cisplatin

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Alkylating agents and antimetabolites remain prominent in a wide variety of cancer chemotherapy protocols on the basis of more selective effects on the faster than normal mitotic cycles of malignant cells. Among new platinum-containing anticancer molecules, the lead compound cisplatin (Cis-platinum (II) diammine dichloride) has been known to possess beneficial anticancer properties in terms of specific interaction of cisplatin and DNA strands. Cisplatin (Rosenberg *et al.* 1969) has been encouraged for treatment of head and neck, bladder, and cervical cancers (Loehrer & Einhorn 1984), as well as breast cancer (Smith & Talbot 1992). Although the prevalent incidence of lung, gastric, breast, colorectal and prostate cancers place them in top ranks of the most common cancers in the civilized countries, many of these advanced cancers are unresponsive to chemotherapy. Platinum-based drugs would be a potent choice in these situations, but they frequently cause substantial side effects, such as nausea, vomiting, nephropathy and hypomagnesaemia due to damage of renal tubules (Lajer & Daugaard 1999). Furthermore, in addition to hearing loss and peripheral neuropathy, myelosuppression is one of the most devastating suppressive side-effects (Prestayko *et al.* 1979) leading to immunocompromized states. Therefore, any reduction of

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the side effects of cisplatin would be valuable. Thus, we explored the effect of BioBran/MGN-3 on protection of weight loss of mice under tolerable maximal doses of cisplatin. Others have briefly commented on the antistress and antifatigue effects of fermented rice bran (Kim *et al.* 2001) or some beneficial effect of BioBran/MGN-3 on some adverse actions of anticancer drugs in rats (Jacoby *et al.* 2000).

The experiment protocol was approved by the Animal Research Ethics Board at McMaster University in Ontario, Canada and as follows. BALB/c female mice (4 week-old) purchased from Charles River Canada were acclimatized for a week. They were weighed and separated into seven groups of five in accordance with minimal variation of weight difference within each group. The average weight of all mice studied (17.43g with standard deviation±0.51g) at the beginning of the present experiment was designated as 100% of body weight. Five mice were housed in each standard metabolic cage in a conventional air-conditioned room. Free access to lab chow (LabDiet®) and water was provided. Light and darkness cycles were 12 hr.

Cisplatin and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Since cisplatin is less soluble in water or phosphate-buffered saline (PBS), DMSO was used as a vehicle for solubilization of cisplatin (0.1% V/V).

Characteristics of BioBran/MGN-3 used in the present experiment were as follows. Defatted extract of rice bran was modified by digestion with glycosidases such as amylase, galactosidase and glucuronidase derived from Shiitake mushroom (*lentis edodes*) mycelia. The end-product of BioBran/MGN-3 was highly water-soluble. It was composed of polysaccharides such as mainly arabinoxylan hemicellulose and of protein (13.2%) determined by the method of Lowry *et al.* (1951). It has been produced by the Daiwa Pharmaceutical Company, Tokyo, Japan and sold by the name of BioBran/MGN-3 (Ghoneum 1998) in North America. The product (US Patent 5560914) was a kind gift from Dr. Hiroaki Maeda of Daiwa Pharmaceutical Company.

One week before cisplatin administration, BioBran/MGN-3 was administered to two groups of mice daily by gavaging in a volume of 0.1 m*l* at a concentration of 10 mg/m*l* of BioBran/MGN-3 (dry weight) in water or by intraperitoneal injection in a volume of 0.1 m*l* at the same concentration of BioBran/MGN-3 in phosphate-buffered saline. The dose of 1 mg of BioBran/MGN-3 per mouse was calculated from that recommended for human usage (50 mg/kg). One shot of cisplatin was administered in a volume of 0.1 m*l* at the concentration of 15 mg/kg of cisplatin in phosphate-buffered saline containing 0.5% DMSO as a vehicle intraperitoneally. Two groups of mice received a gavage of water or intraperitoneal administration of phosphate-buffered saline and one week later cisplatin was administered to the both groups. Mice given tap water for oral administration and mice given phosphate-buffered saline with or without DMSO as a vehicle for cisplatin as intraperitoneal administration groups were designated as three separate control groups. The body weight of individual mouse was monitored daily. In comparison with the average weight of all mice at the start of the experiment at an arbitrary 100%, the percentage of body weight of individual mice was plotted.

Weight loss after intraperitoneal injection of cisplatin occurred next day in both groups with and without administration of BioBran/MGN-3. At the 5th day after cisplatin treatment, the greatest of weight loss was obseved in both groups with or without BioBran/MGN-3 orally as well as intraperitoneally. The most of weight loss occurred in mice given cisplatin without BioBran/MGN-3 administration. Although loss of body weight appeard to be close to 20% of standard body weight of mice in the groups of cisplatin treatment, no mice died, nor did any show diarrhoea or rectal bleeding both frequent side effects of cisplatin.

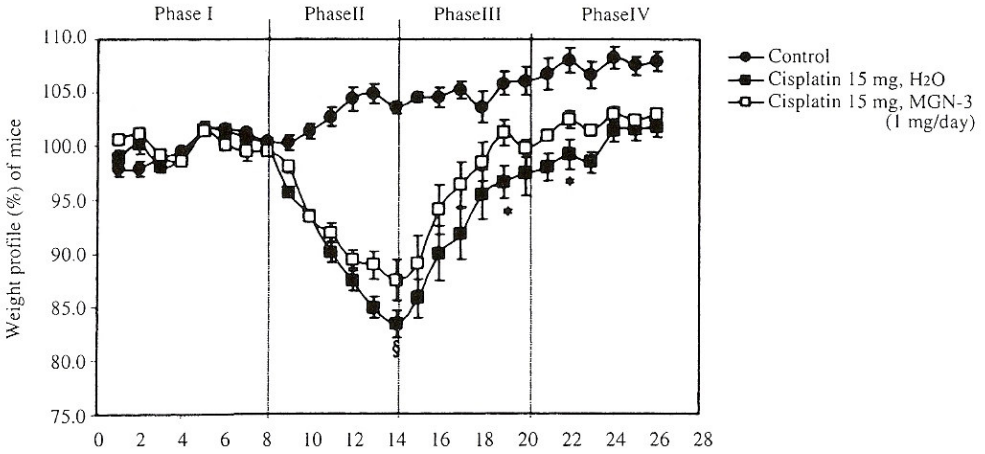

Fig. 1 Comparison of profile of reduced percentage of body weight of mice given cisplatin (15 mg/kg) intraperitoneally on the eighth day with (open rectangles) or without oral intake of MGN-3 (1 mg/day) since the first day (closed rectangles).

Hundred percent of mouse weight of the ordinate means an average weight of whole mice used at the start of the experiment (17.43 g±0.51 g).

Control means neither treatment of cisplatin nor MGN-3.

The abscissa shows experimental days from the start of administration of MGN-3.

Reduced percentage of body weight of mice shows statistically significant differences between the groups with and without oral intake of MGN-3 on the point of maximal reduction of body weight and in the late recovery phase.

Bars = the mean and standard errors of five mice in each group. § $P<0.001$, * $P<0.05$.

As shown in **Fig. 1** and **2**, weight gain of mice started on the same day 14 after a shot of cisplatin in both groups and recovery pace of weight gain of groups of mice given BioBran/MGN-3 orally as well as intraperitoneally was faster than that of the groups of control.

The severity of weight loss of mice by cisplatin was dose-dependent (data not shown). BioBran/MGN-3 given orally as well as intraperitoneally showed accelerated protection against severe loss of body weight of mice due to cisplatin. **Fig. 1** shows statistically significant difference between the curve of weight loss due to cisplatin in oral intake group of BioBran/MGN-3 and that in water intake group by ANOVA analysis ($P<0.05$) in phase II, III and IV. **Fig. 2** shows statistically significant difference ($P<0.05$) between the curve of weight loss due to cisplatin in the intraperitoneal administration group of BioBran/MGN-3 and that in phosphate-buffered saline group by ANOVA analysis in phase II, III and IV. There was no significant difference in protective effect of BioBran/MGN-3 on weight loss when the groups of oral and intraperitoneal administration of BioBran/MGN-3 were compared by ANOVA analysis. These results indicate that beneficial substances in BioBran/MGN-3 in terms of protective effect on weight loss of mice due to cisplatin were equally effective by oral administration of BioBran/MGN-3 as by intraperitoneal administration.

In order to detect absorbed compounds with BioBran/MGN-3 in tha serum from the gut mucosa of mice treated with BioBran/MGN-3, we raised polyclonal antibodies against BioBran/MGN-3 in BALB/c mice. A volume of 0.1 m*l* of BioBran/MGN-3 solution (1 mg/m*l*) emulsified with the same volume of complete

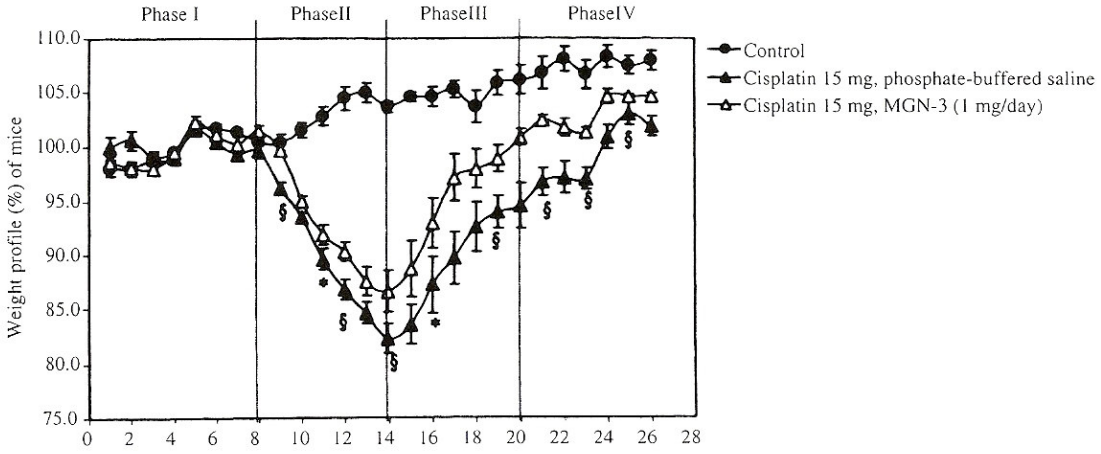

Fig. 2 Comparison of profile of reduced percentage of mouse weight of mice given cisplatin (15 mg/kg) intraperitoneally on the eighth day with (open triangles) or without intraperitoneal administration of MGN-3 (1 mg/day) since the first day (closed triangles).

Hundred percent of mouse weight of the ordinate means an average weight of whole mice used at the start of the experiment (17.43 g±0.51 g).

Control means neither treatment of cisplatin nor MGN-3.

Reduced percentage of body weight of mice shows statistically significant differences between the groups with and without intraperitoneal administration of MGN-3 in the reducing phase after cisplatin treatment and through the recovery phase.

Bars = the mean and standard errors of five mice in each group. §$P<0.001$, *$P<0.05$.

Freund's adjuvant was injected intraperitoneally followed by a boost immunization of 0.1 m*l* of BioBran /MGN-3 solution (1 mg/m*l*) emulsified with the same volume of incomplete Freund's adjuvant. Since BioBran/MGN-3 may be composed of polysaccharides and polysaccharides, immunoreactive substances of both combounds among BioBran/MGN-3 in the serum should be detected in mice treated by BioBran /MGN-3.

To do this, we purchased Covalink microwell plates for quantitation of polypeptides as well as polysaccharides, and conventional polystylene plates (Polysorb) for quantitation of polypeptides from NUNC (Missisauga, Ontario, Canada) for enzyme-linked immunosorbent assay (ELISA) in accordance with the method reported by Zielen *et al.* (1996). To assure that this ELISA system is valid, **Fig.3** shows significant difference in titers of mouse polyclonal antibodies against BioBran/MGN-3 with (+) or without (−) digestion of BioBran/MGN-3 by proteinase K for polypeptides. Peroxidase-conjugated rabbit antibody against mouse IgG antibodies as a second antibody for optical density by 492 nm of wave length by a ELISA reader was purchased from Medical Biological Laboratories (Nagoya, Japan). These data permitted detection of polypeptide as well as polysaccharide compounds of BioBran/MGN-3 on the Covalink microtiter plate.

Immunoreactive compounds of absorbed BioBran/MGN-3 in the mouse serum treated orally by the bran can be detected by absorption procedure of immunological method by using these polyclonal antibodies against BioBran/MGN-3. Thus, immunoreactive substances of BioBran/MGN-3 in sera of mice must be absorbed by the polyclonal antibodies against BioBran/MGN-3 after incubation at 37℃ for 60 min. and centrifugation (15,000 rpm) for 30 min at 4℃. Serial sera were taken from three mice before oral adminis-

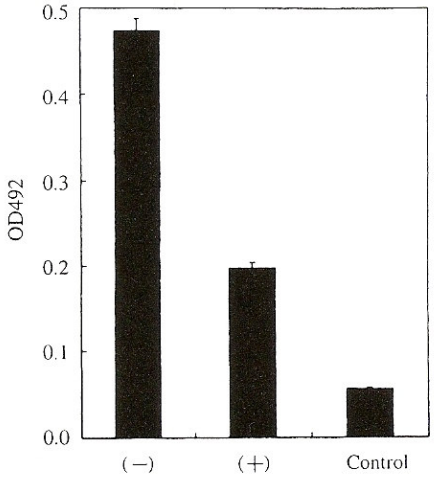

Fig. 3 Polyclonal antibodies against MGN-3 raised by us were tested by ELISA using Covalink microtiter plates.

MGN-3 was digested by proteinase K to eliminate protein antigens.
The left histogram (-) shows titer of polyclonal antibodies against whole MGN-3 without proteinase K digestion and indicates that of both protein and polysaccharide antigens in MGN-3.
The middle histogram (+) shows titer of polyclonal antibodies against MGN-3 after proteinase K digestion and indicates that of only polysaccharide antigens in MGN-3.

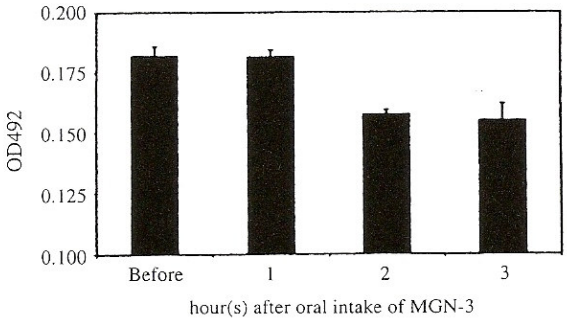

Fig. 4 To summarize absorption data of immunoreactive substances of MGN-3 in the mouse serum after oral intake of MGN-3, OD values before and 1 hr after oral intake are the same level indicating absence of immunoreactive substances of MGN-3 in the serum followed by decrease of OD value indicating existence of them in the mouse serum at 2 and 3 hr after oral intake of MGN-3.

The decrease of serum titers of polyclonal antibodies against MRB was statistically significant (Wilcoxon test $P<0.01$).

tration of BioBran/MGN-3 (10 mg/kg) and three points of time sequence 1 to 3 hr after oral intake of BioBran/MGN-3. Immunoreactive substances of BioBran/MGN-3 in sera of mice treated by BioBran /MGN-3 in two different dilution (100× and 1000×) before and after absorption with the polyclonal anti-

bodies against BioBran/MGN-3 were measured by ELISA in terms of consistency of adsorption procedure for Covalink microtiter plates. **Fig. 4** shows significant decrease of titers of the polyclonal antibodies against BioBran/MGN-3 after absorption with each serum at 2 and 3 hr after oral intake of BioBran/MGN-3. This decrease of titers of polyclonal antibodies against BioBran/MGN-3 was statistically significant (Wilcoxon test $P<0.01$) and indicated that immunoreactive substances of BioBran/MGN-3 must exist in the sera of mice treated orally with BioBran/MGN-3. Whether these substances were the active components responsible for the protective effect on weight loss due to cisplatin remains to be determined. Nevertheless, the present results clearly show that oral intake of BioBran/MGN-3 was effective on reduction of weight loss of mice due to cisplatin. The polyclonal antibodies against BioBran/MGN-3 developed in the present study could identify promising contents of BioBran/MGN-3 closely related to protective function against adverse effect of anticancer drugs.

It has been reported that defatted rice bran hemicellulose increases the peripheral blood lymphocyte count in rats and that the ratio of helper/inducer T cells to suppressor/cytotoxic T showed a decrease in rats given 10% hemicellulose diet (Takenaka & Itoyama 1993). Modified arabinoxylan from rice bran (BioBran /MGN-3) has been shown to enhance the activity of natural killer cells after oral intake of BioBran/MGN-3 in man (Ghoneum 1998). α-Glucan extracted from rice bran by ethanol has potent antitumour activity (Takeo *et al.* 1988), whereas various polysaccharides belonging to β-glucan from mushroom, such as *Lentinus*, *Schizophyllum* and *Grifola*, are resposible for the antitumour effects (Borchers *et al.* 1999).

These reports did not show that components of rice bran in the serum from the gut were absorbed. This is the first demonstration that immunoreactive components of BioBran/MGN-3 in terms of polypeptides and polysaccharides were definitely absorbed from the gut into the blood after oral intake of BioBran/MGN-3.

It has previously been reported that BioBran/MGN-3 is effective in lowering serum lipids and on taste preference in streptozotocin-induced diabetic rats (Ohara *et al.* 2000). This is a potential benefit of BioBran /MGN-3 compated to emetic side effects of cisplatin. Despite not knowing how BioBran/MGN-3 may play a beneficial role in the protection against cisplatin-induced weight loss, our results encourage the performance of a clinical trial of BioBran/MGN-3 as a so-called functional food to verify its protective effect on the quality of life of advanced cancer patients (Harrap 1995). Further characterization of the effective components of BioBran/MGN-3 is needed to achieve the best anticancer effect as well as protective effect against anticancer drugs.

Acknowledgements

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The Effect of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on Cisplatin and Doxorubicin Induced Toxicity in the Rat

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Key words:
MGN-3, Chemotherapy, Chemoprotectants, Rats, Cisplatin, Doxorubicin

Abstract

BioBran/MGN-3 is derived from rice bran and is produced by the partial hydrolysis of the water soluble hemicellulose fraction of rice bran by carbohydrases derived from *Lentinus edodes* mycelia. It is a biological response modifier producing an increase in natural killer cell activity in immunocompromised patients. The aim of the study was to evaluate orally administered BioBran/MGN-3 against gross pathological changes and weight loss produced by a single intraperitoneal dose of cisplatin or doxorubicin by daily oral dosing of 5 or 50 mg/kg BioBran/MGN-3. Male Sprague-Dawley rats received either vehicle or BioBran/MGN-3 prior to and after a single dose of cis-platinum or doxorubicin. Rats were observed for clinical signs daily for 11 days and body weights were recorded every other day. All animals were euthanized and necropsied on Day 11. Lethality was observed only in rats receiving cisplatin (50% with cis-

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platin alone reduced to 10% in rats receiving BioBran/MGN-3 5 mg/kg, and 40% after BioBran/MGN-3 50 mg/kg). Rats receiving BioBran/MGN-3 at 5 or 50 mg plus cis-platinum or doxorubicin had a statistically significant greater weight gain than that observed with the chemotherapeutic agent alone. Rats receiving BioBran /MGN-3 appeared healthier; gained weight and had a lower incidence of diarrhea and gross intestinal pathology. BioBran/MGN-3 was effective at maintaining body weight after a toxic dose of either chemotherapeutic agent and protected against gross gastrointestinal pathological changes and diarrhea. BioBran/MGN-3 may have potential for improving "quality of life "of patients receiving chemotherapy.

Introduction

Chemotherapeutic therapy for cancer is often associated with adverse effects[1]. Historically, chemotherapeutic agents have been selected on the basis of established toxicity to cancer cell lines and rapidly growing tumors in rodents and not on the basis of sophisticated intervention in tumor specific biology. This strategy usually leads to agents, which have toxicity toward normal cells and tissues that share characteristics with tumor cells, such as high cell turnover. Gastrointestinal mucosal cells are among the most sensitive cells and chemotherapeutic agents often have debilitating effects due to lesions throughout the gastrointestinal tract. This may be manifested as gastrointestinal mucosal lesions and hemorrhage. These often lead to potentially life-threatening hemorrhage and perforation. Because of these, and pathological effects on other organ systems, there has been great interest in developing concomitant therapy which will protect normal tissue from the effects of chemotherapeutic agents without interfering with their anti-cancer activity.

MGN-3 (BioBran, Lentin Plus) is a hemicellulose complex containing arabinoxylan as a major component [US Pat. 5560914][2]. It is a water-soluble product of the hydrolyzing hydrolysis of rice bran using multiple enzymes from mycelia of edible mushrooms. Arabinoxylan is obtained from the enzyme reaction under constant conditions and is highly active and stable. The enzymatic digestion of rice bran to hemicelluloses such as arabinoxylan compounds appears to enhances the intrinsic immune stimulating activity due to their greater water solubility and bioavailability. This product is made by a unique and patented process in which Shiitake mushrooms enzymes (basidiomycetes mycelia extract) produce a unique and natural blend of hemicelluloses. There is no measurable mushroom content in the end product. The blend of hemicellulose compounds is generically called BioBran/MGN-3. It is categorized as a food supplement or functional food and is available as a powder, tablet a or capsule in the United States.

Materials and Methods

Sprague-Dawley derived, albino rats (Ace Animals, Inc., Boyertown, PA) were singly housed in suspended stainless steel caging with mesh floors. The animal room was temperature controlled and had a 12-hour light/dark cycle. Rats were fed Purina Rodent Chow #5012 and filtered tap water was supplied *ad libitum* by an automatic water dispensing system. Following an acclimation period of 13 days, eighty healthy male rats were selected for test based on body weights and randomly assigned (10 rats/group) to each of the following eight test groups:

Group No.	Test Substance (mg/kg)	Chemotherapeutic Agent (mg/kg)
1	MGN-3 5 PO	Vehicle
2	MGN-3 50 PO	Vehicle
3	MGN-3 Control	Cisplatin 9 IP
4	MGN-3 5 PO	Cisplatin 9 IP
5	MGN-3 50 PO	Cisplatin 9 IP
6	MGN-3 Control	Doxorubicin 10 IP
7	MGN-3 5 PO	Doxorubicin 10 IP
8	MGN-3 50 PO	Doxorubicin 10 IP

PO: oral, IP: intraperitoneal

Individual doses were calculated based on the initial body weights. The animals from all groups received oral administration of BioBran/MGN-3 or vehicle control (water) daily for 11 days beginning on Day 0, using a stainless steel ball-tipped gavage needle attached to an appropriate syringe. The test substance was administered as a 0.1% (5mg/kg dose) or 1% (50mg/kg dose) w/w suspension in distilled water. The chemotherapeutic agent (cisplatin or doxorubicin) or vehicle was administered to each animal by a single intraperitoneal injection on day 3 only. Doses of chemotherapeutic agents were chosen based on a pilot study using several different doses of cisplatin and doxorubicin.

All groups of rats were observed for signs of gross toxicity and/or behavioral changes daily for 11 days. Consistency of feces was monitored throughout the study. Body weights were recorded on days 0 (pre-dose of BioBran/MGN-3), 3, 5, 7, 9 and 11. On day 11, all animals were euthanized by CO_2 inhalation and necropsied. Gross appearances of major organs of the thoracic and abdominal cavities were evaluated and the presence of gastrointestinal damage was noted. The livers from each animal as well as any gross lesions noted during necropsy examination were excised and preserved in 10% neutral buffered formalin.

Histological examinations were performed on the livers of all animals. Eighty slides of rat livers were submitted to Pathco, Inc. for histopathologic examination. The slides were read in a "blind fashion". Following examination, the slides were put into their respective dose groups. All statistical analyses were done using Graph Pad In Stat® (Version 3.00 for Win 95). The statistical significance of body weight changes was analyzed using a one-way Analysis of Variance (ANOVA) and the Bonferroni Multiple Comparison Test for determining group differences with a $p<0.05$ considered satistically significant. Quantal data such as lethality, lesions and diarrhea was analyzed using a 2×2 contingency table and the chi-square test with a $p<0.05$ considered significant.

Results

Table 1 shows the effects of treatments on body weight over the duration of the study. Rats receiving either BioBran/MGN-3 at a dose level of 5 or 50 mg/kg PO for 11 daily doses showed a typical increase in body weight (approximately +72%) while rats receiving cisplatin 8 mg/kg IP or doxorubicin 10 mg/kg IP on Day 3 showed a significantly smaller increase in body weight (-1.5% for cisplatin and +30% for doxorubicin). Rats receiving BioBran/MGN-3 at 5 or 50 mg/kg PO plus cisplatin or doxorubicin had a significantly greater weight gain than that observed with the chemotherapeutic agent alone. BioBran/MGN-3 5

Table 1 Effect of MGN-3 on loss of body weight induced by cisplatin and doxorubicin

Treatment	N	Day 0	Day 3	%	Day 5	%	Day 7	%	Day 9	%	Day 11	%
MGN-3 5 mg/kg PO + Vehicle IP	10	156.4 ±1.7	198.8 ±1.8	127.2 ±0.02	216.9 ±2.7	138.8 ±0.02	226.3 ±2.9	144.8 ±0.02	243.6 ±2.9	153.2 ±0.04	269.1 ±3.7	172.2 ±0.03
MGN-3 50 mg/kg PO + Vehicle IP	10	156.5 ±4.2	194.9 ±3.8	124.8 ±0.02	208.8 ±4.2	133.8 ±0.02	219.7 ±4.7	140.8 ±0.03	235.1 ±4.7	148.3 ±0.04	259.7 ±4.7	166.5 ±0.03
MGN-3 Control PO + Cisplatin 9 mg/kg IP	6	161.3 ±0.8	207.0 ±1.3	128.3 ±0.01	184.3 ±2.0	114.3 ±0.01	162.0 ±4.7	100.4 ±0.03	143.2 ±5.9	84.9 ±0.04	159.2 ±10.5	98.5 ±0.06
MGN-3 5 mg/kg PO + Cisplatin 9 mg/kg IP	9	154.0 ±2.9	194.9 ±3.8	126.6 ±0.01	180.4 ±6.7	117.3 ±0.03	169.1 ±12.1	109.8 ±0.08	163.6 ±17.4	104.2 ±0.11	172.2 ±20.0	115.5* ±0.13
MGN-3 50 mg/kg PO + Cisplatin 9 mg/kg IP	7	157.4 ±3.2	197.9 ±4.1	125.7 ±0.02	201.0 ±6.6	128.0 ±0.05	204.1 ±12.6	130.2 ±0.09	211.4 ±17.5	130.2 ±0.11	225.3 ±22.4	144.0* ±0.15
MGN-3 Control PO + Doxorub. 10 mg/kg IP	10	161.1 ±3.3	197.1 ±6.8	122.1 ±0.03	197.6 ±6.6	122.5 ±0.02	191.0 ±8.5	118.3 ±0.04	196.4 ±10.2	118.2 ±0.03	210.4 ±10.7	130.2 ±0.05
MGN-3 5 mg/kg PO + Doxorub. 10 mg/kg IP	10	158.1 ±2.1	200.1 ±1.7	126.7 ±0.01	203.0 ±4.1	128.5 ±0.02	204.7 ±8.4	129.5 ±0.05	213.8 ±10.1	131.8 ±10.1	231.6 ±12.0	146.6* ±0.08
MGN-3 50 mg/kg PO + Doxorub. 10 mg/kg IP	10	156.8 ±2.4	201.2 ±2.9	128.4 ±0.01	200.0 ±5.3	127.6 ±0.03	197.0 ±8.2	125.5 ±0.04	207.1 ±8.53	130.0 ±0.06	225.3 ±10.7	143.5* ±0.06

*Statistically different from Control + Chemotherapeutic treatment group-p<0.05 ANOVA + Bonferroni Multiple Comparison Test

mg/kg produced a +11% increase in body weight in cisplatin treated rats and a +46% increase in doxorubicin treated rats. BioBran/MGN-3 50mg/kg produced a +44% increase in body weight in cisplatin treated rats and a +43% increase in doxorubicin treated rats.

Cisplatin caused deaths in 50% of rats treated (**Table 2**). Lethality of cisplatin was decreased to 10% in rats treated with 5 mg/kg BioBran/MGN-3 and 40% in groups treated with 50 mg/kg. Doxorubicin did not cause death after a single dose.

One hundred percent of rats receiving a single dose of 9 mg/kg cisplatin IP showed signs of diarrhea on days 7 to 11 (**Table 2**). BioBran/MGN-3, 5 mg/kg decreased the incidence to 50%, and 50 mg/kg decreased the incidence to 40%. Cisplatin produced gross gastrointestinal mucosal pathology in 70% of treated rats. This was decreased to 40% after 5 mg/kg BioBran/MGN-3 and 50% after 50 mg/kg BioBran /MGN-3. Doxorubicin produced diarrhea in only 20% of rats and the incidence was decreased to 0% after BioBran/MGN-3 5mg/kg and 10% after 50mg/kg. Doxorubicin produced gross gastrointestinal mucosal pathology in 50% of rats. Incidence was decreased to 10% after 5 mg/kg BioBran/MGN-3 and 30% after 50 mg/kg BioBran/MGN-3.

There were few changes in the liver parenchyma in any dose group. BioBran/MGN-3 5 and 50 mg/kg groups were very similar with very fine vacuolization within the cytoplasm of hepatocytes that were slightly more prominent in the periportal area. This was all within the range of normal. All other groups receiving either cisplatin or doxorubicin had varying degrees of chronic/active inflammation of the liver capsule.

Table 2 Effect of MGN-3 on the occurrence of spontaneous deaths, gross intestibnal lesion and diarrhea

Treatment	D%	GI Path %	Dia. %
MGN-3 5 PO + Vehicle	0	0	0
MGN-3 50 PO + Vehicle	0	0	0
MGN-3 Control + Cisplatin 9 IP	50	70	100
MGN-3 5 PO + Cisplatin 9 IP	10#	40	50#
MGN-3 50 PO + Cisplatin 9 IP	40	50	40#
MGN-3 Control + Doxorubicin 10 IP	0	50	20
MGN-3 5 PO + Doxorubicni 10 IP	0	10#	0
MGN-3 50 PO + Doxorubicin 10 IP	0	30	10

Statistically significant $p<0.05$ chi-square test

D-Spontaneous deaths

GI Path-Gross damage to gastrointestinal tract indicated by perforations, hemorrhagic spots, mucosal abnormalities, or excess fluid accumulation

Dia.-Incidence of diarrhea or soft stools

This was characterized by a thickened capsule due to a proliferation of young fibroblasts, and an infiltrate of acute and chronic inflammatory cells. This change is consistent with an intra-peritoneal injection of an irritating material. The inflammation of the capsule was slightly more severe in rats injected with doxorubicin than cisplatin. There was no necrosis or degenerative changes observed in the liver parenchyma of any dose group. The centrilobular hypertrophy seen in several livers was quite subtle, and may well be due to secondary changes associated with peritonitis rather than a direct compound effect. Because liver parenchymal changes were so subtle with the administration of chemothrapeutic agents, no attempt was made to evaluate the benefits of administering BioBran/MGN-3 on histologic examination.

Discussion and Conclusions

BioBran/MGN-3 was shown in this study to protect rats given an acutely toxic dose of cisplatin or doxorubicin. The endpoints chosen for evaluation were prevention of the effects of these agents on body weight, stool consistency, gross pathology of the gastrointestinal tract and mortality. Liver histology was also studied, but consistent severe pathology was not observed with the single dose of cisplatin or doxorubicin administered. Significant protection was observed on body weight. It is not clear from this study whether this was due to increased food intake or prevention of the catabolic effects of the chemotherapeutic agent. Protection against gastrointestinal mucosal lesions and diarrhea were also observed, as was a decreased mortality due to cisplatin. BioBran/MGN-3 administered at 5 mg/kg PO appeared to be more effective than at 50 mg/kg PO. No obvious explanations are apparent to explain the greater efficacy of the lower dose of BioBran/MGN-3. The peak efficacious dose may actually be lower than the lowest dose tested and would require further testing. The effects on weight gain, lesion formation and diarrhea appear to be plateauing. It is only the lack of protection of lethality from cisplatin that the 50 mg/kg dose is significantly less than the 5 mg/kg dose.

Cisplatin and doxorubicin produce well-documented pathology when used as chemotherapy for malignant

diseases[3, 4]. Severe cardiac and renal pathology are common. This study was not designed to evaluate these adverse effects since a single dose of cisplatin or doxorubicin was not sufficient to produce significant pathologic changes in the liver. However, there were signs of gross pathology when gastrointestinal mucosa was evaluated. In this study, cisplatin produced more severe adverse effects than did doxorubicin. A study using a multiple dosing protocol with somewhat lower doses of chemotherapeutic agents would be required to evaluate potential protection against pathologic changes in the liver, kidneys and heart.

The mechanism by which BioBran/MGN-3 protects against the toxicity of doxorubicin and cisplatin is unknown. BioBran/MGN-3 has been reported to increase NK cell activity in immunocompromised patients and possess scavenging activities against superoxide anion radicals and hydroxyl radicals[2]. Reactive oxygen species produced during metabolism of doxorubicin are purported to play an important role in the pathogenesis of experimental doxorubicin nephropathy in rats[3]. Ghoneum and Jewitt[8] have shown that BioBran/MGN-3 in an *in vitro* system produced an increased production of tumor necrosis factor α and interferon α from peripheral blood lymphocytes as well as augmentation of natural killer cell cytotoxic function. This activity has been proposed to explain the effectiveness of BioBran/MGN-3 as a novel biological response modifier. It also suggests its use as a safe alternative or as an adjuvant to existing immunotherapeutic therapy. However, it does not explain its protective effect on gastrointestinal mucosa. There is no indication that the cytoprotective activity is due to an increase in prostaglandin levels in the gastrointestinal mucosa.

Recent advances in chemotherapy have focused on the benefit of high dose regimens, increasing the dose intensity of conventional chemotherapy and using intensified chemotherapy with or without autologous bone marrow rescue[1, 4]. Dose intensity usually increases objective response rates of antineoplastic drugs and might, in some circumstances, improves survival. However, unacceptable acute and/or cumulative toxicity often impairs the proper management of patients, leading to dose reduction or treatment delay, thus reducing the efficacy and potentially the quality of life of patients. Therefore, considerable efforts have been made to manage, to prevent, and to delay many acute, and cumulative treatment-related toxicities. Several chemoprotective compounds have now been extensively investigated, including dexrazoxane, amifostine, glutathione, mesna and ORG2766[5]. Dexrazoxane appears to complex with metal co-factors including iron, to reduce the incidence of anthracycline-induced cardiotoxicity, allowing the delivery of higher cumulative doses of anthracyclines without the expected consequence of cardiomyopathy. Numerous studies have demonstrated that sulfur-containing nucleophiles; including amifostine[6, 7]. These studies have not revealed any evidence of reduction in antitumor efficacy.

There is still a great need for non-toxic agents that have significant protective activity against chemotherapy induced adverse effects[1]. BioBran/MGN-3 appears to be effective in protection against some of the disturbing side effects produced by cisplatin and doxorubicin and may be valuable in improving "quality of life" in-patients receiving chemotherapy. BioBran/MGN-3 is a natural food supplement and now used for genaral health-promotion benefits. It is generally regarded as non-toxic at doses used in humans (0.5-3 grams per day). BioBran/MGN-3 may prove to be useful as an adjunct to cancer chemotherapy.

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Effect of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on Experimental Liver Dysfunction in Rats

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Objective

BioBran/MGN-3 is a partial hydrolysis product obtained by treating water-soluble hemicellulose from rice bran with enzymes from a basidiomycete. It has been shown to have a variety of physiological functions. However, its effect on liver dysfunction has not been fully investigated. In this study, the effect of BioBran/MGN-3 on experimental liver dysfunction in rats induced by galactosamine (GalN) and acetaminophen (AAP) was examined.

Method

Male SLC Wistar clean rats (aged 4 weeks) were used. After 4 days of preliminary raising, the animals were divided into groups. During experiments, they received the standard feed AIN-76 *ad libitum*. The feed composition is shown in **Table 1**.

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Table 1 Composition of the experimental diet (%)

Casein	20
Gelatinized cornstarch	40
Sucrose	25
Corn oil	5
Vitamin mixture*	1
Mineral mixture*	3.5
Cellulose	5
Choline bitartrate	0.2
DL-Methoinine	0.3

*: Composition of the AIN-76™ diet

To induce experimental liver dysfunction, GalN was administered in experiments 1-3 and AAP in experiments 4 and 5.

In experiment 1, rats received intraperitoneal (i.p.) administration of BioBran/MGN-3 at 20, 40, and 80 mg/kg and GalN at 800 mg/kg, 1 hour after the administration of BioBran/MGN-3.

In experiment 2, rats received either BioBran/MGN-3 orally, or a high or low molecular weight fraction of BioBran/MGN-3 i.p., and were given GalN at 800 mg/kg 1 hour after the administration of BioBran /MGN-3.

(Treatment groups)
Group A: D-galN
Group B: D-galN+MGN-3 i.p.
Group C: D-galN+MGN-3 high molecular weight i.p.
Group D: D-galN+MGN-3 low molecular weight i.p.
Group E: D-galN+MGN-3 p.o.
Group F: AAP
Group G: AAP+MGN-3 i.p.
Group H: AAP+MGN-3 p.o.

Experiment 2 (1)

Fractionation of BioBran/MGN-3 by gel filtration

Sephadex-G 25 (Fine) was swollen with distilled water (d.w.) and packed into a column (15 mm×200 mm). A mixture of riboflavin and blue dextran in aqueous solution was applied as markers and elution detected with UV. BioBran/MGN-3 at 10 mg/m*l* in d.w. was also applied to the column and the soluble part fractionated. On elution, BioBran/MGN-3 was separated into high and low molecular weight frac-

tions using the midpoint between the peaks of the above two markers.

Experiment 2 (2)

Preparation and administration of BioBran/MGN-3 solution

In Groups B and G, BioBran/MGN-3 was administered i.p. to rats at 40 mg/kg body weight 7 days after the start of raising for experiments. Group C received i.p. administration of the high molecular weight fraction at a dose equivalent to 40 mg/kg and Group D with the low molecular weight fraction at a dose equivalent to 40 mg/kg. Groups E and H received BioBran/MGN-3 orally at 120 mg/kg.

Experiment 2 (3)

Preparation and administration of D-galN solution

Groups A to E were given an i.p. injection of D-galN at 400 mg/kg 1 hour after BioBran/MGN-3. Rats were fasted for 4 hours before and after administration. D-galN (Sigma) was dissolved at 160 mg/m*l* in PBS (phosphate buffered saline) and the solution was adjusted to pH 7.0 with 2N NaOH and passed through a sterile filter.

Experiment 2 (4)

Preparation and administration of AAP solution

Groups F to H were given an i.p. injection of AAP at 700 mg/kg 1 hour after administration of BioBran /MGN-3. Rats were fasted for 18 hours before and 24 hours after administration. p-Acetamidophenol (Wako Pure Chemical Industries, Ltd.) was dissolved in a 0.9% NaCl solution at 35 mg/m*l* and autoclaved at 120℃ for 10 mins. The solution was cooled to 40℃ just prior to administration. Where precipitation was observed, the material was redissolved by increasing the temperature, and the solution was again cooled to 40℃ before administration.

In experiment 3, rats were received i.p. administration of BioBran/MGN-3 heated, hydrolyzed, or treated with an ion exchange resin and GalN at 800 mg/kg 1 hour after the administration of BioBran/MGN-3.

(Treatment groups)

Group A: Control

Group B: MGN-3 heated (i.p.)

Group C: MGN-3 hydrolyzed (with HCl) (i.p.)

Group D: MGN-3 treated with cation exchange resin (i.p.)

Group E: MGN-3 treated with anion exchange resin (i.p.)

Experiment 3 (1)

Heating of BioBran/MGN-3

BioBran/MGN-3 was dissolved in d.w. at 10 mg/m*l*, heated in boiling water at 100℃ for 5 min, and freeze-dried.

Experiment 3 (2)

Hydrolysis of BioBran/MGN-3

BioBran/MGN-3 was dissolved at 10 mg/m*l* in 1N HCl and hydrolyzed in boiling water at 100℃ for 60 min. The solution was evaporated to dryness under decreased pressure using a rotary evaporator.

Experiment 3 (3)

Ion exchange resin treatment of BioBran/MGN-3

The cation exchange resin was DOWEX 50W-X8 (Dow Chemical). To the resin, 1N NaOH was added

and the mixture stirred. The resin was washed with d.w. and packed into a column (15 mm×100 mm). After a sufficient amount of 1N HCl had flowed through, the column was washed with d.w. An aqueous solution of BioBran/MGN-3 (10 mg/m*l*) was applied and the non-adsorbed components were eluted with d.w. and freeze-dried.

The anion exchange resin was DOWEX 1-X2 (Dow Chemical). To the resin, 1N HCl was added and the mixture stirred. The resin was washed with d.w. and packed into a column (15 mm×100 mm). When a sufficient amount of 1N NaOH had flowed through, the column was washed with d.w. An aqueous solution of BioBran/MGN-3 (10 mg/m*l*) was applied applied and the non-adsorbed components were eluted with d.w. and freeze-dried.

In experiment 4, 1 hour after i.p. or oral administration of BioBran/MGN-3, AAP was given at 700 mg /kg.

In experiment 5, rats received hydrolyzed BioBran/MGN-3 i.p. and AAP was given at 500 mg/kg 1 hour later.

In all experiments, rats were sacrificed for autopsy 24 hours after the administration of GalN or AAP to determine the activities of serum transaminase (GOT and GPT). The latter was performed using a Transaminase CII-Test Wako (Wako Pure Chemical Industries, Ltd.) and activities expressed in international units (IU/L). Differences were analyzed at a significance level of 5% or lower using the Scheffe test.

Results and Discussion

Experiment 1

The increases in serum GOT and GPT activities due to GalN-induced liver dysfunction were significantly inhibited in all BioBran/MGN-3 treatment groups compared with controls. BioBran/MGN-3 produced a significant inhibitory effect on GalN-induced liver dysfunction at 20 mg/kg and the effect was unchanged at higher doses (**Figures 1** and **2**).

Experiment 2

The increase in serum GPT activity due to GalN-induced liver dysfunction was significantly inhibited in all treatment groups receiving BioBran/MGN-3 i.p. or the high or low molecular weight fractions of BioBran/MGN-3, compared with controls. Similar inhibition was observed in rats given BioBran/MGN-3 orally (**Figures 3** and **4**).

Experiment 3

The increase in serum GOT activity due to GalN-induced liver dysfunction was significantly inhibited in the group receiving hydrolyzed BioBran/MGN-3 compared with the control group (**Figure 5**).

Experiment 4

The increase in serum GOT activity due to AAP-induced liver dysfunction was significantly inhibited in the i.p. and oral BioBran/MGN-3 treatment groups compared with the control group (**Figures 6** and **7**).

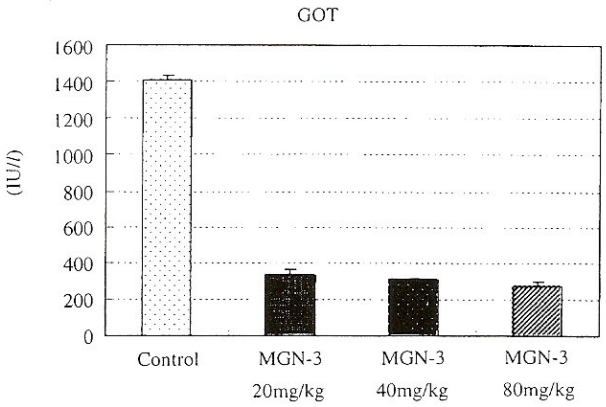

Fig. 1 Effect of MGN-3 on serum GOT activity in galactosamine-induced hepatitis in rats

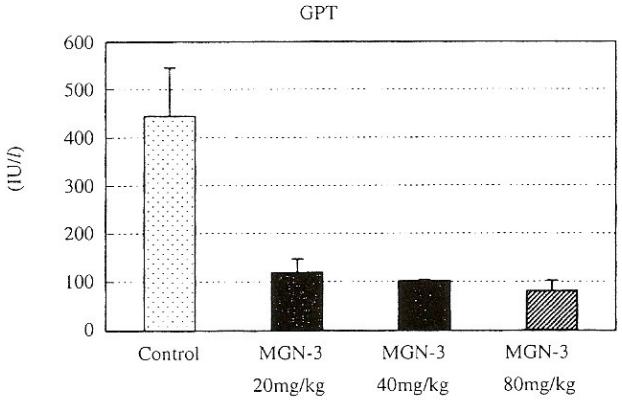

Fig. 2 Effect of MGN-3 on serum GPT activity in galactosamine-induced hepatitis in rats

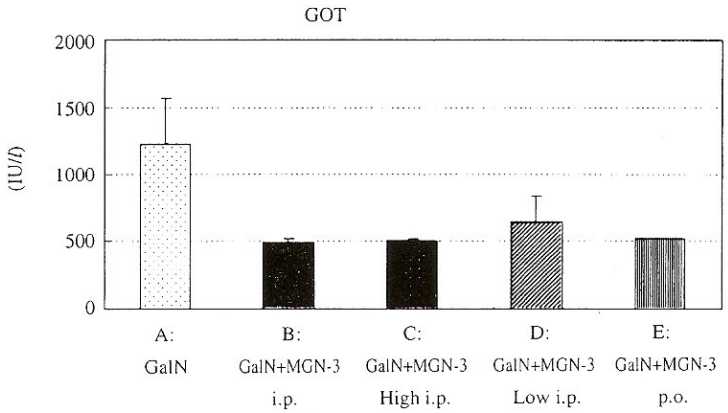

Fig. 3 Effect of MGN-3 on serum GPT activity

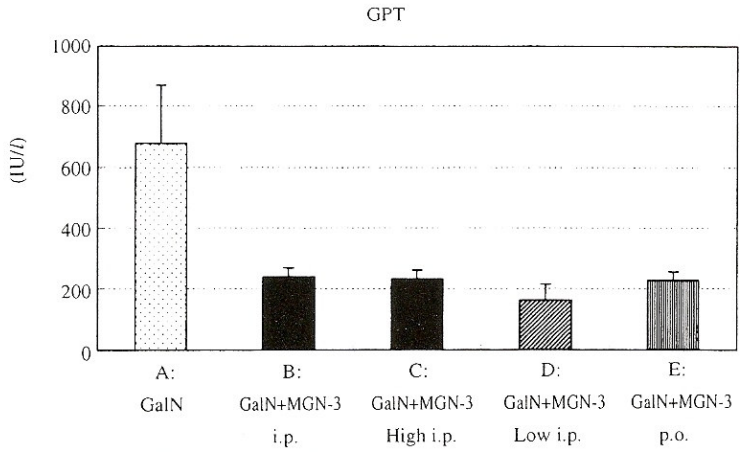

Fig. 4 Effect of MGN-3 on serum GPT activity

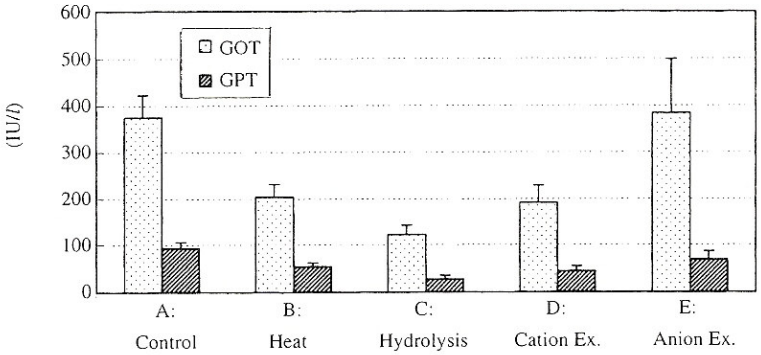

Fig. 5

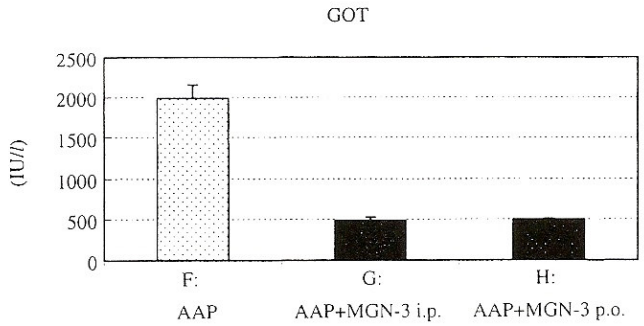

Fig. 6 Effect of MGN-3 on serum GOT activity in galactosamine-induced hepatitis in rats

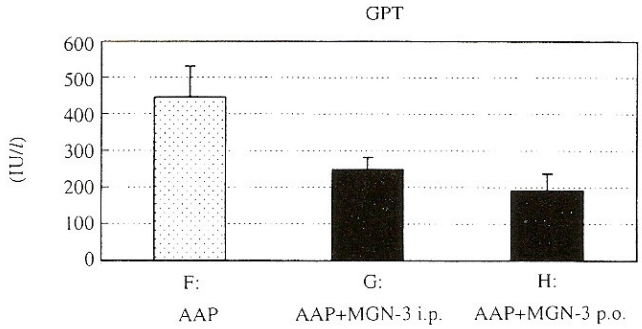

Fig. 7 Effect of MGN-3 on serum GPT activity in galactosamine-induced hepatitis in rats

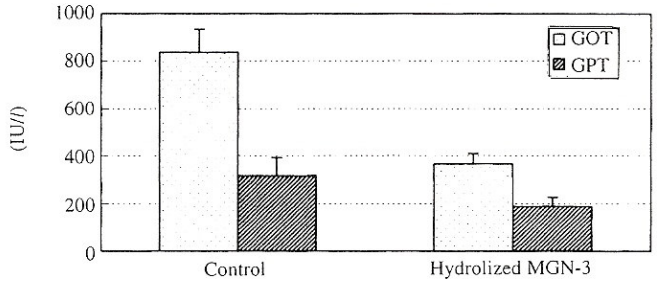

Fig. 8

Experiment 5

Based on the results of experiment 3, the effect of hydrolyzed BioBran/MGN-3 on AAP was studied. Hydrolyzed BioBran/MGN-3 significantly inhibited the increase in serum GOT activity in the treatment group compared with the control group (**Figure 8**).

As stated above, i.p. administration of BioBran/MGN-3 1 hour before treatment with D-galactosamine inhibited D-galN-induced liver dysfunction at an effective dose of 20 mg/kg. This inhibitory effect was obtained with both high and low molecular weight fractions and even after oral administration. Acetaminophen-induced liver dysfunction was also inhibited by i.p. and oral administration of BioBran/MGN-3 1 hour before AAP treatment. The subsequent experiments examined the stability of the active ingredient in BioBran/MGN-3 for inhibition of GalN-induced liver dysfunction during heating and hydrolysis with HCl, and its adsorption to cation and anion exchange resins after acid hydrolysis. The results showed that BioBran/MGN-3 was stable to heating to 100℃ whereas hydrolysis with 1N HCl for 1 hour tended to increase inhibitory activity. The active ingredient in BioBran/MGN-3 could be adsorbed onto an anion exchange resin but not to a cation exchange resin and treatment with either resin slightly decreased its inhibitory action. In addition, the stability to acid hydrolysis of the active component in BioBran/MGN-3 responsible for inhibition of acetaminophen-induced liver dysfunction was studied. As with GalN-induced liver dysfunction, the inhibitory activity on AAP-induced liver dysfunction was stable to hydrolysis by 1N

HCl at 100℃ for 1 hour. These results showed that BioBran/MGN-3 has an inhibitory effect on GalN and AAP induced liver dysfunction and suggested that the active ingredient is not susceptible to hydrolysis with HCl.

A Study Report on the Protective Activity of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Introduction

Modified Alabinoxylan from Rice Bran (BioBran/MGN-3) developed by Daiwa Pharmaceutical Co., Ltd. has been shown to activate rat NK cells and is predicted to have an anticancer effect. To determine whether BioBran/MGN-3 prevents bacterial infection, in this study, mice were inoculated with either virulent or attenuated strains of *Salmonella*, or enterohaemorrhagic *Escherichia coli* O157, and the effect of BioBran/MGN-3 in preventing infection was observed.

Experimental method

Female BALB/cAJc16 mice aged 7 weeks (12 mice per group) were given BioBran/MGN-3 or a placebo (1 mg/mL) orally *ad libitum* (1-2 mL/day/mouse). In the experiment, BioBran/MGN-3 or the placebo (1 mg/mouse: 50 mg/kg) was administered to the mice intraperitoneally either 5 days or 1 day before infection and the mice were then given the same substance (1 mg/mL) orally *ad libitum*

This is a report of collaborative study result conducted with International Medical Center of Japan.

Table 1 Effect of BioBran/MGN-3 on *Salmonella enteritidis* No.11 infection

	No. of survivals at 15days after inoculation			LD_{50}
	3.0×10^1	3.0×10^2	3.0×10^3	
MGN-3	0	0	0	$<3.0\times10^1$
Placebo	0	0	0	$<3.0\times10^1$

No difference was shown between BioBran/MGN-3 and placebo groups

Table 2 Effect of BioBran/MGN-3 on *Escherichia coli* O157: H7 (EHEC-SMR) infection

	MGN-3	Placebo
	①$1.0\times10^2$ CFU/spleen ②$3.0\times10^2$ CFU/spleen	①$1.0\times10^2$ CFU/spleen ②$2.0\times10^2$ CFU/spleen
One day after inoculation	①<100 CFU/liver ②<100 CFU/liver	①<100 CFU/liver ②<100 CFU/liver
	①<100 CFU/ kidney ②<100 CFU/kidney	①<100 CFU/ kidney ②<100 CFU/kidney
	①<100 CFU/spleen ②<100 CFU/ spleen	①<100 CFU/spleen ②<100 CFU/ spleen
Two days after inoculation	①<100 CFU/liver ②<100 CFU/liver	①<100 CFU/liver ②<100 CFU/liver
	①<100 CFU/ kidney ②<100 CFU/kidney	①<100 CFU/ kidney ②<100 CFU/kidney

(1-2mL/day/mouse). One of *Salmonella enteritidis* No. 11, *Salmonella typhimurium* LT2, or streptomycin-resistant enterohaemorrhagic *Escherichia coli* O157:H7 (EHEC-SMR) was inoculated into the abdominal cavity. The mortality rate of the mice infected with *Salmonella enteritidis* No. 11 and *Salmonella typhimurium* was observed. In the case of *Escherichia coli* o157:H7 (EHEC-SMR), the number of bacterial cells present in organs was counted 24 and 48 hours after inoculation.

Results

Salmonella enteritidis No. 11

Mice were given BioBran/MGN-3 or a placebo (1mg/mL) orally *ad libitum* for 5 days and intraperitoneally administered the virulent strain *Salmonella enteritidis* No. 11 in quantities of 3.0×10, 3.0×10^2, and 3.0×10^3 CFU/ mouse. As shown in **Table 1**, all mice in both the placebo and BioBran/MGN-3

Table 3 Effect of oral administration of BioBran/MGN-3 on *Salmonella typhimmurium* LT2 infection mice

Salmonella typhimmurium	Mortality	
	MGN-3	Placebo
1.0×10^{1} CFU/mouse	0	0
1.0×10^{2} CFU/mouse	0	0
1.0×10^{3} CFU/mouse	8	12
1.0×10^{4} CFU/mouse	12	12
1.0×10^{5} CFU/mouse	12	12
1.0×10^{6} CFU/mouse	12	12
LD50	7.0×10^{2} CFU/mouse	4.5×10^{2} CFU/mouse

Table 4 Effect of intraperitoneal administration of BioBran/MGN-3 on *Salmonella typhimmurium* LT2 infection mice

Salmonella typhimmurium	Mortality	
	MGN-3	Placebo
2.0×10^{2} CFU/mouse	0	0
2.0×10^{3} CFU/mouse	0	5
2.0×10^{4} CFU/mouse	12	12
2.0×10^{5} CFU/mouse	12	12
LD50	6.4×10^{3} CFU/mouse	3.0×10^{3} CFU/mouse

groups died. The death rate was 100% after inoculation with only 30 viable cells of this strain of bacteria. BioBran/MGN-3 did not prevent death from infection and there was no significant difference between the survival curves for the two groups.

Escherichia coli O157:H7 (EHEC-SMR)

Mice given BioBran/MGN-3 or a placebo (1 mg/mL) orally *ad libitum* for 5 days were inoculated with *Escherichia coli* O157:H7 (EHEC-SMR) at 1.0×10^{5} and the number of bacterial cells present in organs was measured 24 and 48 hours after inoculation. It is known that the mice were not affected in this experimental system (1). The number of bacterial cells was measured to observe the clearance of the bacteria. **Table 2** shows no difference between the number of bacterial cells for the BioBran/MGN-3 and placebo groups.

Salmonella typhimurium LT2

Mice given BioBran/MGN-3 or a placebo (1 mg/mL) orally *ad libitum* for 5 days were intraperitoneally administered with the attenuated strain *Salmonella typhimurium* LT2 in amounts of between 1.0×10 and 1.0×10^{6} CFU/ mouse. As shown in **Table 3**, all mice in the placebo group inoculated with 1.0×10^{3} CFU/mouse died and the LD_{50} was 4.5×10^{2} CFU/mouse. In the BioBran/MGN-3 group, all mice died after inoculation with 1.0×10^{4} CFU/mouse, but 33% survived 1.0×10^{3} CFU/mouse. The LD_{50} was 7.0×10^{2} CFU/mouse.

The same dose-dependent effect was observed in mice given an increased dose of BioBran/MGN-3 and

then inoculated with 2.0×10^3 CFU/mouse of *Salmonella typhimurium* LT2 (**Table 4**).

Discussion

The preventive effect of BioBran/MGN-3, a derivative of rice bran arabinoxylan developed by Daiwa Pharmaceutical Co., Ltd. against infections, was studied using virulent and attenuated strains of *Salmonella* and enterohaemorrhagic *Escherichia coli* O157. The results showed that BioBran/MGN-3 acted as a biological defense against *Salmonella* injection. *Salmonella* is an intracellular bacterium, suggesting that BioBran/MGN-3 may have the same effect on intracellular *Mycobacterium* tuberculosis and *Listeria*.

The Protective effects of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) against cisplatin

H. I. JACOBY and H. MAEDA

1. Evaluation of BioBran/MGN-3 on cisplatin-induced emesis in the ferret

(1) Purpose

The objective of this study is to evaluate the potential of using BioBran/MGN-3 for protection against cisplatin induced emesis in the ferret.

(2) Procedure

A group of *Mustela putorius furo*, sable ferrets was received from Marshall Farms. The animals were singly housed, two per cage, in suspended stainless steel caging with mesh floors. Litter paper was placed beneath the cages and was changed at least three times per week. The animal room was temperature controlled and had a 12-hour light/dark cycle. The animals were fed Purina Ferret Lab Diet and filtered tap water was supplied *ad libitum* by an automatic watering system.

Following acclimation to the laboratory for 2 days, 25 healthy male ferrets weighing between 890 and 1130 grams were selected for test and distributed into the following five groups of animals:

Table 1 : Test Group

Group #	No. of Animals	Test/Control Article	Dose Level of MGN-3 (mg/kg PO)	Dose Level of Cisplatin (mg/kg IP)	Test Suspension Concentration (%)	
					MGN-3	Cisplatin
1	5	Vehicle Control (0.5% CMC in distilled water)	–	–	–	–
2	5	Vehicle Control (0.5% CMC in distilled water)	–	10	–	0.4
3	5	MGN-3	100	10	2.0	0.4
4	5	MGN-3	50	10	1.0	0.4
5	5	MGN-3	20	10	0.4	0.4

PO=oral IP=intraperitoneally

The ferrets were examined for health and weighed. Individual doses for all administrations were calculated based on these bodyweights, taking into account the concentration of the appropriate suspension. Each test group animal was administered the test article or vehicle control daily for 7 days, as described above, by oral intubation using a gavage cannula. The test article was administered as a w/w suspension in a 0.5% w/w solution of carboxymethylcelluose (CMC) in distilled water. All test animals were dosed at a constant volume of 5 ml/kg. The control groups (Group 1 & 2) received 5 ml/kg of a 0.5% w/w solution of CMC in distilled water and were maintained under similar environmental conditions.

Thirty minutes after the last daily dose (Day 7), either the vehicle or 10 mg/kg cisplatin were administered to each animal intraperitoneally. All animals from Groups 2, 3, 4 and 5 were dosed IP cisplatin at a constant volume of 2.5 ml/kg. Group 1 received 2.5 ml/kg of a 0.5% w/w solution of CMC in distilled water. The ferrets were observed for 6 hours after dosing which included at least 1 hour where no signs of emesis were observed (**Table 1**). The number and time of bouts of retching/vomiting (emesis) was noted and the severity of each by noting the number of individual emetic occurrences. Emetic activity was scored by classifying the response of each ferret on the basis of the occurrence of no emesis, mild to moderate emesis (less than 3 occurrences of emesis) and moderate to severe greater than 3 occurrences of emesis.

All animals were observed daily for signs of gross toxicity and behavioral changes. All ferrets were anesthetized using isofluorane following the observation period.

(3) Results and discussion

All of the ferrets in the group receiving vehicle plus cisplatin showed signs of emesis (retching and vomiting) (**Table 2**). This was moderate to severe in four of five ferrets. Surprisingly, the lowest dose of BioBran/MGN-3 (20 mg/kg) showed the most protection against cisplatin with three of five ferrets showing only mild emesis (retching, but no vomiting). This response was statistically different from vehicle + cisplatin. These ferrets appeared to be almost fully protected against the emetic activity of cisplatin. 50 mg/kg BioBran/MGN-3 also appeared to show some protection, with three of five ferrets showing emesis, however in two there was moderate to severe bouts of retching and vomiting. Only two of five ferrets receiving the highest dose (100 mg/kg PO) of BioBran/MGN-3 showed sign of emesis. However, in these two the emetic responses were moderate to severe.

Overall, BioBran/MGN-3 did protect ferrets from the emetic effects of cisplatin. The most effective dose appeared to be the lowest dose tested. A single dose of BioBran/MGN-3 would be effective and at least for protection form emsis that chronic dosing is not necessary.

BioBran/MGN-3 may be acting as an antagonist to serotonin or neurokinis, both of which are involved with chemotherapy induced emesis. Agents which have this activity are very useful in the treatment of emesis due to chemotherapeutic agents such as cisplatin. It is unknown whether this is a specific activity or wheter BioBran/MGN-3 would also be effective against other emetic agents such as morphine, apomorphine, cholinesterase inhibitors and some phosphodiesterase inhibitors. If it is it may also be useful to counteract the side-effects of agents used in Alzheimer's disease and Perkinson's disease. The antiemetic effect may also be due to an positive effect on the protective mechanisms of the stomach and small intestine. The effect against DSS colitis showed such a protective effect.

Table 2 : Effect of Vehicle and MGN-3 Against Cisplatin Induced Emesis

Treatment	Dose mg/kg PO Daily	Cisplatin 10mg/kg IP N	N	#Ferrets no emesis	#Ferrets Mild-mod Emesis	#Ferrets Mod-Severe emesis
Vehicle	----	−	5	5	0	0
Vehicle	----	+	5	0	1	4
MGN-3	20	+	5	2	3	0*
MGN-3	50	+	5	3	0	2
MGN-3	100	+	5	2	1	2

*Statistically significant difference from Vehicle + Cisplatin group $p < 0.05$ Fisher Exact test

2. Evaluation of the effect of BioBran/MGN-3 on cisplatin-induced toxicity in rats

(1) Purpose

To evaluate the effect of orally administered BioBran/MGN-3 against gastrointestinal pathology produced by a single administration of cisplatin in rats.

(2) Procedure

A group of male Sprague-Dawley rats was received from Ace Animals, Inc., Boyertown, PA. The animals were grouped housed singly in suspended stainless steel wire mesh cages. The animal room was temperature controlled and had a 12-hour light/dark cycle. The animal were fed Purina Rodent Chow #5012 and filtered tap water supplied *ad libitum.*

Following acclimation to the laboratory for 4 days, 50 healthy male rats weighing between 167 and 194 grams were selected for test and distributed into the following five groups of animals:

Individual doses were calculated based on initial body weights. Each test group animal was administered the test article or vehicle control daily for 10 days, as described above, by oral intubation using a gavage cannula. The test article was administered as a w/w suspension in a 0.5% w/w solution of carboxymethylcelluose (CMC) in distilled water. All test animals were dosed at a constant volume of 5 ml/kg. The control groups (Group 1 & 2) received 5 ml/kg of a 0.5% w/w solution of CMC in distilled water and were maintained under similar environmental conditions. Approximately two hours after the Day 7 daily dose, each animal from Group 2 through 5 received a single intraperitoneal injection of 8 mg/kg cisplatin (Sigma Chemical, St. Louis, MO, Lot #014K0993). Group 1 was injected with 5 ml/kg of saline.

All rats were weighed prior to administration (Day 1) and on Days 4, 7, 9 and 11. All animals were

observed for signs of gross toxicity and/or behavioral changes daily (**Table 3**).

Table 3 : Test Group

Group #	No. of Animals	Test/Control Article	Dose Level of MGN-3 (mg/kg PO)	Dose Level of Cisplatin (mg/kg IP)	Test Suspension Concentration (%)	
					MGN-3	Cisplatin
1	10	Vehicle Control (0.5% CMC in distilled water)	–	–	–	–
2	10	Vehicle Control (0.5% CMC in distilled water)	–	8	–	0.16
3	10	MGN-3	100	8	2.0	0.16
4	10	MGN-3	50	8	1.0	0.16
5	10	MGN-3	20	8	0.5	0.16

PO=oral IP=intraperitoneally

On Day 11, all animals were euthanized by CO_2 inhalation. Gross necropsis were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined for changes in gross appearance. The entire gastrointestinal tract was evaluated for the presence of damage and the number of lesions found in the stomach, small intestine and colon were counted.

(3) Results and discussion

BioBran/MGN-3 produced significant dose related protection against cisplatin induced gastrointestinal toxicity and pathology (**Table 4**). This did not achieve statistical significance because of the sample size, however, there was a definite dose related protection against cisplatin-induced gastrorintestinal lesions and diarrhea. There were no effects on weigh loss due to cisplatin at any dose of BioBran/MGN-3. BioBran/MGN-3 was an effective protective agent against cisplatin-induced adverse effects in the 50 and 100 mg/kg PO dose groups. However, once a day dosing may not provide sufficient levels for protection against this chemotherapeutic agent, and therefore, the recommendation is to try twice a day dosing starting on the day cisplatin is administered to provide around the clock protection.

Table 4 : Protection of MGN-3 against GI Lesions and Diarrhea

Treatment Daily	Dose mg/kg PO	Cisplatin 8 mg/kg IP	N	#Norm. GI	% Pro	#GI Lesions	% Pro	#Diarrhea	% Pro
0.5%CMC	----	–	10	10	----	0	----	0	----
0.5%CMC	----	+	10	1	----	7	----	7	----
MGN-3	100	+	10	5	56	5	29	1	86
MGN-3	50	+	10	3	33	7	0	4	43
MGN-3	25	+	10	1	0	9	0	4	43

Norm – Normal appearing organs % Pro-percent protection as compared to Group 2

The mechanism of the protective activity may be due to interaction and inhibition of pathological factors released into the gastrointestinal tract by cisplatin and other chemotherapeutic agents. This may be due to some intrinsic effect on the immune system which is adversely effected by most chemotherapeutic agents. The protective effect is not due to interactions with levels of prostaglandins since BioBran/MGN-3 was not

effective in protecting against gastric mucosal lesions produced by a non-steroidal anti-inflammatory agent which inhibits mucosal synthesis of prostaglandins. The gastrointestinal mucosal effect combined with the positive effect against cisplatin-induced emesis suggests its usefulness as a supplement to be given concomitantly with chemotherapeutic agents to relieve some of the disturbing side effects of this therapy.

II-5-1

Evaluation of the Effects of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) in Preventing Asthma and Relieving Symptoms in a Mouse Model of Asthma

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Objective

The effects of BioBran/MGN-3 in preventing asthma and relieving symptoms was studied in a mouse model of asthma induced with toluene diisocyanate (TDI).

Experimental method

1. Mouse model of asthma

Female BALB/c mice received 1 mL of a 10% TDI/ethyl acetate solution intranasally daily for 7 days to produce antigen sensitisation. Three weeks later, the same solution was administered similarly twice weekly and a total of 24 challenges performed over a period of 12 weeks. Changes in the levels of IgG1 and IgE type antibodies against TDI, thickening of the mucous membrane, and level of nasal secretions were assessed. This experimental model, which has been developed in our laboratory, has greater similari-

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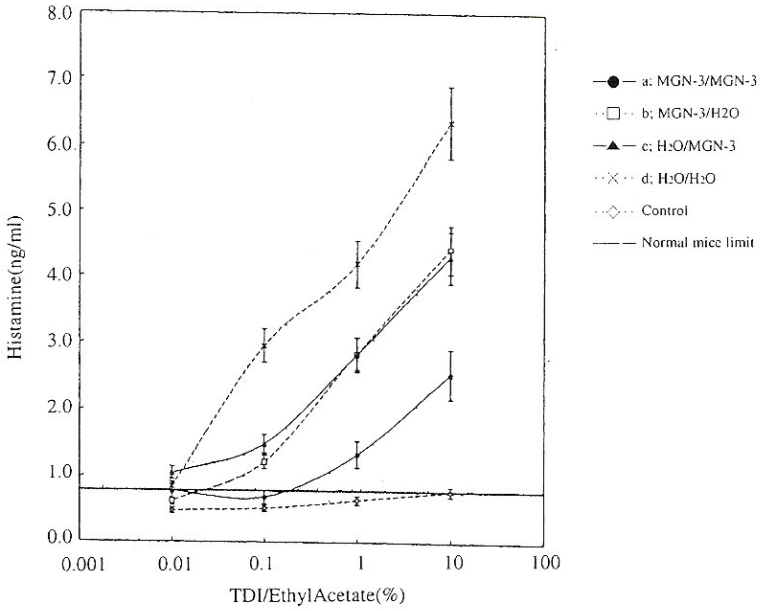

Fig.1 Serum level of histamine after challenge with various concentration of TDI

ties to human asthma than others producing short-term antigen sensitisation.

2. Administration

BioBran/MGN-3 (Daiwa Pharmaceutical Co., Ltd.) was dissolved at 2 g/L in drinking water and mice allowed to drink it *ad libitum*.

3. Experimental groups

a: One group of mice received one month of pretreatment, TDI sensitisation and challenge (the MGN-3/MGN-3 group).

b: A second group received one month of pretreatment and TDI sensitisation (the MGN-3/H2O group).

c: A third group received only the challenge with TDI (the H2O/MGN-3 group).

d: A fourth group of mice received no treatment (the H2O/H2O group).

4. Evaluation

Blood histamine concentration, eosinophil count in bronchoalveolar lavage fluid (BALF), TDI ear lobe application test, and serum levels of IgG1, IgG2a, and IgE antibodies against TDI during the challenges were measured.

5. Statistical analysis

ANOVA was used.

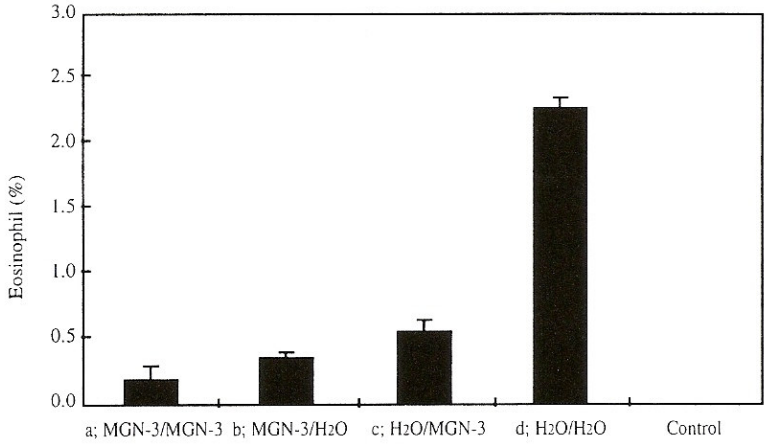

Fig.2 Percentage of eosiophils in BALF from TDI mice

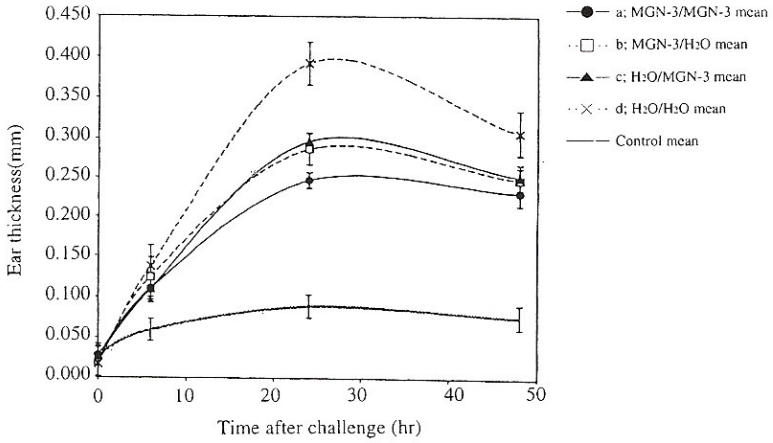

Fig.3 Ear thickness* of mice after TDI challenge

※ Ear thickness = right ear (TDI challenge) − left ear (vihecle)

Results

The peak blood concentration of histamine 7 minutes after challenge with 10% TDI was 2.5 ± 0.53, 4.2 ± 0.75, 4.3 ± 0.78, and 6.4 ± 0.87 mg/mL for the MGN-3/MGN-3, MGN-3/H_2O, H_2O/MGN-3, and H_2O/H_2O groups, respectively. Groups treated with MGN-3 had significantly lower values than those that were nontreated. In challenge experiments using 0.01%, 0.1%, 1%, and 10% TDI solutions, the sensitivity of the MGN-3 treatment groups was 10-100 times lower than in the nontreated groups (**Fig.1**). The

eosinophil counts in BALF were 5-10 times lower in the MGN-3 treatment groups than in the nontreatment group (**Fig.2**) and ear lobe thickness was significantly less in the MGN-3 treatment groups following TDI application (**Fig.3**).

Conclusion

BioBran/MGN-3 produced clear effects in preventing asthma and relieving symptoms in this mouse model of asthma induced with TDI. BioBran/MGN-3 had no effect on the production of IgG1 and IgE antibodies induced through Th2 cells, suggesting an inhibitory action on mast cells.

II-5-2

Inhibitory Effects of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on the Progression of Atopic Dermatitis in NC Mice

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Summary

BioBran/MGN-3 is an extract of arabinoxylan from rice bran. We investigated the immunoregulatory effects of BioBran/MGN-3 using NC mice, which naturally develop atopic dermatitis-like skin lesions together with high levels of serum IgE. BioBran/MGN-3 was administered orally to 5 NC mice, which were then immunised three times with ovalbumin (OVA). Blood samples were collected biweekly before and after immunisation. Levels of total IgE, as well as OVA-specific IgE, in the sera measured by specific ELISA were significantly decreased in BioBran/MGN-3 treated mice compared with untreated animals. Furthermore, atopic dermatitis-like skin lesions did not develop in any of the mice which received BioBran /MGN-3, whereas all those without the treatment developed lesions. We conclude that BioBran/MGN-3 has inhibitory effects on the progression of the atopic dermatitis-like skin lesions in NC mice.

This article is a revision of a presentation given at the 11th International Congress of Immunology (2001).

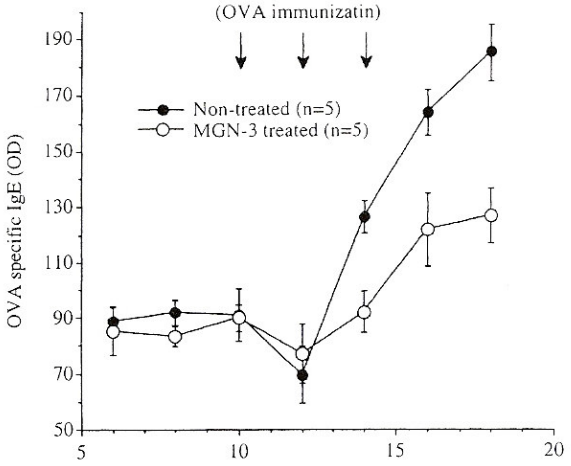

Fig.1 MGN-3 has inhibitory effects on the increse of serum OVA specific IgE concentration in NC mice

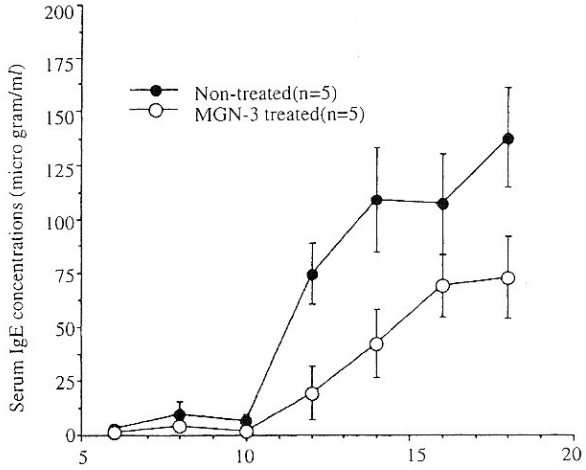

Fig.2 MGN-3 has inhibitory effects on the increse of serum IgE concentration in NC mice

Experimental Methods

[Animals]

4 week-old female NC/Nga mice were used. NC/Nga mice originated from Japanese fancy mice (Nishiki-Nezumi) and were established as an inbred strain in 1955. Reported biological characteristics include liver and kidney esterase like DBA/2 mice, high susceptibility to X-irradiation, and to anaphylactic shock after ovalbumin (OVA) immunisation.

[Methods]

0.5 % of BioBran/MGN-3 was mixed with commercial stock diet (Oriental Yeast Co., Ltd.) and administered orally to 5 NC mice throughout the study. 5 NCga mice without BioBran/MGN-3 treatment were used as control. OVA was injected intraperitoneally (i.p.) three times with 2 week intervals between immunisations. Blood samples were taken biweekly. Total serum IgE and OVA specific IgE were measured by specific ELISA. Mice were photographed biweekly to assess the progression of atopic dermatitis-like lesions.

Results

1) BioBran/MGN-3 inhibited the increase of total IgE in sera of NC mice (**Fig.1**).
2) BioBran/MGN-3 inhibited the increase of OVA-specific IgE in sera of NC mice immunized with OVA (**Fig.2**).
3) BioBran/MGN-3 had inhibitory effects on the progression of atopic dermatitis-like skin lesion in NC mice.

Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) Sensitizes Human T Cell Leukemia Cells to Death Receptor (CD95)-Induced Apoptosis

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Key words:
Apoptosis, MGN-3, Caspase, Mitochondria, HUT 78, Bcl-2

Abstract

BioBran/MGN-3, an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from Shiitake mushrooms, is an effective biological response modifier that increases NK cell activity, and potentiates the activity of conventional chemotherapeutic agents. In this study, we investigated the effect of BioBran/MGN-3 on death receptor-induced apoptosis in the human leukemic HUT 78 cell line. HUT 78 cells were pre-treated with BioBran/MGN-3, and then were incubated with the agonistic antibody against death receptor (Fas, CD95). Apoptosis was determined by the propidium iodide technique using FACScan. Activation of caspase 3, caspase 8, and caspase 9 was determined by flow cytometry. Mitochondrial membrane potential was measured with $DIOC_6$ dye using FACScan. Expression of CD95 and Bcl-2 were measured by flow cytometry. In a dose-dependent manner, BioBran/MGN-3 enhanced anti-CD95 anti-

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body-induced apoptosis. Increased cell death was correlated with increased depolarization of mitochondrial membrane potential and increased activation of caspase 3, caspase 8, and caspase 9. BioBran/MGN-3 treatment had no effect on the level of expression of CD95, but it caused down regulation of Bcl-2 expression. These results suggest that BioBran/MGN-3 increases the susceptibility of cancer cells to undergo apoptosis mediated by death ligands, which may be relevant for anti-cancer activities.

Introduction

BioBran/MGN-3 is a denatured hemicellulose which is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from the Shiitake mushrooms. 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[1]. We have previously reported that BioBran/MGN-3 augments NK, T, and B cell functions both *in vitro* and *in vivo*[1-5]. We have recently demonstrated a direct effect of BioBran/MGN-3 on tumor cell growth. BioBran/MGN-3 arrested the growth of cutaneous squamous cell carcinoma (SCC13) cell line in conjunction with an increase in intracellular levels of IL-10 and IL-12 as compared to control untreated cells[6]. In addition, when BioBran/MGN-3 was administered in conjunction with conventional chemotherapeutic agents, it was highly effective in inducing remission of cancer in animal models[7].

Apoptosis (programmed cell death) is a physiologic form of cell death that plays an important role in normal development, tissue homeostasis, and pathological situations[8,9]. There are two major pathways of apoptosis: the death receptor pathway and a mitochondrial (intrinsic) pathway[10-12]. CD95 is a death receptor that belongs to the tumor necrosis factor receptor (TNF R)/nerve growth factor receptor (NGFR) gene superfamily[13,14]. Oligomerization of CD95 by CD95 ligand (CD95L) or agonistic anti-CD95 antibody activates the apoptotic pathway by recruiting adapter protein Fas associated death domain (FADD)[14,15]. FADD then recruits caspase 8 to the death receptor complex (DISC). Auto activation of caspase 8 at the DISC is followed by activation of effector caspases, including caspase 3[16]. In certain cell types, termed type II, activated caspase 8 has been shown to cleave a Bcl-2 family member, Bid, resulting in the production of truncated Bid. This affects mitochondria and releases cytochrome *c*[17]. Cytochrome *c* couples with adaptor protein Apaf-1 to activate caspase 9. Caspase 9 then activates caspase 3. Caspase 3 acts on several substrates to produce the morphological and biochemical changes in apoptosis.

Death receptors are expressed in a variety of tumor cells, but a number of tumor cells are resistant to death ligands, despite expressing substantial amounts of CD95 on their surface. This suggests that the expression of death receptors is not sufficient to allow an apoptotic response[17,18]. Recent research has focused on identifying agents that increase the susceptibility of cancer cells to undergo apoptosis mediated by death receptors. This study was undertaken to determine whether BioBran/MGN-3 can also increase the susceptibility of cancer cells to anti-CD95 antibody-induced apoptosis.

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 (β-1, 3-glucan and activated hemicellulose). BioBran/MGN-3 was freshly prepared by dissolving in distilled H_2O at concentration of 30 g/m*l*. BioBran

/MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd, Tokyo, Japan.

1) Cell culture and test exposures

The human T cell leukemic HUT 78 cell line (ATCC, Manassas, VA) was maintained in a suspension culture at 37℃ 5% CO_2/95% air in RPMI-1640 (GIBCO/BRL, Grand Island, NY) supplemented with 10% FBS (GIBCO/BRL), 2mM glutamine, 100U penicillin and 100 μg/m*l* of streptomycin. For experimentation, cells in log phase growth were cultured with BioBran/MGN-3 (100-1000 μg/m*l*) for 3-24 h and then were stimulated with agonistic anti-Fas (CD95) antibody for an additional 24 h. Following exposure to anti-CD95 antibody, cells were washed with PBS, and then were used for different assays described below.

2) Apoptosis

Cellular DNA content was measured following extraction of degraded DNA from apoptotic cells by propidium iodide (PI) staining, as described by Darzynkiewicz et al.[19]. Briefly, cells (1×10^6 ml^{-1}) were fixed in 70% ethanol, washed with PBS and were re-suspended in the DNA extraction buffer (0.2 M Na_2HPO_4 with 0.1 M citric acid pH 7.8). Following extraction, cells were washed and incubated in DNA staining solution (20 μg/m*l* PI in PBS containing 50 μg/m*l* RNase A). Cells were stained for 30 min at room temperature in the dark and were analyzed by FACScan (Becton Dickinson, San Jose, CA). After exclusion of necrotic debris, the sub G0/G1 peak was used to quantify apoptosis.

3) Intracellular activity of caspases 8, 9 and 3

The method is based on carboxyfluorescein labeled fluromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase positive (+) cells are distinguished from caspase negative (−) cells with aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with fluorescein labeled FMK-peptide inhibitors (FAM-LETD-FMK for caspase 8, FAM-LEHD-FMK for caspase 9, and FAM-DEVD-FMK for caspase 3; Intergen Company, NY). After 1 h incubation, the cells were washed to remove unbound caspase, and cells that contained bound inhibitor were quantified using a FACScan flow cytometer.

2. Mitochondrial potential $\Delta\psi_m$

Variations of the mitochondrial transmembrane potential $\Delta\psi_m$ during apoptosis was studied using 3′3′-dihexyloxacarbocycnine dye [$DIOC_6$ (3)] (Molecular Probes, Eugene, OR). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$. 5×10^5 cells/m*l* were incubated with 0.5 μM $DIOC_6$ (3) for 30 min at 37℃. Cells were transferred on ice for FACS analysis. Forward and side scatters were used to gate and exclude cellular debris using a FACScan. Cells were excited at 488 nm and green fluorescence was collected on FL1 at 530 nm. Five thousand cells were analyzed. Data was acquired and analyzed using Cell Quest software (Becton Dickinson).

3. Expression of CD95 and Bcl-2

Cells were stained with FITC-labeled anti-human CD95 antibody or isotype control IgG (B.D.Biosciences) and the surface expression of CD95 was determined by flow cytometry. For detection of Bcl-2, cells were first fixed and permeabilized with ice-cold 70% methanol. They were then stained with FITC labeled anti-Bcl-2, or isotype control (Dako Corp, Carpintaria, CA). Cells were washed and analyzed by FACScan. The percentages of cells expressing CD95, Bcl-2, and mean fluorescence intensity (an indicator of density of the molecules/cell) were determined.

Statistical analysis. A two-tailed student's test was used to determine the significance of differences between experimental and control groups. Statistical analysis of histograms was performed by the Kolmogorov-Smirnov statistics. AD value of >0.2 is considered statistically significant.

Results

1. BioBran/MGN-3 sensitizes HUT 78 cells to death receptor-mediated apoptosis

Leukemia cell line (HUT 78) was pre-treated with BioBran/MGN-3 and then incubated with agonistic anti-CD95 antibody. Twenty-four hours post-incubation, apoptosis was determined by the PI technique, using a FACScan flow cytometer. Specific apoptosis was calculated as the percentage of experimental apoptosis-percentage of spontaneous apoptosis. The data in **Fig.1 (a)** shows a representative histogram plot and **Fig.1 (b)** shows data from three independent experiments. BioBran/MGN-3 alone at concentrations of 100-1000 μg/m*l* had minimal effect on apoptosis (specific apoptosis 2.5-4.5%). Anti-CD95 antibody induced apoptosis in 29% of HUT 78 cells (specific apoptosis = 20%). However, when leukemic cells were pre-treated with BioBran/MGN-3, followed by the anti-CD95 antibody, a significant increase in the number of apoptotic cells (specific apoptosis 35-42%) ($P<0.01$) was noted. This represents a 200% increase as compared to anti-CD95 antibody alone. Lower concentration <100 μg/m*l* did not sensitize HUT 78 cells to anti-CD95 antibody-induced apoptosis.

2. BioBran/MGN-3 increases activation of caspase 8, caspase 9, and caspase 3

Apoptosis via the death receptor (CD95) is mediated by activation of the caspase cascade. In order to determine the steps in CD95 mediated apoptosis that were affected by BioBran/MGN-3, we examined the activation of proximal caspases (caspase 8, caspase 9) and the executioner caspase (caspase 3). HUT 78 T cells were exposed to BioBran/MGN-3 for 24 h and were incubated with anti-CD95 antibody for 6 h. The proportion of cells with active caspase 8, caspase 9, and caspase 3 were determined with a Caspatag caspase detection kit using FACScan. **Fig. 2** shows data from three experiments for activation of caspases. The data shows that anti-CD95 antibody induced the activation of caspase 8, caspase 9, and caspase 3 in HUT 78 cells. It also demonstrates that the proportion of cells with increased active caspase 8, caspase 9, and caspase 3 was higher in BioBran/MGN-3 treated cells than in untreated control cells. Moreover, the mean fluorescence intensity of caspase 8 and caspase 3 was significantly higher in BioBran/MGN-3 pre-treated cells followed by anti-CD95 antibody in comparison with untreated cells. This would suggest that pre-exposure to BioBran/MGN-3 led to increased activation of proximal and executioner caspases.

3. BioBran/MGN-3 does not alter the level of expression of the death receptor CD95

To determine the possibility that the increased susceptibility of BioBran/MGN-3 treated cells is due to up regulation of a death receptor, CD95 expression on HUT 78 cells treated with and without BioBran/MGN-3 was determined. The percentage of cells expressing CD95 and the density of CD95 on BioBran/MGN-3 treated cells was similar to that of untreated cells.

4. BioBran/MGN-3 disrupts mitochondrial membrane potentials

Stimulation of the CD95 receptor leads to the disruption of the mitochondrial membrane potential[20, 21] and to the release of mitochondrial contents. This results in activation of caspase 9 and caspase 3. To further

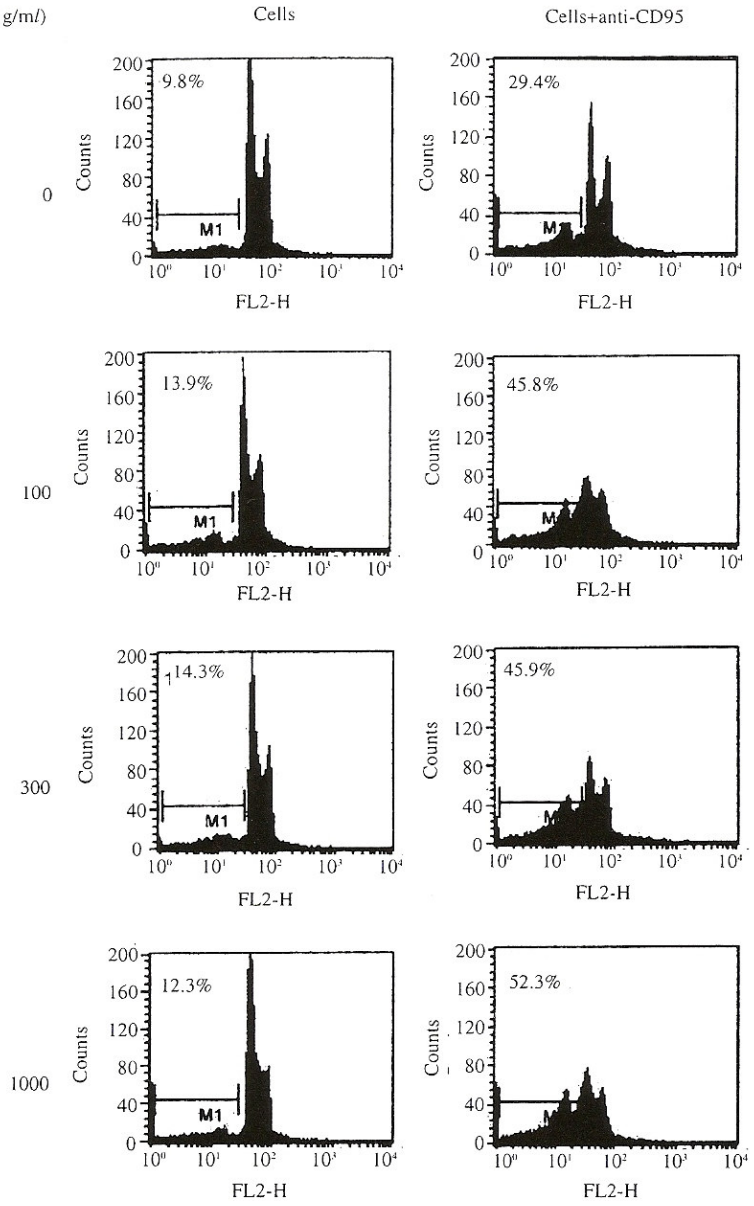

Fig.1 Effect of MGN-3 on anti-CD95 antibody-induced apoptosis

Cells (HUT 78) were pre-treated with MGN-3 for 3 h and then were incubated with agonistic anti-CD95 antibody. Apoptotic cells were determined by PI technique using FACScan flow cytometer.

(a) A representative histogram showing increased apoptosis and (b) represents the mean±SD of three experiments. Combination = MGN-3 at 100, 300, 1000 μg/ml followed by anti-CD95 antibody.

*Significant at $P<0.01$.

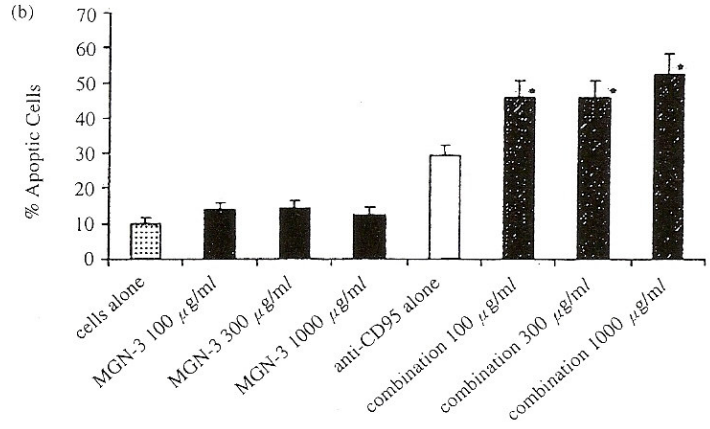

Fig. 1 (*continued*)

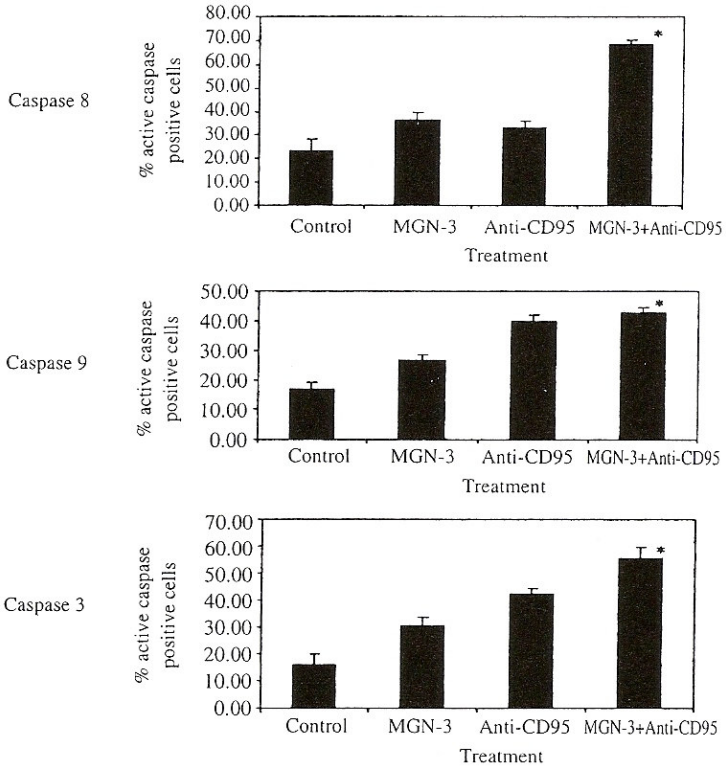

Fig. 2 Increased activation of caspases 3, 8, and 9

Cells were treated with MGN-3 and were incubated with anti-CD95 antibody, and intracellular active caspases 3, 8, and 9 were determined with caspatag caspases 3, 8, and 9 determination kit using FACScan.

Data represent the mean±SD of three experiments.

*Significant at $P<0.01$.

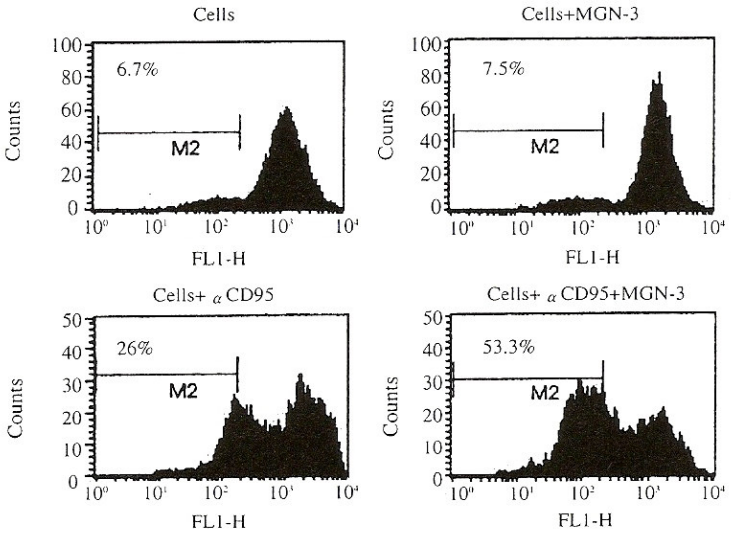

Fig. 3 Effect of MGN-3 on mitochondrial potential

HUT 78 cells were incubated with or without anti-CD95 antibody in the presence or absence of MGN-3 for 2 h. The cells were then stained with DIOC6 and were subjected to flow cytometry analysis to determine the $\Delta\psi$m. The numbers in the histogram represent the proportion of cells exhibiting decreased membrane potential.

characterize the sensitizing action of BioBran/MGN-3 on the death receptor pathway of apoptosis, we determined mitochondorial membrane potentials. HUT 78 cells treated with or without BioBran/MGN-3 were exposed to anti-CD95 antibody, and mitochondrial potential was determined by flow cytometry using membrane potential sensitive DIOC6 (3) dye. The data in **Fig. 3** shows that a significantly higher proportion of BioBran/MGN-3 pre-treated HUT 78 cells exhibit decreased membrane potential as compared to untreated cells. BioBran/MGN-3 alone had no significant effect on mitochondrial membrane potential.

5. BioBran/MGN-3 downregulates Bcl-2 expression

Bci-2 is an anti-apoptotic molecule that is shown to protect the cells from apoptosis induced by diverse agents[22, 23]. To further investigate the effect of BioBran/MGN-3, we tested its effect on Bcl-2 expression. The data in **Table 1** shows that BioBran/MGN-3 caused a significant decrease in the level of expression of this anti-apoptotic protein.

Discussion

Susceptibility to apoptosis is a prerequisite for successful treatment of cancer cells by natural killer cells, cytotoxic T cells, chemotherapy, or radiation threapy. In this study, we have established the fact that BioBran/MGN-3, a biological response modifier (BRM), sensitizes human leukemic HUT 78 cells to anti-CD95 antibody-induced apoptosis. This conclusion is based on the following evidence: BioBran/MGN-3 on its own, at 100-1000 μg/m*l*, resulted in a 3-4% apoptotic cell death, while CD95 stimulation caused a 20% apoptosis. When cells were treated with both BioBran/MGN-3 followed by the anti-CD95 antibody,

Table 1 Effect of MGN-3 on the expression of Bcl-2

Cells treated with	Bcl-2 positive cells (%)	MFC
None	100	740
MGN-3	100	559*

HUT 78 cells (1×10^6 cells/m*l*) were cultured in the absence or presence of MGN-3 (1000 μ g/m*l*) for 24 h.
Expression of Bcl-2 was determined by staining the cells with anti-human Bcl-2 antibody and flow cytometry.
MFC = mean fluorescence channel number.
* Significantly different from control untreated cells $D = 0.30$.

35-42% apoptic cells were observed. This represents a 200% increase, as compared to using the anti-CD95 antibody alone (**Fig. 1 (a)** and **(b)**). This was correlated with the increased number of cells having active caspase 8, caspase 9, and caspase 3 in BioBran/MGN-3 pre-treated cells that were subsequently exposed to anti-CD95 antibody, as compared to cells exposed to anti-CD95 antibody alone.

The recommended dose of BioBran/MGN-3 for a human weighing 60kg is 3g per day by month[2]. Studies in experimental animals have shown that doubling of this dose is well tolerated without toxic manifestation in terms of body weight of mice (Personal Communications). Currently, there is not a significant body of research on the effect of natural BRMs against cancer cell apoptosis. Fan et al.[24] demonstrated that a lipoprotein fraction of rice bran induces apoptosis and growth inhibition in cultured human endometrial adenocarcinoma cells (Sawano). Miyoshi et al.[25] reported that rice bran agglutinin (RBA) induces growth arrest and apoptosis in human monoblastic leukemia U937 cells. Han et al.[26] showed that mycelial extracts of Coprinus disseminatus (pers. Fr.) induce apoptosis in the human cervical carcinoma cells via activation of caspase 3. Sigounas et al.[27] studied the effect of *dl*- α -tocopherol (vitamin E) on different cancer cell lines and they found that *dl*- α -tocopherol induced apoptosis in erythroleukemia, prostate, and breast cancer cells with different degrees of sensitivity. In another study, apoptic activity of Chinese herbal preparation PC SPES was examined. Halicka et al.[28] found that treatment with PC SPES resulted in an increased percentage of cells with fractional DNA content in histiocytic lymphoma U937 and prostate adenocarcinoma PC 3 lines. They reported that apoptosis occurred through the down-regulation of the expression of Bcl-2.

To investigate the mechanism by which BioBran/MGN-3 modulates CD95-mediated apoptosis, the effect of BioBran/MGN-3 on known effector molecules in anti-CD95 antibody-induced apoptosis was examined. In this study we have shown that BioBran/MGN-3 up regulates the activation of proximal caspase 8, and downstreams caspase 9 and executioner caspase 3. BioBran/MGN-3 also caused a significant decrease in the level of expression of anti-apoptotic protein Bcl-2, which is shown to protect the cells from apoptosis induced by diverse agents[22, 23]. In addition, BioBran/MGN-3 treated cells, upon activation with anti-CD95 antibody, showed a significant decrease in mitochondrial membrane potential. This may play a critical role in the sensitization induced by BioBran/MGN-3, since Bcl-2 exerts its antiapoptotic effect by maintaining the integrity of mitochondria and preventing the release of proapoptotic molecules from its intermembrane space[22, 23].

The mechanism(s) by which BioBran/MGN-3 increases the activation of caspases and downregulates

Bcl-2 expression is not known. We have previously shown that BioBran/MGN-3 increases the production of tumor necrosis factor α (TNF- α) and interferon- γ (IFN- γ)[5]. In tumor cells, both TNF- α and IFN- γ induce or modulate cell death via activation of caspase[10, 11, 29, 30]. It is possible that BioBran/MGN-3 increases activation of caspases in HUT 78 cells via inducing the production of TNF- α and IFN- γ.

Defects in apoptosis pathways contribute to the resistance of tumor cells to chemotherapeutic agents, radiation, and to cellular immune responses (NK and cytotoxic T cell-mediated apoptosis). Currently, there is much interest in identifying natural products that can boost host cell-mediated immune responses against cancer, as well as agents that increase the susceptibility of cancer cells to undergo apoptosis. In this study, we have shown that BioBran/MGN-3, a BRM extracted from rice bran, sensitizes the leukemia cells to apoptosis mediated by the anti-CD95 antibody. We are currently investigating the sensitizing activities of BioBran/MGN-3 against a range of additional leukemic cell lines. The precise machanism(s) of action of BioBran/MGN-3 remains to be investigated. Nevertheless, our results suggest that BioBran/MGN-3 could be used to improve the treatment of certain human leukemias.

Acknowledgements

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Modified Arabinoxylan Rice Bran BioBran/MGN-3 Enhances Yeast-induced Apoptosis in Human Breast Cancer Cells *In Vitro*

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Abstract

We have recently reported that phagocytosis of killed *Saccharomyces cerevisiae*, baker's yeast, induced apoptosis in human breast cancer cell lines MCF-7 and ZR-75-1 and HCC70. In this study we have evaluated the effect of treatment with BioBran/MGN-3, a modified arabinoxylan from rice bran, on phagocytosis and yeast-induced apoptosis in breast cancer cells. Cancer cells were cultured with yeast at a ratio of 1: 10 in the absence or presence of BioBran/MGN-3, and the percentages of phagocytic and apoptotic cancer cells were examined by flow cytometry and by cytospin preparations. Cancer cells treated with BioBran /MGN-3 exhibited increased percentages of attachment (200%) and uptake of yeast (313%) by MCF-7 cells at 0.5 hr, as compared with cells without BioBran/MGN-3. In addition, treatment with BioBran/MGN-3 resulted in a 2 fold increase in the percentage of apoptotic MCF-7 cells. 2.5 fold for ZR-75 cells and 1.8 fold for HCC70 cells. BioBran/MGN-3 effect was dose-dependent and associated with increased activation of caspases 8 and 9 in MCF-7 cells, and caspases 8, 9 and 3 in HCC70 cells. This data demonstrates that BioBran/MGN-3 accelerates phagocytosis-induced apoptosis of cancer cells, which may represent a

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novel therapeutic strategy for the treatment of breast cancer.

Introduction

In the past 30 years researchers have learned a great deal about the diagnosis of cancer, however, its treatment still represents a serious obstacle. In the field of cancer therapeutics, medical oncologists have had to rely mainly on surgery, chemotherapy and radiation for cancer treatment. Both chemotherapy and radiation therapy are toxic, immune-suppressive, mutagenic and carcinogenic [1-4]. With respect to immunotherapy, many biological response modifiers (BRMs) designed to activate the host immune response have also been shown to produce severe side-effects [5]. The need for a new cancer therapy with minimal or no side-effects is greatly warranted. In the present study, we introduced a novel approach to breast cancer therapy using modified arabinoxylan rice bran (BioBran/MGN-3), to accelerate apoptosis in breast cancer cells (BCCs) post phagocytosis of yeast *in vitro*.

Induction of apoptosis post phagocytosis of microorganisms is a well established phenomenon in professional phagocytic cells. Phagocytosis of *Escherichia coli*, *C. albicans* and *Mycobacterium tuberculosis* induce apoptosis by neutrophils [6-8]. Other studies showed that phagocytosis of *Staphylococcus aureus* also induced apoptosis in monocytes [9]. Phagocytosis by different types of cancer cells has been reported; these include leukemia, fibrous histiocytoma, dermatofibroma, cervical cancer and lymphatic tumor cells. These cancer cells exhibit phagocytic activities against red blood cells, white blood cells, blood platelets, bacteria, and *Candida albicans* [10-19]. BCCs also exhibit phagocytic activity against erythrocytes [20], gelatin matrix [21,22], and yeast [23]. We hypothesized that tumor cells acquire phagocytic properties during the course of malignancy. Interestingly, we recently demonstrated that BCC lines underwent apoptosis post phagocytosis of heat-killed yeast [24]. Clearly, factors that accelerate apoptosis in cancer cells post ingestion of microorganisms are in great need of exploration.

BioBran/MGN-3 is an arabinoxylan from rice bran that has been modified by carbohydrate hydrolyzing enzymes from shiitake mushrooms [25]. We have previously reported that BioBran/MGN-3 enhances NK cell activity [26], increases the production of TNF-α by human peripheral blood lymphocytes [27], and sensitizes human leukemia cells to death receptor [CD95]-induced apoptosis [28]. In this study we tested the ability of BioBran/MGN-3 to accelerate apoptosis in BCCs after co-culture with heat-killed *S. cerevisiae*. Results show that BioBran/MGN-3 enhanced both phagocytosis of yeast by BCCs and subsequent apoptosis of cancer cells.

Materials and Methods

1. Cell culture media

Tumor cells were maintained in our laboratory in the following media: (DMEM) [4.5 mg glucose per m*l*, 10% fetal calf serum, and 1% antibiotics (100 U penicillin and 100 μg/m*l* streptomycin)] and complete medium (CM) [RPMI-1640 supplemented with 10% fetal calf serum and 1 % antibiotics (as above)].

2. Tumor cell lines

Three human breast cancer cell (BCC) lines were used in the present study. These cell lines were MCF-7, ZR-75-1 and HCC70. Human macrophage cell line (U973) was used as the control. All cell lines were

purchased from ATCC, Manassas VA, USA.

3. BioBran/MGN-3

BioBran/MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from Shiitake mushrooms and which contains polysaccharides (β-1, 3-glucan and activated hemicellulose). BioBran/MGN-3 was freshly prepared by dissolving in distilled H_2O at a concentration of 30 mg/L. BioBran/MGN-3 was provided by Daiwa Pharmaceutical Co., Ltd., Tokyo, Japan.

4. Preparation of *S. cerevisiae*

Commercially available Baker's and brewer's yeast, *S. cerevisiae*, was used. Yeast suspensions was washed once with phosphate-buffered saline (PBS) and was incubated for 1 hr at 90℃ to kill yeast. Following washing, yeast cells were quantified using a hemocytometer and cell suspensions were adjusted at 1×10^7 cells/m*l*.

5. Phagocytic assay

Phagocytosis was assessed by cytospin preparation and flow cytometry. Phagocytic assay using cytospin preparations was done as previously described with slight modifications [19,29]. In brief, yeast was mixed with tumor cells at a 10:1 ratio (yeast to tumor cell). For this purpose, a 0.5 m*l* tumor cell suspension in CM containing 1×10^6 cells/m*l* was mixed with 0.5 m*l* yeast suspension containing 1×10^7 organisms/m*l*. 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 5% CO_2. After 0.5 and 2hr incubation, the mixtures were thoroughly re-suspended to detach loosely attached yeast from tumor 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, stained with 4 % Giemsa for 15 min and were examined using oil immersion and a light microcroscope fitted with a 60× objective (Nikon, Tokyo, Japan).

Flow cytometric assay for phagocytosis: Tumor cells (1×10^6) were incubated in round-bottomed tubes with propidium iodide-labeled, heat-killed yeast at a cancer cell to yeast ratio of 1:10. After 1 hr incubation at 37 ℃, samples were analyzed by FACScan flow cytometer. To distinguish between cell-bound and internalized, fluorescent yeast, quenching was performed by the addition of trypan blue dye. Trypan blue quenches the fluorescence of cell-bound but not internalized yeast.

6. Assessment of attachment and uptake of yeast by tumor cells

Assessment of attachment of yeast by tumor cells was calculated as the percentage of 200 tumor cells that attached to one or more yeast, and the attachment index was calculated using the following formula:

Attachment index = % Tumor cells attaching to×number yeast / 100 cells: 1000

The assessment of uptake of yeast by tumor cells was calculated as the percentage of 200 tumor cells that ingested one or more yeast, and the phagocytic index was calculated using the following formula:

Phagocytic index = % Phagocytizing tumor cells × number yeast / 100 cells: 1000

7. Assessment of tumor cell survival and apoptosis

Flow cytometry analysis was used to examine the percentage of dead cancer cells. Cancer cells were cultured with yeast cells at a 1:10 ratio and the percentage of dead cancer cells was examined by the pro-

pidium iodide (PI) technique using flow cytometry. Briefly, cells (1×10^6/m*l*) were incubated with 50 μg /m*l* of PI for 25 minutes at room temperature. Cells were acquired by FACScan (Becton Dickinson, San Jose, CA, USA) and analyzed by CellQuest software.

Percentage of apoptotic cancer cells was also determined in cytospin preparations that were used for phagocytic assay. Apoptosis is morphologically defined by membrane blebbing and chromatin condensation [30,31]. These criteria were used to identify the apoptotic cancer cells.

To study the effect of BioBran/MGN-3 on apoptosis, tumor cells were incubated with or without BioBran /MGN-3 (500 μg/m*l*) in the presence or absence of yeast for 2 hr and apoptosis was determined as described above.

8. Intracellular activity of caspases 8, 9 and 3

The method is based on carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase positive (+) cells are distinguished from caspase negative (-) cells with aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with fluorescein-labeled FMK-peptide inhibitors (FAM-DEVD-FMK for caspase 3, FAM-LETD-FMK for caspase 8, FAM-LEHD-FMK for caspase 9, Intergen Company, NY). After 1 hr incubation, the cells were washed to remove unbound caspase, and cells that contained bound inhibitor were quantified using a FACScan flow cytometer.

Statistical Analyses

In order to compare the means of treatment 1 (yeast), treatment 2 (BioBran/MGN-3) and combination of treatments 1 and 2, we used the analysis of variance design.

Results

1. Effect of BioBran/MGN-3 on attachment of yeast to MCF-7 cells

Figures 1a & **b** represent data of 3 experiments. Tumor cells were co-cultured with yeast in the absence or presence of BioBran/MGN-3 and the percentage of attachment and attachment index were examined at 0.5 hr and 2 hr. At 0.5 hr, the percentage of MCF-7 cells exhibiting binding was 27%. Treatment of MCF-7 cells with BioBran/MGN-3 significantly increased the level of attachment (54%) representing a 2 fold increase. This proportion was inversed at 2 hr (**Figure 1a**). The attachment indices at 0.5 hr for MCF-7 cells cultured with yeast in the absence or presence of BioBran/MGN-3 were 4.2 and 15.1, respectively. This represents a 3.6-fold increase associated with BioBran/MGN-3 treatment. Again this proportion was inversed at 2 hr (**Figure 1b**). Changes in the level of attachment by BioBran/MGN-3 treatment are illustrated by cytospin preparations. At 10 minutes many MCF-7 cells attached to 1 or 2 yeast (**Figures 1c** & **d**) while BioBran/MGN-3 treated tumor cells are attached to an increased number of yeast (**Figures 1e** & **f**).

2. Effect of BioBran/MGN-3 on phagocytosis of yeast by MCF-7 cells

Results in **Figure 2** show that yeast was phagocytized by MCF-7 cells. This was confirmed by flow cytometric analysis which clearly distinguishes cell-bound and internalized yeast (**Figure 2a-c**). Cytospin

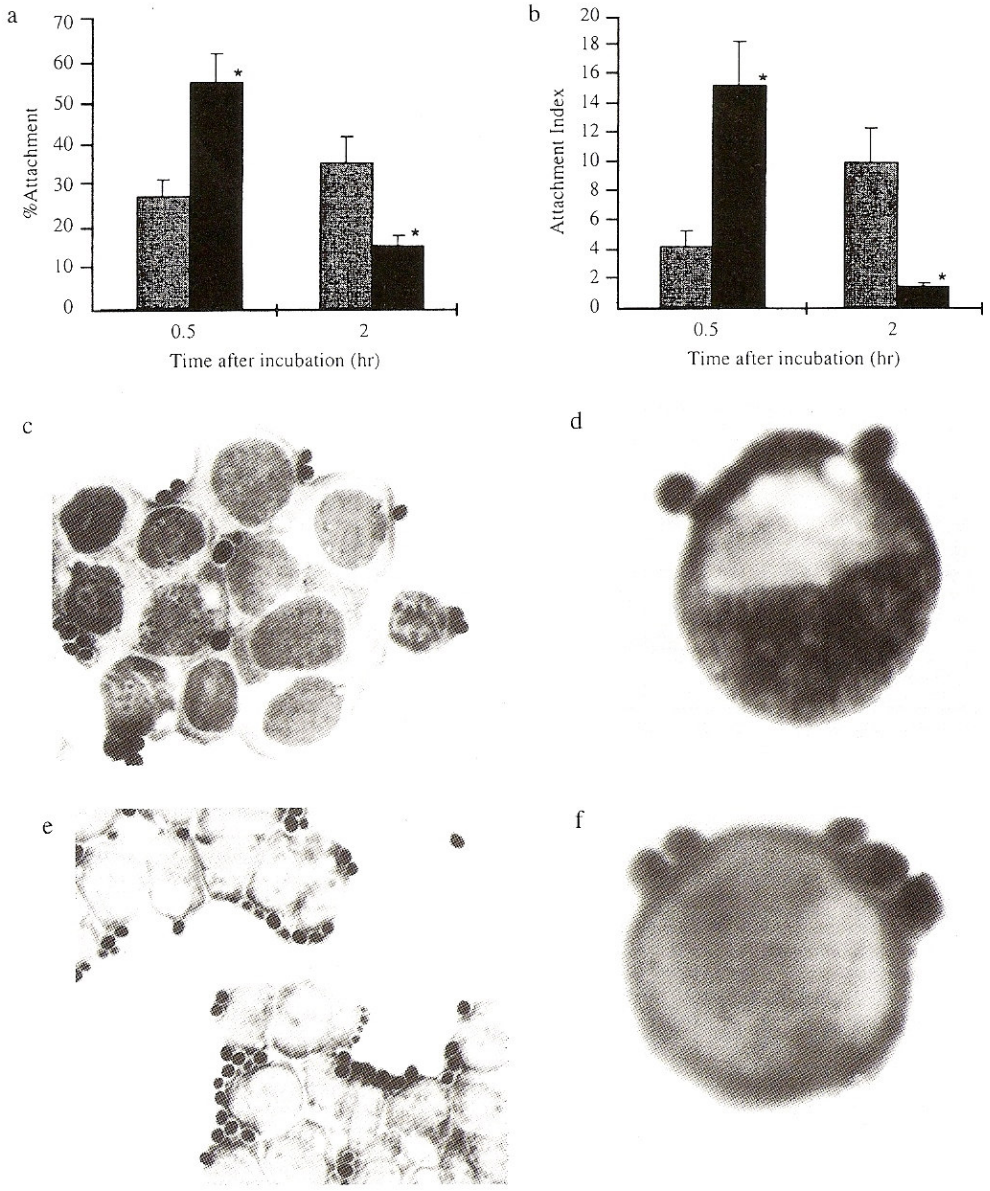

Fig.1 Action of MGN-3 on the attachment of MCF-7 cells to yeast and attachment index.

Tumor cells were cultured with yeast in a ratio of 1:10 in the presence (■) or absence of MGN-3 (▨).

The percentage (%) of attachment (a) and the attachment index (b) were determined at 0.5 and 2 hr.

Data represent the mean ±SD of 3 different experiments.

*$P < 0.001$ as compared to MCF-7 cells and yeast.

Figs.1c-f Giemsa stained cytospin preparations showing MCF-7 cells attached to one or two yeast, examined at 10 min. post culture of MCF-7 cells with yeast (c & d) and attached to several yeast at 10 min post culture of MCF-7 cells with yeast in the presence of MGN-3(e & f). c&e ×740. d&f ×1000.

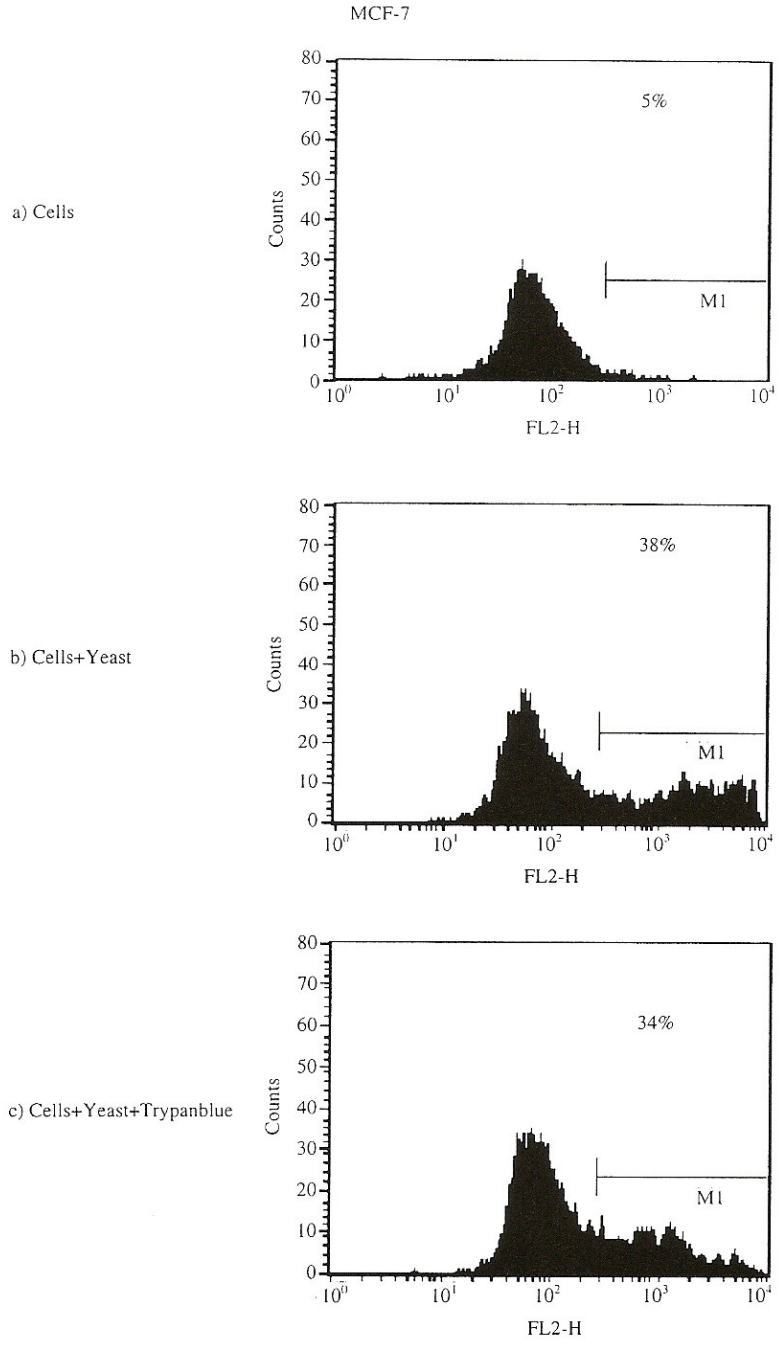

Fig. 2 Phagocytosis of yeast by MCF-7 cells.

a) Flow cytometric analysis which distinguishes cell-bound and internalized yeast.

Tumor cells were incubated with propidium iodide-labeled, heat-killed yeast.

Graph is a representative dot blot, showing the percent of MCF-7 cells alone, cells + yeast, and cells + yeast + trypan blue.

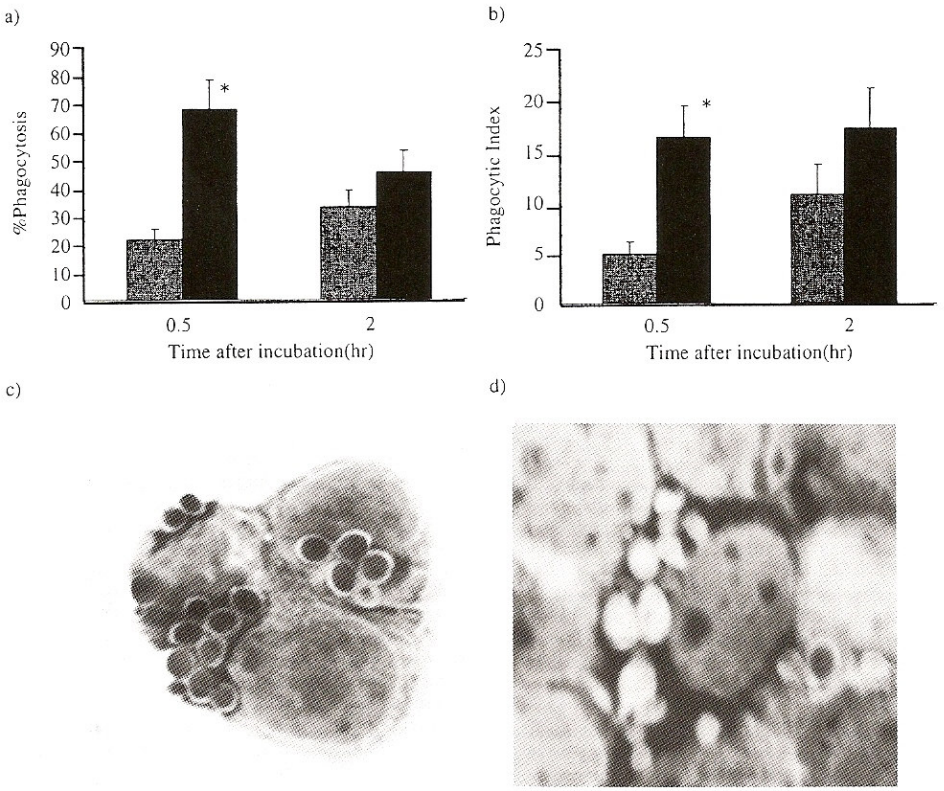

Fig. 3 Effect of MGN-3 on the percent of phagocytosis of yeast by MCF-7 cells and phagocytic index.

Tumor cells were incubated with yeast in a ratio of 1:10 in the presence (■) or absence of MGN-3 (▒).

The percentage (%) of phagocytic tumor cells (a) and the phagocytic index (b) were determined at 0.5 and 2 hr.

Data represent the mean ±SD of 3 different experiments.

*P < 0.01 as compared to MCF-7 cells and yeast.

Fig. 3c & d Cytospin preparations showing MCF-7 cells treated with MGN-3 phagocytizing several yeast examined at 0.5 hr (c).

Notice presence of several vacuoles of digested yeast at 1hr (d).

Giemsa ×1000.

preparations showed BioBran/MGN-3 treatment for 0.5 hr resulted in a significant increase in the percentage of phagocytosis of yeast by MCF-7 cells (72%), as compared to cells and yeast (23%). At 2 hr, the phagocytic activity of BioBran/MGN-3 treated MCF-7 cells had declined (**Figure 3a**). The phagocytic indices for MCF-7 cells and BioBran/MGN-3 treated MCF-7 cells were 5 and 17 respectively, representing a 3.4-fold increase by BioBran/MGN-3 treatment. At 2 hr, the phagocytic index of BioBran/MGN-3 treated MCF-7 cells was maintained, while that of MCF-7 cells and yeast rose to 11% (**Figure 3b**). Changes in the phagocytic activity of MCF-7 cells, post treatment with BioBran/MGN-3, are illustrated by cytospin preparations. At 0.5hr, MCF-7 cells showed an increased level of phagocytosis of yeast. Notice the presence of the vacuole surrounding the phagocytosed yeast; this indicates the yeast has been ingested by cancer cells (**Figure 3c**). Also note the presence of several vacuoles inside cancer cells (**Figure 3d**) indicating

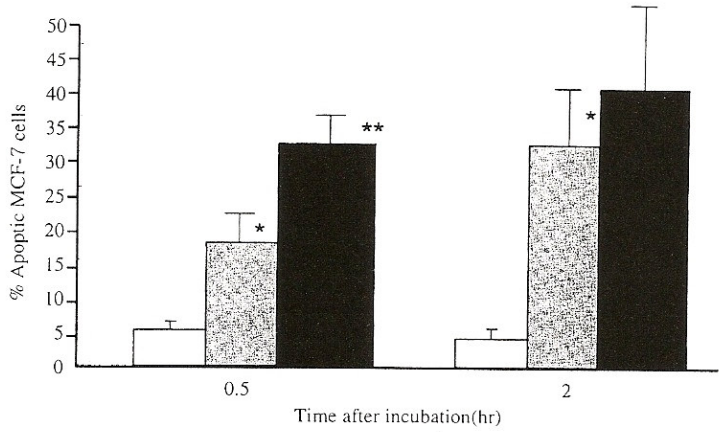

Fig. 4 Effect of MGN-3 on apoptosis of MCF-7 as defined in cytospin preparations.
Tumor cells were incubated with yeast at a ratio of 1:10 in the presence (■) or absence of MGN-3 (▒) and the percentage (%) of apoptic tumor cells was determined at 0.5 and 2 hr in cytospin preparation.
Apoptic MCF-7 cells alone (□) were also examined.
Data represent the mean ±SD of 3 different experiments.
*P < 0.05, as compared to control untreated cells. **P < 0.05, as compared to cells + yeast.

that the yeast has been digested.

3. Effect of BioBran/MGN-3 on apoptosis of MCF-7 cells

A. Measurement by cytospin preparations: The effect of BioBran/MGN-3 on phagocytosis-induced apoptosis of MCF-7 cells was measured in cytospin preparation. Tumor cells were cultured with yeast at a ratio of 1:10 in the presence or absence of BioBran/MGN-3. Results depicted in **Figure 4** show that the background apoptosis in MCF-7 cells was 5%. At 0.5 hr post phagocytosis, 18.7 % of MCF-7 cells underwent apoptosis. Treatment with BioBran/MGN-3 significantly increased the percentage of apoptosis of MCF-7 cells (32%). At 2 hr the percentages of apoptotic MCF-7 cells continued to rise post treatment with yeast alone (31%) and yeast in the presence of BioBran/MGN-3 (39.3%).

B. Morphological examination of apoptic MCF-7 cells: Giemsa-stained cytospin preparations show apoptotic BioBran/MGN-3-treated MCF-7 cells, post phagocytizing yeast. At 0.5 hr cancer cells illustrate membrane blebbing and early nuclear condensation (**Figure 5a** & **b**). Preparation also showed cells with severe chromatin condensation. **Figure 5** shows several apoptic cells (**Figure 5c**) and a separate cell (**Figure 5d**) with a very small nucleus, which was situated in an eccentric position. Finally, nuclear fragmentation occurs (notice red-stained fragments in **Figure 5e**) and these fragments subsequently disappear (**Figure 5f**). Apoptic MCF-7 cells acquire the trypan blue stain (**Figure 5g**). Notice yeast, stained blue, inside the apoptic MCF-7 cells.

C. Flow cytometry analysis: Survival of MCF-7 cells was further examined by flow cytometry. Results in **Figure 6** show that phagocytosis of yeast caused a significant decrease in MCF-7 cell survival (21.4% dead cells as compared to control 3.4%). BioBran/MGN-3 treatment further increased tumor cell demise (34.7%). BioBran/MGN-3 alone caused a substantial increase in cell death (10.9%) as compared to back-

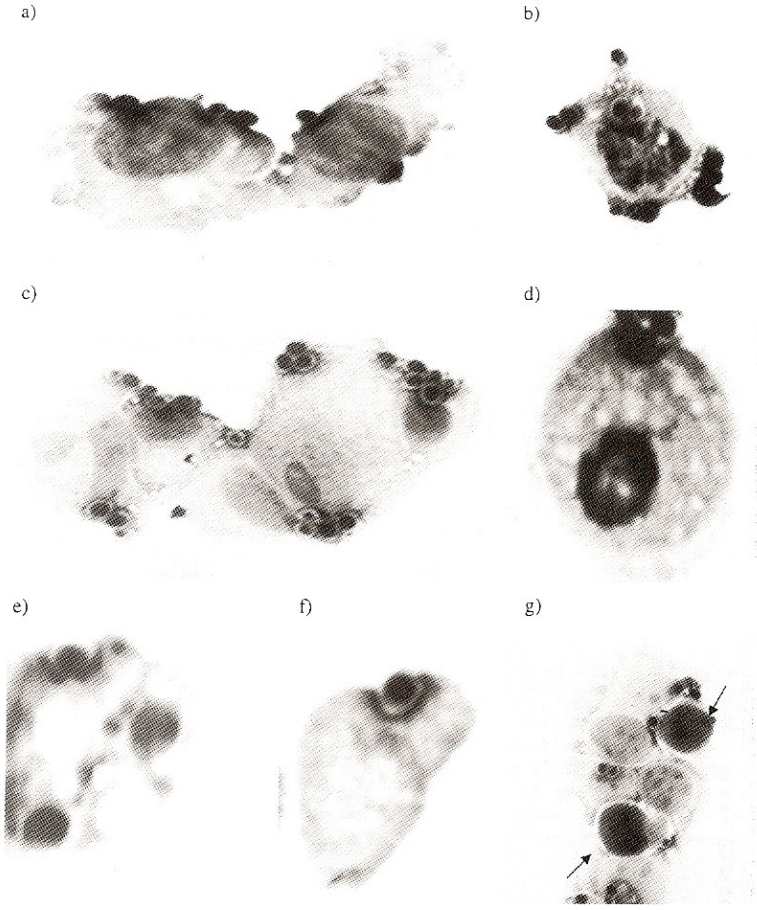

Fig. 5(a - g) Morphological examination of apoptotic MGN-3-treated MCF-7 cells using Giemsa-stained cytospin preparations.

Notice tumor cells exhibiting membrane blebbing and early nuclear condensation (a & b).
Preparation also showed cells with severe nuclear condensation which was located in an eccentric position (c & d).
Finally, nuclear fragmentation occurs (stained red) (e).
Nucleus then disappears (f).
Notice dead tumor cells stained with trypan blue (g).
Also, notice yeast (stained blue) has been ingested inside the tumor cells.
Figs. 5a, c&g ×400. b, d, e&f ×1000

ground of MCF-7 alone (3.4%). The effect of BioBran/MGN-3 on phagocytosis-induced cell death was found to be dose-dependent (**Figure 7**). BioBran/MGN-3 at 100 μg/m*l* showed an increase of MCF-7 apoptic cells (35.4%), as compared to MCF-7 cells and yeast. At 500 μg/m*l*, it further increased (40.1%), however, at 1000 μg/m*l*, it began to decline (33.04%).

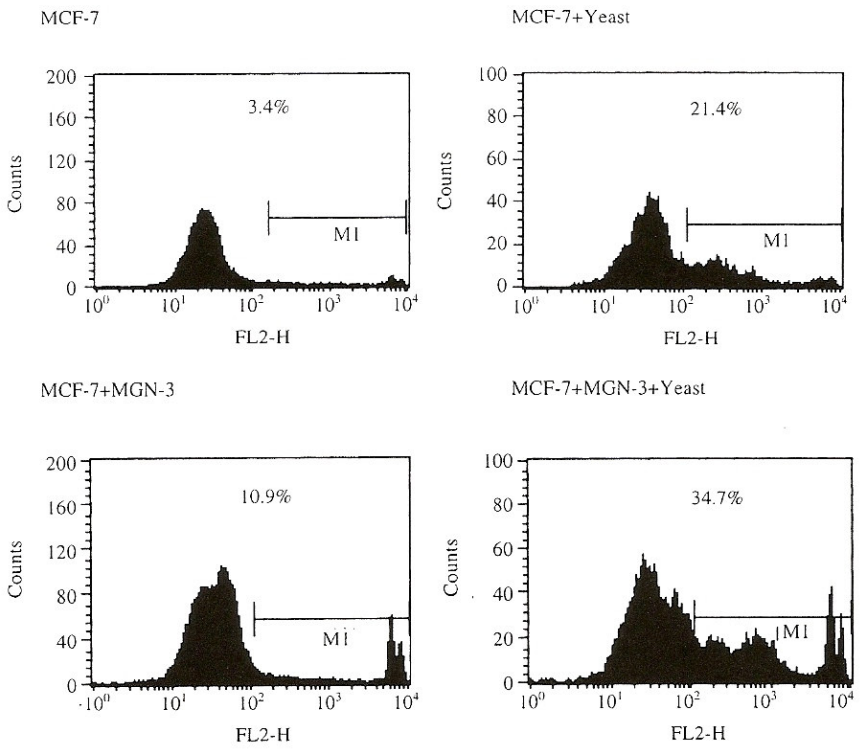

Fig. 6 Percent of dead MCF-7 cells as determined by flow cytometry.

MCF-7 cells were cultured with yeast at the ratio of 1:10 in the presence or absence of MGN-3 (500 μg/ml) for 2 hr, and cell survival was determined by flow cytometry using propidium iodide (PI) technique. In this technique dead cells pick up PI and florescence.
The number in the histgrams represents the percent of dead cells.

4. Effect of BioBran/MGN-3 on apoptosis of other BCC lines

We examined the accelerative effect of BioBran/MGN-3 on apoptosis of other BCCs post phagocytosis of yeast. Survival in ZR-75 and HCC70 cell lines, was examined at 0.5 hr post culture with yeast. Results in **Figure 8** demonstrated that phagocytosis of yeast resulted in increased percentages of dead cancer cells: ZR-75 (21%) and HCC70 cells (11%). BioBran/MGN-3 treatment caused a significant increase in the expression of apoptic ZR-75 cells (48%), while the effect was less noticeable in HCC70 cells (20%). BioBran/MGN-3 alone demonstrated an appreciable increase in percent apoptotic ZR-75 cells (11%) and HCC70 (10%), as compared to background of cancer cells alone (3.5-5%).

5. Activation of caspases

A. Effect of yeast phagocytosis: To determine whether the decreased survival of tumor cells is due to yeast-induced necrosis or apoptosis, activation of caspases was determined. MCF-7 and HCC70 cells were incubated with yeast for 2 hr and activation of caspases 8, 9 and 3 was determined by flow cytometry. **Figure 9** is a representative of histograms that illustrate the yeast induced activation of both caspase 8

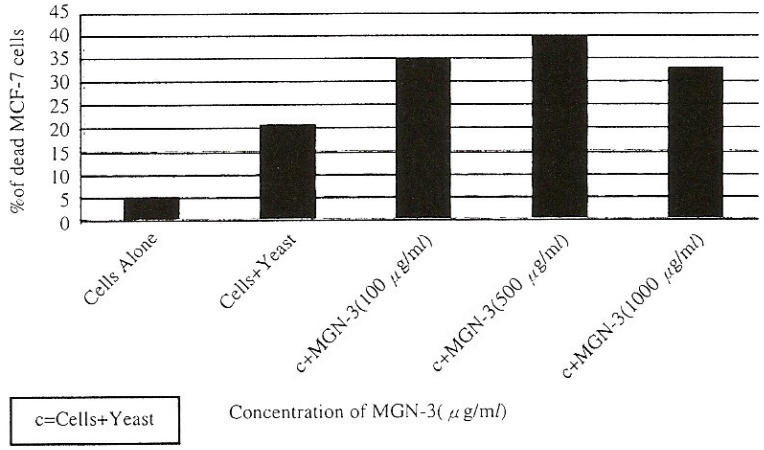

Fig. 7 Action of MGN-3 at different concentrations on apoptosis of MCF-7 cells.

MCF-7 cells were cultured with yeast at the ratio of 1:10 in the presence of MGN-3 (100-1000 μg/ml). Cancer cell survival was determined by flow cytometry using propidium iodide (PI) technique. In this technique dead cells pick up PI and florescence.

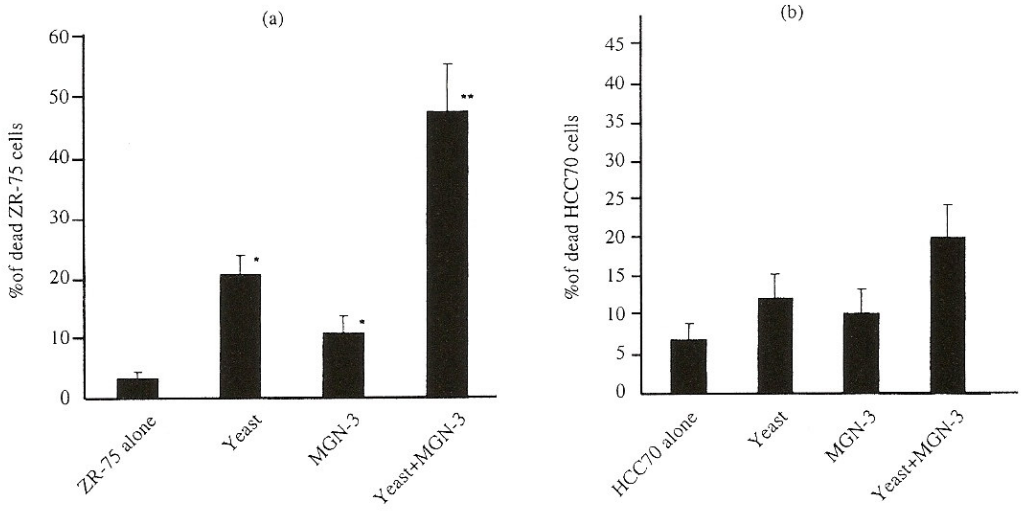

Fig. 8 Effect of MGN-3 on phagocytosis-induced apoptosis in ZR-75 and HCC70 cells as determined by flow cytometry.

Cancer cells were cultured with yeast at the ratio of 1:10 for 0.5 hr in the presence or absence of MGN-3 (500 μg/ml), and survival of ZR-75 cells (a) and HCC70 cells (b) was determined by flow cytometry using the propidium iodide (PI) technique. In this technique dead cells pick up PI and florescence.

*P < 0.05 as compared to control untreated cells. **P < 0.01 as compared to cells + yeast or cells + MGN-3.

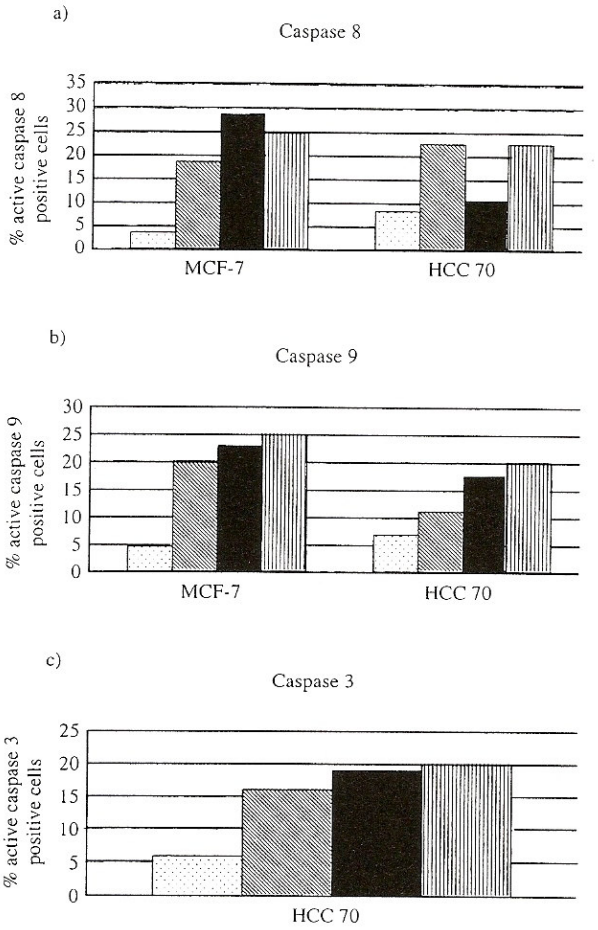

Fig. 9 Activation of caspases in MCF-7 and HCC70 cells post treatment with MGN-3.

Cancer cells were incubated with yeast in the ratio of 1:10 for 2 hr in the presence and absence of MGN-3 (500 μg/ml). Intracellular active caspase 8 (Fig. 9a), caspase 9 (Fig. 9b), and caspase 3 (Fig. 9c) were determined with casp glow caspases determination kit using FACScan.

Activation of caspases was examined in the following groups: cells alone (▨), cells + yeast (▩), cells + MGN-3 (■), and cells + yeast + MGN-3 (▥).

and caspase 9 in MCF-7 cells, suggesting that death of MCF-7 cells is due to apoptosis. A similar observation was made in HCC70 cells where an increase in caspase 8, 9, and 3 post culture of the cells with yeast was observed.

B. Effect of BioBran/MGN-3: In order to determine the steps in yeast-mediated apoptosis that are affected by BioBran/MGN-3, we examined the activation of caspase 8 and caspase 9. MCF-7 cells were co-cultured with yeast in the presence or absence of BioBran/MGN-3 for 2 hr. The proportion of cells with active caspases 8 and 9 was determined with a Caspatag caspase detection kit, using FACScan. The proportions of MCF-7 cells with active caspase 8 and caspase 9 were 3.5% and 4.9%, respectively. Phagocytosis of yeast

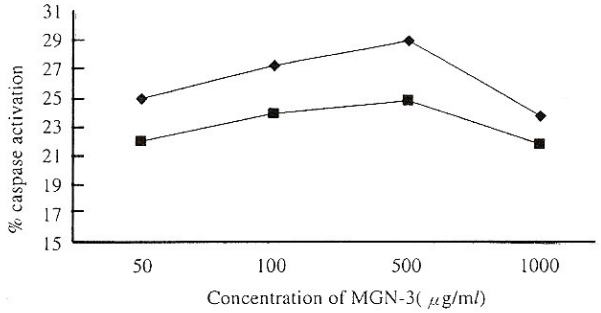

Fig. 10 Activation of caspases 8 and 9 of MCF-7 cells post treatment with MGN-3 at different concentrations.
MCF-7 cells were incubated with yeast in the ratio of 1:10 and treated with MGN-3 at different concentrations (50-1000 μg/ml). Intracellular active caspase 8 (◆) and caspase 9 (■) were determined with casp glow caspases 8&9 determination kit using FACScan.

by MCF-7 cells resulted in an increased proportion of cells exhibiting active caspase 8 (18.9%), and caspase 9 (20%). MGN-3 enhanced phagocytosis-induced activation of caspase 8&9. The action of BioBran /MGN-3 on induction and enhancement of apoptosis in HCC70 cells was also associated with activation of caspases (**Figure 9**). BioBran/MGN-3 has a negligible effect on the activation of caspase 8 in HCC70 cells, but it increased the proportion of cells with active caspase 9 and caspase 3.

C. Dose- dependant effect of BioBran/MGN-3: The effect of BioBran/MGN-3 on the level of caspases of MCF-7 cells was shown to be dose-dependent. The data demonstrates that the proportion of cells with increased active caspase 8 was higher in BioBran/MGN-3 (500 μg/ml) treated cells (29.2%), as compared to untreated control (18.9%). A similar trend of activation with caspase 9 was noted (**Figure10**).

Discussion

Results of this study reveal that modified arabinoxylan rice bran (BioBran/MGN-3) accelerates the level of phagocytosis of yeast by MCF-7 cells and significantly enhances apoptosis by MCF-7 cells. Several investigators reported phagocytosis by different types of cancer cells of nonphagocytic origin [10-19] including breast cancer cells (BCCs). These BCCs demonstrated their ability to phagocytize latex beads and fluorescent Matrigel [21,22]. We extended these studies and showed that MCF-7 cells are also able to phagocytize yeast [23], and that phagocytosis subsequently resulted in apoptosis in BCCs [24]. Several studies demonstrated that the expression of apoptosis in the human professional phagocytic cells can be modified in response to phagocytosis of microorganisms [6-9]. There is a great need to further study the factors that accelerate the expression on apoptosis in BCCs, post ingestion of microorganisms.

Recent studies demonstrate that several natural biological response modifiers (BRMs) induce apoptosis against a variety of cancer cell lines. These include: HL-60 cells by [6]-gingerol and [6]-paradol (ginger derivatives) [32], erythroleukemia, prostate, and BCC lines by *dl*-alpha-tocopherol (vitamin E) [33], histiocytic lymphoma U937, and prostate adenocarcinoma PC 3 lines by PC SPES (a Chinese herbal preparation) [34], cervical carcinoma cells by Coprinus disseminatus [35], and human endometrial adenocarcinoma cells by a lipoprotein fraction of rice bran [36]. Moreover, the growth of human BCCs (MCF-7 and MDA-231) has

been inhibited by ergosterol (extract of baker's yeast) [37].

BioBran/MGN-3 is a polysaccharide and its main chemical structure has been identified as an arabinoxylan with a xylose in its main chain and arabinose polymer in its side chain [25]. Earlier studies revealed that BioBran/MGN-3 enhances human NK cell activity *in vivo* [26], increases T and B cell proliferation [25], and increases TNF-α and IFN-γ production [27]. In addition, BioBran/MGN-3 is able to induce cancer cell apoptosis [28]. In this study, BioBran/MGN-3 demonstrated an additional anticancer characteristic that accelerates yeast phagocytosis-induced apoptosis in cancer cells. BioBran/MGN-3 increases the levels of attachment and phagocytosis as early as 0.5 hr post culture MCF-7 cells with yeast. The mechanism(s) by which BioBran/MGN-3 induces this effect is not fully understood, but it could be attributed to changes in cancer cell receptors that are involved in attachment/phagocytosis. Whether the receptors found in professional phagocytic cells such as mannose, Fc and C3 receptors [38] are the same ones that govern phagocytosis of yeast by cancer cells is under investigation.

BioBran/MGN-3 also accelerates the expression of apoptosis in BCCs post phagocytizing yeast. Giemsa-stained cytospin preparations show apoptotic BioBran/MGN-3-treated MCF-7 cells, post phagocytizing yeast. Cancer cells illustrate membrane blebbing and nuclear condensation. This is followed by nuclear fragmentation that subsequently disappears, and apoptic cells acquire the trypan blue stain. It was noted, in the three BCC lines used, that the percentage of apoptosis in cancer cells co-cultured with yeast in the presence of BioBran/MGN-3 was higher than that of either yeast or BioBran/MGN-3 treatment alone. This could be attributed to a synergistic apoptic effect of yeast alone in addition to the direct apoptic effect of BioBran/MGN-3. The mechanism by which BioBran/MGN-3 enhances apoptosis in BCCs after phagocytizing yeast may involve the FAS/FAS Ligand system. The apoptic effect of FasL post phagocytosis of microorganisms has been investigated recently. Baran et al [9] reported release of FasL from monocytes post phagocytosis of *Staphylococcus aureus*, and these FasL induce apoptosis of phagocytic monocytes and, to some extent, the bystander cells. We have recently investigated the effect of BioBran/MGN-3 on death receptor-induced apoptosis in the human leukemic HUT-78 cell line [28]. HUT-78 cells that were pre-treated with BioBran/MGN-3 and then with the agonistic antibody against death receptor (Fas, CD95) revealed enhanced anti-CD95 induced apoptosis.

Alternatively, BioBran/MGN-3 may exert its effect through activation of caspases. Data from the present study showed that BioBran/MGN-3 treatment of MCF-7 cells up regulates the activation of proximal caspases 8 and 9; this occurred in a dose-dependent manner that was maximized at 500 μg/m*l*. The mechanism by which BioBran/MGN-3 activates caspases may be due to its increased production of tumor necrosis factor a (TNF-α), and interferon-γ (IFN-γ) [27]. Both cytokines are known to induce tumor cell death, or modulate cell death via activation of caspases [39-41]. MCF-7 cells do not express caspase 3 [42]; this suggests that a caspase 3 independent pathway that causes the DNA fragmentation in MCF-7 cells may exist. In addition, studying the mitochondria membrane potential and Bcl level may be helpful in investigating the mechanism by which BioBran/MGN-3 causes apoptosis of MCF-7 cells.

Data in the present study reveal a differential response among BCC lines towards the augmentory effect of BioBran/MGN-3 on enhancing apoptosis in cancer cells, post culture with yeast. Metastatic BCCs, such as MCF-7 and ZR-75, are more responsive than HCC70. The reason for this phenomenon is not known, but it could be attributed to the difference in the mechanisms of apoptosis in these cell lines. BioBran /MGN-3 increased activation of caspases 8 and 9 in MCF-7 cells, a cell line that lacks caspase 3. On the other hand, HCC70 cells treated with BioBran/MGN-3 demonstrated an increase in caspases 3 and 9 but

not caspase 8. This suggests that treatment with BioBran/MGN-3 caused apoptosis in MCF-7 cells through both intrinsic and extrinsic pathways. On the other hand, apoptosis in HCC70 cells may occur only through mitochondrial activation. A similar apoptic pathway was noted in mouse macrophages upon ingestion and digestion of *E. coli*, activating caspases 3 and 9, but not caspase 8 [43].

The National Cancer Institute has frequently highlighted the critical need for cancer treatments with greater specificity for cancer cells and less toxicity for normal tissue; it strongly encourages the exploration of such therapies. In the present study, we introduced a novel approach to BCC therapy using *S. cerevisiae*, a heat-killed non-pathogenic yeast, and BioBran/MGN-3, a safe product made from arabinoxylan derived from rice bran. We believe that the results of the present study may have implications for the treatment of breast cancer.

Acknowledgements

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II-7-1

Effects of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) on Serum Lipids and Taste Preference in Streptozotocin-induced Diabetic Rats

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Key words:
Dietary Fiber, Serum Lipids, Taste preference, Diabetic Rat

Abstract

The present study was designed to determine whether or not the administreation of BioBran/MGN-3 could improve streptozotocin (STZ)-induced diabetes. Taste preferences were also compared in diabetic and control rats. Male Sprague-Dawley rats were divided into control and diabetic groups. A single STZ injection, 65 mg per kg body mass i.p., induced diabetes. Rats were given free access to commercial diet and water for 2 months and BioBran/MGN-3 (0.5 g per kg body mass) was administered daily by stomach tube. Two-bottle-choice preference tests between aqueous solutions of either 5 mM citric acid, 27 mM monosodium glutamate, 0.016 mM quinine, or 0.82 mM saccharin in deionized water were conducted in the experimental period. Blood was collected and serum levels of glucose, insulin, triglycerides, total cholesterol, HDL-cholesterol, urea nitrogen, total protein, albumin, and zinc were measured. Serum triglycerides and

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total cholesterol decreased with the administration of BioBran/MGN-3, although serum insulin and glucose remained low and high, respectively. Water intake was also reduced by the BioBran /MGN-3, which suggests that polyuria induced by STZ improved. Diabetic rats showed significant aversion to citric acid and quinine when compared with control rats. BioBran/MGN-3 can be useful as a dietary fiber supplement for the treatment of diabetes. In addition, high taste sensitivity for sourness and/or bitterness is a characteristic of STZ-induced diabetes.

Introduction

Changes in concentrations of plasma lipids including cholesterol are complications frequently observed in patients with diabetes mellitus[1,2] and certainly contributes to the development of vascular disease in these patients. Many studies have been performed using diabetic animal models such as streptozotocin (STZ)-induced diabetic rats to clarify the mechanism by which the diabetic state induces hypercholesterolemia, or to confirm successful treatment of this disease[3-6].

Beneficial actions of diets high in fiber on the amelioration of diabetic symptoms are well documented. For example, certain dietary fibers, especially soluble ones, lower plasma cholesterol and maintain blood glucose concentrations within a suitable range[7-9]. Mechanisms underlying these effects are not fully understood, but a delay of gastric emptying[10], interferences with intestinal absorption of cholesterol and glucose, and inhibition of digestive enzymes[11] are thought to be caused. Most of these studies were conducted with large amounts of fiber consumption, usually >20g/day. Since, it is thought to be difficult to achieve such intakes of fiber from foods alone, fiber supplements are needed[12]. There are also questions as to whether or not fiber plays a significant practical role[13].

On the other hand, disturbances of taste preference have been reported in diabetes, both in experimental animals[14-16] and in humans[17-19]. Threshold values for sweetness are higher in diabetic patients than in normal subjects. However, there is a paucity of data available concerning other tastes, such as bitterness, saltiness, or sourness, with diabetes. In addition, few studies have been reported in the past on taste impairment and diabetes[19].

Newly manufactured dietary fiber, BioBran/MGN-3, effectively enhances natural killer cell activity and has an immunotherapeutic effect in the treatment of cancer patients[20,21]. However, the improvement of diabetic symptoms following BioBran/MGN-3 supplementation has not been investigated. Accordingly, the present study was designed firstly to examine the therapeutic effect of BioBran/MGN-3 on diabetes and secondly to measure taste preference of diabetic rats.

Materials and Methods

1. Animals

Eight-week-old male Sprague Dawley rats were obtained from Japan SLC (Hamamatsu, Japan). Animals were acclimated for 1 week and housed in individual stainless steel wire mesh cages (21×24×20 cm) in a well-ventilated room at 22±1℃ with the relative humidity of 40 to 60% and a 12-hour light/dark cycle. Diabetes was induced by a single intraperitoneal injection of STZ (65 mg/kg) (Wako, Richmond, VA, USA), dissolved in 20mM citrate buffer pH 4.5. Non-Diabetic control rats were injected the buffer only. Rats were fed commercial stock diet (MF : Oriental Yeast Co., Ltd, Osaka, Japan) and water ad libitum

throughout the experimental period (60 days). Individual body mass was measured daily. Blood samples were collected by decapitation on Day 60, centrifuged at 600×g for 10 minutes, separated sera were protected from light and stored at -20℃ pending analysis. The experimental protocol was approved by the Animal Care and Use Committee of Kobe Women's University.

2. Dietary fiber supplement

BioBran/MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from *Basidiomycetes* mycelia. It is a hemicellulose that contains β-1,4 xylopyranose. MGN-3 is commercially known as BioBran (Daiwa Pharmaceutical Co., Ltd., Tokyo, Japan).

BioBran/MGN-3 mixed with 0.5% sodium alginate as a suspension stabilizer in order to prevent plugging of the syringe by the supplement, was administered (0.50 g/kg body mass) daily by stomach tube. Rats administered 0.5% sodium alginate alone were used as a vehicle control.

3. Preference tests

Two-bottle-choice preference tests were performed for 8 hours using an aqueous solution of either 5 mM citric acid (sour) on Days 49 and 59; 0.82 mM sodium saccharin (sweet) on Days 50 and 56; 0.016 mM quinine sulfate (bitter) on Days 51 and 55; or 27 mM monosodium glutamate (savory that is a taste quality represented typically by glutamates and 5'-nucleotides) on Days 53 and 58, and deionized water. The position of the flavored solutions in the cage was alternated after each measurement period. Taste preferences, expressed as percentages, were calculated according to the following formula:

$$\text{Preference (\%)} = \frac{\text{Vol. (m}l\text{) Test solution consumed}}{\text{Vol. (m}l\text{) Test solution + Vol. (m}l\text{) Water consumed}} \times 100$$

Presented data are means of two taste tests. The number of animals per group varied from 5 to 6 for each taste test. The same animals were used in all experiments.

4. Biochemical analysis

Serum samples were analyzed for glucose, insulin, triglycerides, total cholesterol, HDL-cholesterol, albumin, urea nitrogen, and zinc by commercial kits obtained from Wako Pure Chemicals (Code #271-31401, 305-11511, 276-69801, 272-64901, 274-67401, 274-24801, 279-36201 and 431-14901 respectively). Total serum protein concentration was measured using a colorimetric method, Coomassie protein assay reagent (Pierce, Rockford, Illinois, USA: Code #23200).

5. Data analysis

Data are expressed as mean±SEM. These data were analyzed statistically by one-way analysis of variance[22]. A probability of 0.05 or less indicated significant difference.

Results

Following injection with STZ, these animals displayed the expected symptoms of insulin-dependent diabetes mellitus, i.e. hyperglycemia of more than 30 mmol/L within 48 hours, which persisted throughout the 60 day study period, depression of body mass gain (**Table 1**), and polydipsia (**Table 2**). Insulin con-

Table 1 Body mass and serum biochemical values in MGN-3 fed non-diabetic and STZ-diabetic rats*

	Non-Diabetic		STZ-Diabetic	
	−MGN-3	+MGN-3	−MGN-3	+MGN-3
n	6	5	5	6
Body Mass (g)	496 ± 6^{a}	471 ± 7^{a}	228 ± 11^{b}	276 ± 29^{b}
Glucose (mmol/L)	8.31 ± 0.27^{b}	7.70 ± 0.21^{b}	34.00 ± 2.09^{a}	33.84 ± 2.13^{a}
Insulin (μ U/ml)	40.1 ± 4.8^{a}	37.6 ± 6.3^{a}	0.0 ± 0.0^{b}	2.1 ± 1.3^{b}
Triglycerides (mmol/L)	2.32 ± 0.19^{c}	1.98 ± 0.25^{c}	23.82 ± 6.15^{a}	11.74 ± 3.26^{b}
Total Cholesterol (mmol/L))	2.08 ± 0.09^{b}	1.94 ± 0.14^{b}	5.71 ± 1.52^{a}	3.11 ± 0.73^{b}
HDL-Cholesterol (mmol/L)	1.45 ± 0.09	1.25 ± 0.08	1.78 ± 0.30	1.74 ± 0.35
Urea Nitrogen (mmol/L))	11.1 ± 1.4^{b}	15.6 ± 1.7^{b}	33.2 ± 3.2^{a}	27.3 ± 3.2^{a}
Total Protein (g/dl)	6.43 ± 0.16^{a}	6.82 ± 0.23^{a}	4.45 ± 0.19^{b}	5.35 ± 0.17^{a}
Albumin (g/dl)	3.84 ± 0.03^{a}	3.74 ± 0.08^{a}	2.54 ± 0.06^{b}	2.64 ± 0.10^{b}
Zinc (μmol/L)	9.15 ± 0.35^{c}	9.79 ± 0.65^{bc}	14.74 ± 1.18^{a}	11.83 ± 1.22^{b}

*Values are mean±SEM.

In each row, values not sharing a common superscript letter are significantly different at $p\leqq0.05$.

centrations in serum of diabetic rats were significantly lower than that in non-diabetic controls. Administration of BioBran/MGN-3, however, did not improve the hyperglycemia and hypoinsulinemia induced by STZ.

As shown in **Table 1**, serum triglycerides and total cholesterol levels were significantly higher in STZ-diabetic rats than in non-diabetic control animals. HDL-cholesterol levels, however, were unaffected by STZ injection or BioBran/MGN-3 administration. In diabetic animals, BioBran/MGN-3 reduced the rise in serum triglycerides and total cholesterol levels. In addition, serum urea nitrogen increased significantly and total protein and albumin levels decreased in STZ-diabetic rats compared to non-diabetic rats. When BioBran/MGN-3 was administered to diabetic rats, total protein levels recovered to near that of non-diabetic animals, although urea nitrogen was unaffected. Serum zinc cencentration in STZ-diabetic rats were significantly higher than non-diabetic rats. Administration of BioBran/MGN-3 lowered serum zinc concentrations in STZ-diabetic rats. In non-diabetic animals, BioBran/MGN-3 administration had no effect on any of these parameters.

As shown in **Table 2**, total volume intake (intake of deionized water plus intake of flavored solution) for period of 8 hours increased significantly in the STZ-diabetic rats than in the non-diabetic rats. STZ-diabetic rats without BioBran/MGN-3 drank siginificantly more water than rats given BioBran/MGN-3. In the two-choice tests, all except non-diabetic control rats significantly preferred monosodium glutamate and all except non-diabetic given BioBran/MGN-3 rats significantly preferred saccharin. Whether BioBran /MGN-3 was administered or not, STZ-diabetic rats had a significantly decreased preference for citric acid and quinine solutions compared to non-diabetic rats.

Discussion

The present study was designed to investigate actions of BioBran/MGN-3 on serum biochemical param-

Table 2 Total volume intake (intake of deionized water + intake of flavored solution) for 8 hours and percent preference (intake of flavored solution / total volume intake) in MGN-3 fed non-diabetic and STZ-diabetic rats*

	Non-Diabetic		STZ-Diabetic	
	−MGN-3	+MGN-3	−MGN-3	+MGN-3
5mM Citric Acid Total Intake (ml/100gBM)	0.9±0.2^{c}	1.1±0.1^{c}	28.4±4.3^{a}	15.0±2.3^{b}
Preference (%)	36.8±7.4^{b}	44.0±6.2^{a}	8.9±2.8^{c}	12.4±2.9^{c}
27mM Monosodium Glutamate Total Intake (ml/100gBM)	1.2±0.2^{c}	1.3±0.1^{c}	32.7±4.8^{a}	19.3±2.9^{b}
Preference (%)	40.3±6.7^{b}	67.1±6.0^{a}	68.0±7.3^{a}	69.3±9.5^{a}
0.016mM Quinine Sulfate Total Intake (ml/100gBM)	0.8±0.1^{c}	0.7±0.1^{c}	25.4±3.9^{a}	14.6±2.0^{b}
Preference (%)	25.2±6.8^{b}	35.4±9.7^{a}	17.9±9.0^{c}	10.1±3.4^{d}
0.82nM Sodium Saccharin Total Intake (ml/100gBM)	1.0±0.2^{c}	1.4±0.2^{c}	27.6±4.6^{a}	14.0±1.8^{b}
Preference (%)	62.1±5.7^{a}	50.4±7.5^{b}	62.6±8.4^{a}	60.0±10.6^{a}

*Values are mean±SEM for 5-6 rats per group.
In each row, values not sharing a common superscript letter are significantly different at $p \leqq 0.05$.

eters and taste preference in STZ-induced diabetic rats and normal non-diabetic rats. A couple of days after a single injection of STZ rats showed clear signs of diabetic symptoms. Water intake increased markedly, rats failed to gain body mass, and blood glucose levels reached values of more than 30 mmol/L. These factors remained fairly constant for 60 days of subsequent testing.

Serum lipids in STZ-induced diabetic rats, especially, triglycerides and total cholesterol, were elevated significantly, together with significantly elevated serum glucose and decreased insulin concentrations. However, the administration of BioBran/MGN-3 markedly lowered triglycerides and total cholesterol concentrations. Although these results are consistent with previous reports[23], there is considerable difference in the consumption of dietary fiber. The fiber level we used was only 0.5 g/kg/day and this amounts to approximately one-fifth to one-tenth of the usual supplement. Therefore, differing mechanisms other than the delay of gastric emptying[10] and/or interferences with intestinal absorption of cholesterol and inhibition of digestive enzymes[11] may account for improvements in serum lipids profile in diabetic rats, although the machanism by which BioBran/MGN-3 administration lowered serum triglycerides and total cholesterol was not specifically addressed in this study.

Serum glucose and insulin levels in STZ-induced diabetic rats remained as they were whether BioBran /MGN-3 was given or not. This means that the destruction of pancreatic β-cells by STZ in the current study was severe. The effect of destruction of fewer pancreatic β-cells remains to be examined.

Diabetes mellitus is characterized by derangement in metabolism not only of glucose and fat but also of protein[24]. However, protein has always received less attention than fat and glucose, both for alterations in its metabolism and in its nutritional implications. The present study examined the influence of diabetes on

urea nitrogen, total protein, and albumin in serum. Results show that there were significant reductions in concentrations of total proteins and albumin and an elevation in the concentration of urea nitrogen. Significant improvement in concentration of total protein was observed by supplementation with BioBran /MGN-3, while concentrations of urea nitrogen and albumin remained unaffected. Gougeon et al.[24] stated that protein intake as well as carbohydrate intake should be restricted in diabetes to normalize protein metabolism, which may normalize glycemia, and prevent typical symptoms of diabetic renal disease, albuminuria[25]. It is possible that treatment with BioBran/MGN-3, instead of protein restriction, can be used to improve protein metabolism.

A number of investigators have reported changes in consumption of various flavored substances in diabetes. Most of these studies were conducted with sweet compounds, and found a reduction in the preference for sweet tasting compounds by the diabetic[16, 17, 19]. Preference tests performed in this experiment indicate that the STZ-induced diabetic condition alters sensitivity to sour and bitter among various tastes. To the best of our knowledge, this is the only study in which preference for acid and bitter compounds have been elevated in diabetic rats. Although rats in the present experiment did not avoid sweet, mean preference scores were not different compared to the non-diabetic control. This is possibly due to elevated water intake, 27.6 m*l*/100 g BM/8 hours (**Table 2**).

Mechanisms underlying changes in consumption patterns for various tastes in diabetes are unknown, but several possibilities such as changes in salivary composition or flow rate need to be considered. It is also possible that the alteration of zinc concentration in blood could have some effect on taste acuity due to a decrease in taste receptors (**Table 1**), since the importance of zinc on taste acuity is well recognized in humans[26] and animals[27, 28].

These data clearly show that the administration of BioBran/MGN-3 reduced water intake and the rise in serum triglycerides, total cholesterol, and markedly elevated serum total protein in diabetic rats. The precise mechanism by which BioBran/MGN-3 improves lipid and protein metabolism in diabetic rats is unclear. It may be possible that BioBran/MGN-3 improves host response to resist diabetes, considering BioBran/MGN-3 is known to enhance natural killer cell activity[20, 21].

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Modified Arabinoxylan from Rice Bran (BioBran/MGN-3) Improves Glucose Tolerance in NIDDM Adult Rats Given Streptozotocin as Neonates

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Key words:
Modified Rice Bran, Glucose Tolerance, Diabetic Rat

Abstract

The effect of BioBran/MGN-3 on glucose tolerance was studied in adult non-insulin dependent diabetes mellitus (NIDDM) rats that had been caused by intraperitoneal injection of 100 mg per kg streptozotocin (STZ) at 1.5 days of age.

Following weaning, the animals were divided into 3 groups, 5 control rats fed a 1.7% cellulose diet, 7 diabetic rats fed a 1.7% cellulose diet and 8 diabetic rats fed a 1% BioBran/MGN-3 (+0.7% cellulose) diet. Rats had free access to one of experimental diets and water for 60 days. Oral glucose tolerance tests were performed at 8 weeks of age. Trunk blood was collected and plasma levels of insulin, glucose, triglycerides, total cholesterol, HDL-cholesterol, total protein, albumin and urea nitrogen were measured.

Plasma glucose levels in diabetic rats fed the 1.7% cellulose diet increased significantly faster and to

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higher levels than those of the normal control in an oral glucose tolerance test. In the NIDDM rats fed the BioBran/MGN-3 diets, the rapid rise of plasma glucose levels was depressed. Among the various other biochemical measurements only the plasma total cholesterol levels were significantly reduced by the administration of BioBran/MGN-3.

BioBran/MGN-3 holds promise as a dietary fiber supplement for the treatment of diabetes.

Introduction

Dietary fiber, based on its physicochemical nature, is known to have preventive actions in various disorders (Tsuji 1988). For example, there have been many reports on the curative and preventive effects of dietary fiber on diabetes mellitus (Anderson et al. 1991; Goswamy and Mani 1985; Morgan et al. 1990). Water-soluble dietary fiber lowers plasma cholesterol and maintains blood glucose concentrations within a suitable range (Guévin et al. 1996; Yuan et al. 1998; Groop et al. 1993; Sugano et al. 1988; Aoe et al. 1988). Mechanisms underlying these effects are not fully understood. The delay of gastric emptying (Leclère et al. 1994) and the slowdown of digestion and absorption of nutrients, associated with a modification in the viscosity of gastric and intestinal lumen (Schneeman 1982), and inhibition of the secretion of gut hormones (Morgan 1979) are thought to be important. Theses studies were mostly conducted using large amounts of fiber consumption, usually >20g/day. Therefore some authors consider it to be difficult to achieve such intakes of fiber from foods alone and fiber supplements are needed (Horton and Napoli 1996). There are also questions as to whether or not fiber plays a significant role (Nutall 1993).

On the other hand, newly manufactured dietary fiber from rice bran, BioBran/MGN-3, is used for general health promotion benefits, as a natural food supplement. This is also recognized to have an immunotherapeutic effect in the treatment of cancer patients (Ghoneum 1998; Ghoneum 1998).

Our previous study demonstrated that the administration of BioBran/MGN-3 improves streptozotocin (STZ)-induced diabetes (Ohara et al. 2000). Serum levels of triglycerides and total cholesterol were reduced by the administration of BioBran/MGN-3. Water intake was also reduced by the BioBran/MGN-3, which suggests that polyurea induced by STZ improved.

The present study was designed to examine the effect of BioBran/MGN-3 on glucose tolerance in the adult rat model of NIDDM as a result of neonatal administration of STZ.

Materials and Methods

1. Animals and Diets

Pregnant albino Wistar rats were obtained from Japan SLC (Hamamatsu, Japan). Animals bred in our colony were fed ad libitum with a commercial stock diet (MF; Oriental Yeast Co. Ltd., Osaka, Japan).

Male rats were made diabetic by a single intraperitoneal injection of 100 mg/kg STZ (Wako, Richmond, VA) (Blondel et al. 1989; Weir et al. 1981; Malaisse et al. 1991), dissolved in 20 mM citrate buffer pH 4.5 at 1.5 days of age. Non-diabetic control rats were injected with the buffer only. Animals were weaned on day 28, housed in individual stainless steel wire mesh cages (21×24×20 cm) in a well-ventilated room at 22±1℃ with the relative humidity at 40 to 60% and a 12-hour light/dark cycle. At eight weeks following weaning, diabetic rats were divided into 2 groups, those fed a 1.7% cellulose diet and those fed a 1% BioBran/MGN-3 diet (+0.7% cellulose) (**Table 1**). Non-diabetic control rats were also fed the 1.7% cellu-

Table 1 Composition of experimental diets

Ingredient	Cellulose diet (g/kg)	MGN-3
PEP*	80	0
α-Cornstarch	815	815
Mineral mixture**	35	35
Corn oil	50	50
Vitamin mixture***	10	10
Cellulose powder	10	—
MGN-3	—	10
Total dietary fiber	17	17

*PEP=Purified egg protein. (Taiyo Kagaku Co. Ltd., Mie, Japan)

**g/kg mixture; $CaHPO_4 \cdot H_2O$, 145.6; KH_2PO_4, 257.2; NaH_2PO_4, 93.5; NaCl, 46.6; Ca-lactate, 350.9; Fe-citrate, 318; $MgSO_4$, 71.7; $ZnCO_3$, 1.1; $MnSO_4 \cdot 5H_2O$, 0.3; KI, 0.1. (Oriental Yeast Co. Ltd., Osaka, Japan)

***g/kg mixture; all-rac-α-tocopheryl acetate, 5.0; cholecalciferol, 0.0025; menadione, 5.2; retinol acetate, 1.0; ascorbic acid, 30.0; calcium pantothenate, 5.0; choline chloride, 200.0, cyanocobalalmin, 0.0005; D-biotin, 0.02; folic acid, 0.2; inositol, 6.0; nicotinic acid, 6.0; pyridoxine hydrohloride, 0.8; p-aminobenzoic acid, 5.0; riboflavin, 4.0; thiamin hydrochloride, 1.2; Sufficient cellulose powder was added to make up 1 kg. (Oriental Yeast Co. Ltd., Osaka, Japan)

lose diet. Individual body weight and diet and water intakes were measured daily. The food and water intakes were measured by taking the weight of the day before from the next day. Animals were fed experimental diets and water ad libitum throughout the experimental period (60 days).

2. Dietary Fiber Supplement

BioBran/MGN-3 is a water soluble hemicellulose B that is enzymatically treated by carbohydrases complex, including α·β-glucosidase, α·β-galactosidase and β-1,4-glucosidase, from forest mushroom, *Lentinus edodes*. The main ingredient is an arabinoxylan; which specifies saccharides by anthronesulfuric acid method; 55-56%, protein by Lowry method; 10-20%, crude ash; 6-12% and moisture; less than 7%. MGN-3 is commercially known as BioBran (Daiwa Pharmaceutical Co. Ltd., Tokyo Japan).

3. Oral Glucose Tolerance Tests

Oral glucose tolerance tests were performed on day 58 after 20 hours of fasting. Blood was withdrawn from the tail vein at 0, 30, 60 and 120 minutes following the administration of 2g/kg glucose by stomach tube. Blood samples were immediately centrifuged at 4℃, and plasma was stored at -20℃ pending the analysis of glucose concentrations.

4. Plasma Biochemical Analysis

As anesthetics have an action of elevating blood glucose levels (unpublished observation), especially ether, collection of blood should be done by without anesthesia. Then the blood was collected by decapitation on day 60 after 20 hours of fasting. Blood was centrifuged at 600 ×g for 10 minutes. The separated plasma was protected from light and stored at -20℃ pending analysis.

Table 2 Body weight, food intake, feed efficiency and water intake in non-diabetic control rats fed the experimental 1% cellulose, diabetic rats fed 1.7% cellulose and diabetic fed 1% MGN-3 (+0.7% cellulose) diets for 60 days

Dietary group	n	Body weight (g)			Cumulative food intake (g)	Feed efficiency (%)	Cumulative water intake (g)
		Initial	Final	Body weight gain			
Non-diabetic control rats fed 1.7% cellulose	5	218±8[a]	337±12[a]	130±3	1143±30	10.9±0.3	1051±32[a]
Diabetic rats fed 1.7% cellulose	7	182±5[b]	297±10[b]	115±6	1085±19	10.6±0.4	924±34[b]
Diabetic rats fed 1% MGN-3(+0.7% cellulose)	8	180±9[b]	293±14[b]	113±14	1066±31	10.6±0.4	935±31[b]

Values are mean±SEM for 5-8 rats.
In each column, values not sharing a common superscript letter are significantly different at $p<0.05$.
Feed efficiency is body weight gain divided by food intake.

Plasma samples were analyzed for glucose (Trinder 1969), insulin (Mukoujima 1979), triglycerides (Spayd et al. 1978), total cholesterol (Allain et al. 1974), HDL-cholesterol (Ash and Hentschel 1978), albumin (Doumas et al. 1971), and urea nitrogen (Kanai and Kanai 1983) by commercial kits (Wako Pure Chemicals Co. Ltd., Osaka, Japan). Total protein concentration was measured using a color-imetric method, Coomassie protein assay (Pierce, Rockford, IL).

5. Data Analysis

Data are expressed as mean±SEM. All data were tested statistically using one-way analysis of variance (ANOVA) followed by Scheffé *F* test of multiple comparisons between pairs of means as appropriate at a significance level of 5%.

Results

1. Body Weight, Food Intake, Feed Efficiency and Water Consumption in Rats Fed Experimental Diets for 60 Days

Although the NIDDM rats showed the retardation of growth, body weight gain during the experimental period was not statistically different between groups (**Table 2**). Also, food intakes and feed efficiencies were not significantly different. Water consumption in the rats fed control diet was significantly more than that of diabetic rats, but these were within normal range.

2. Oral Glucose Tolerance Test

At any time following the administration of glucose, plasma glucose levels in the NIDDM rats were significantly higher than those in the non-diabetic controls (**Fig. 1**). In the NIDDM rats fed BioBran/MGN-3 diets, the significant and rapid rise of plasma glucose levels due to STZ was attenuated at 30 min.

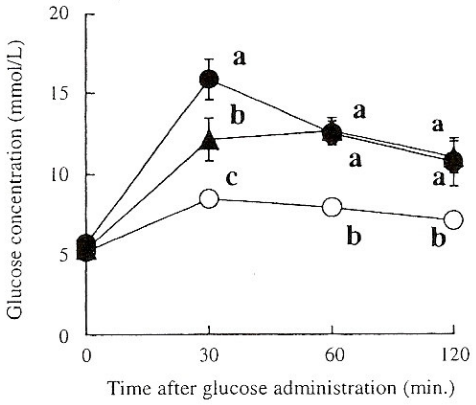

Fig.1 Glucose tolerance responses to glucose (2g/kg stomach tube) in non-diabetic male Wistar rats fed 1% cellulose diet (open circle), diabetic rats fed 1% cellulose diet (closed circle) and diabetic rats fed 1% MGN-3 (+0.7% cellulose) diet (triangle), measured on day 58 after 20 hours of starvation.

Each plotted point represents the mean for 5-8 rats and the vertical bars denote ±SEM.

At each time, values not sharing a common superscript letter are significantly different at $p<0.05$.

Table 3 Plasma biochemical values in fasting non-diabetic control rats fed the experimental 1.7% cellulose, diabetic rats fed 1.7% cellulose and diabetic rats fed 1% MGN-3 (+0.7% cellulose) diets for 60 days

	n	Glucose (mmol/L)	Insulin (μU/m*l*)	Triglycerides (mmol/L)	Total cholesterol (mmol/L)	HDL-cholesterol (mmol/L)	Urea nitrogen (mmol/L)	Total protein (g/d*l*)	Albumin (g/d*l*)
Non-diabetic control rats fed 1.7% cellulose	5	6.91±0.25	10.99±3.21	1.80±0.17	1.87±0.08^{b}	1.40±0.11	9.9±0.7^{b}	5.67±0.08	4.00±0.11
Diabetic rats fed 1.7% cellulose	7	7.81±0.4	9.60±2.2	1.89±0.23	2.24±0.14^{a}	1.70±0.14	13.3±1.3^{a}	5.71±0.1	4.09±0.03
Diabetic rats fed 1% MGN-3 (+0.7%cellulose)	8	7.10±0.7	15.82±1.8	2.10±0.15	1.97±0.11^{b}	1.56±0.10	13.4±0.9^{a}	5.61±0.1	4.14±0.06

Values are mean±SEM for 5-8 rats per group.

In each column, values not sharing a common superscript letter are significantly different at $P<0.05$.

3. Plasma Biochemical Analysis

Insulin concentration in the plasma of cellulose-fed diabetic rats were lower than those in non-diabetic controls, but these tended to be higher with administration of BioBran/MGN-3 although these values were not significantly different (**Table 3**). Plasma glucose levels in the fasting state were not different between groups. Among other biochemical measurements, total cholesterol and urea nitrogen were significantly higher in the NIDDM rats than those in the non-diabetic controls. The increase in total cholesterol levels

was significantly attenuated by the administration of BioBran/MGN-3.

Discussion

β-Cells of the pancreas in the animals injected STZ were destroyed selectively, which led to insulin-deficient state (Steiner 1970; Hoftiezer and Carpenter 1973). As the β-cells have an ability to regenerate when the animals are 1 to 2 days after birth, severe insulin deficiency may not occur following injection of STZ to neonatal animals. In the case of rats, insulin content of the pancreas recovers to 50% of the control level within 2 weeks (Cantenys et al. 1981; Aratan-Spire et al. 1984). Plasma insulin concentration is normal after weaning and glucose concentration increases a little, but responses to glucose tolerance worsen significantly. The animals are known to resemble to human NIDDM. The results of the present study indicate that the rats had less severe NIDDM (**Table 2**). We infer that their pancreatic β-cells continued partial function. Although growth rates in the diabetic rats were significantly retarded, plasma glucose and insulin concentrations were close to normal. Water consumption was also normal, although normal rats drank significantly more water than the diabetic rats.

The results of this study indicate that BioBran/MGN-3 can modify the response to a glucose tolerance test in mildly NIDDM rats (**Fig. 1**). Many researchers have observed an improvement of postprandial glycemic response after incremental amounts of non-purified dietary fiber in NIDDM subjects (Guévin et al. 1996; Del Toma et al. 1988)

Generally, the main mechanisms cited to explain the effects of dietary fiber on the digestion and absorption of glucose are associated with a modification in the viscosity of gastric and intestinal lumen. The more that viscous dietary fiber exists in the gastric and intestinal lumen, the less effective is digestion and absorption. As a result, the reduction of postprandial blood glucose response would be expected.

Other aspects that could modify the viscosity of dietary fiber, such as soluble-in-soluble ratio and the total quantity of fiber (Guévin et al. 1996) have been reported.

BioBran/MGN-3 used in this study, is previously hydrolyzed by mushroom hydrases and the particle sizes become smaller than unpurified rice bran. Then, there is a possibility that portions of low molecular weight in this dietary fiber can be transported through the enterocyte brush border, and that these work directly on the pancreas and/or the receptor site of insulin action. Moreover, the improvement of BioBran /MGN-3 on diabetes observed by the addition of these amounts to approximately one-fifth or one-tenth the weight of other usual supplements. Therefore, differing mechanisms than the modification in the viscosity of gastric and intestinal lumen may account for improvements in glucose tolerance tests in diabetic rats.

Total cholesterol in the plasma of the diabetic rats in the present study clearly increased (**Table 3**). Plasma triglycerides and HDL-cholesterol concentrations in the diabetic rats also tended to increase, but was not significant. The addition of BioBran/MGN-3 attenuated the total-cholesterol increase in the plasma of the diabetic rats. Generally, a cholesterol lowering effect of dietary fiber is well known (Sugano et al. 1988; Demigné et al. 1998; Aoe et al. 1988), and has been believed to occur by inhibiting micellation of cholesterol, because soluble dietary fiber binds to bile acid and/or modifies enterohepatic circulation of bile acid. These studies are usually conducted with a diet containing over 5% dietary fiber (Sugano et al. 1988; Nagata et al. 1995). On the contrary, we added only 1% BioBran/MGN-3 to the diet. Therefore the mechanisms for reducing cholesterol levels in the plasma of the present study are thought to be quite dif-

ferent from other studies.

Rice bran is a byproduct of rice polishment and is usually removed from food because of its unpleasant taste. They are widely believed to be useless, although part of them is used for fertilizer and feeds for animals. However, rice bran contains many functional materials. The present study demonstrated that one of these functions is hypoglycemic activity.

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