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.

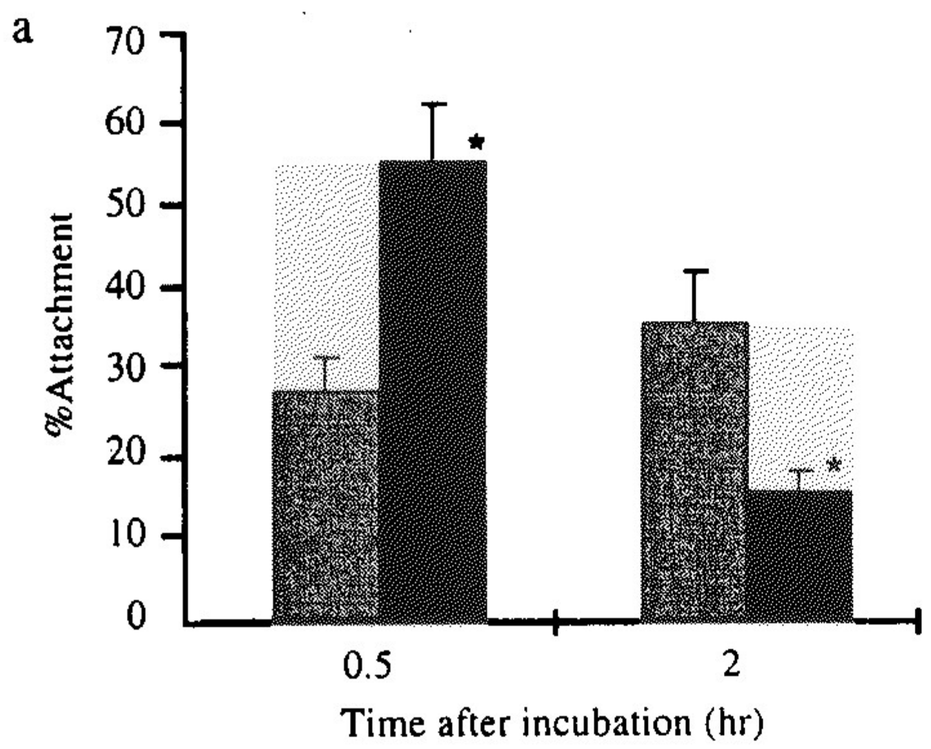
Results

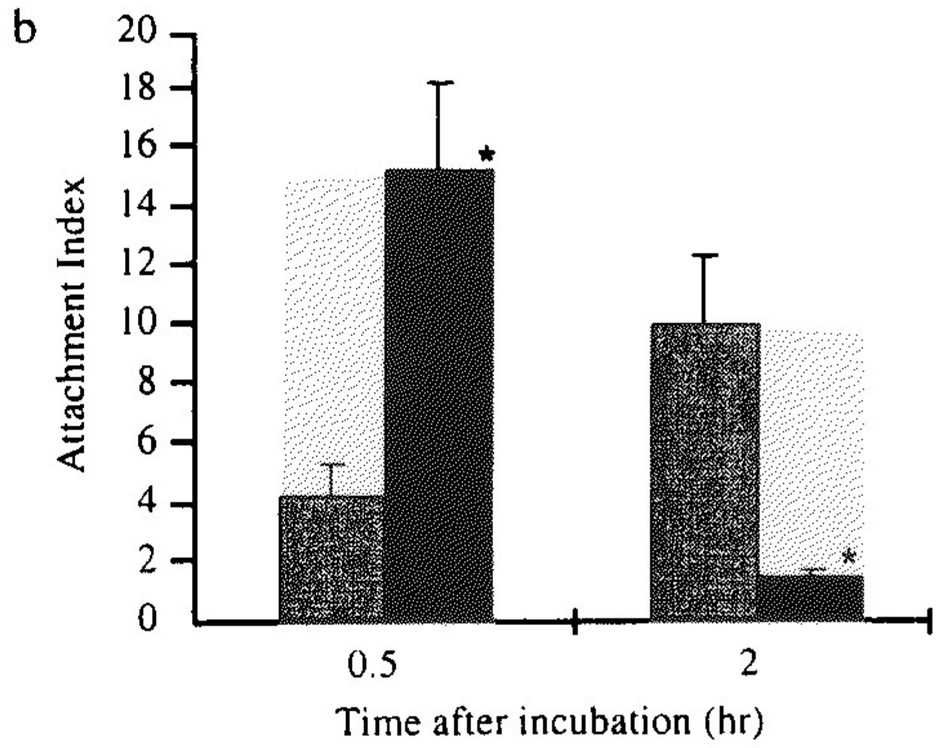
1. Effect of BioBran/MGN-3 on attachment of yeast to MCF-7 cells

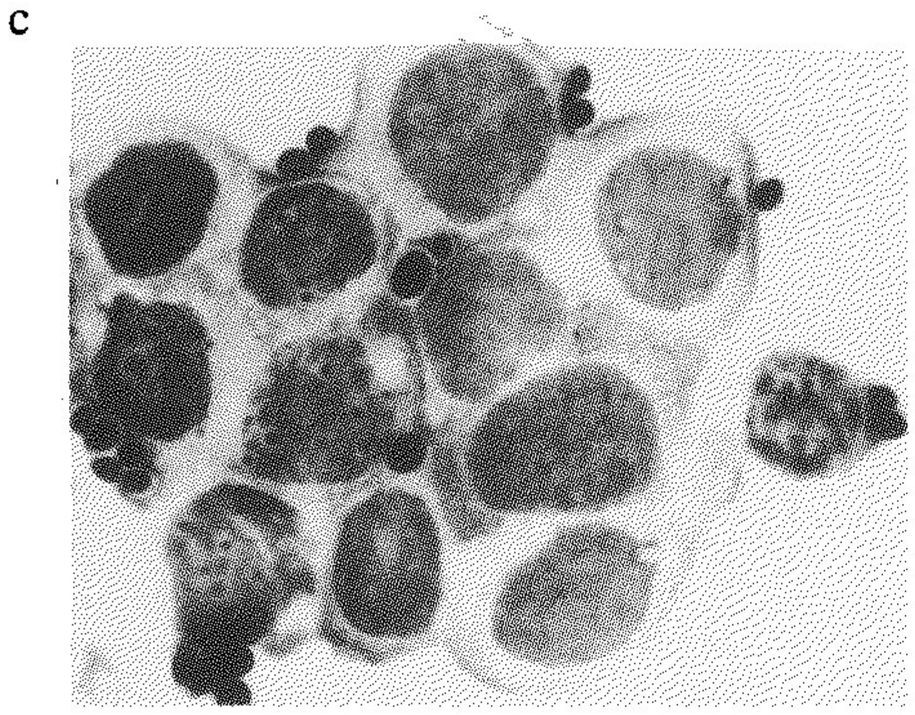
Figures 1a & b represent data of 3 experiments. Tumor cells were co-cultured with yeast in the absence or presence of BioBran/MGN-3 and the percentage of attachment and attachment index were examined at 0.5 hr and 2 hr. At 0.5 hr, the percentage of MCF-7 cells exhibiting binding was 27%. Treatment of 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**).

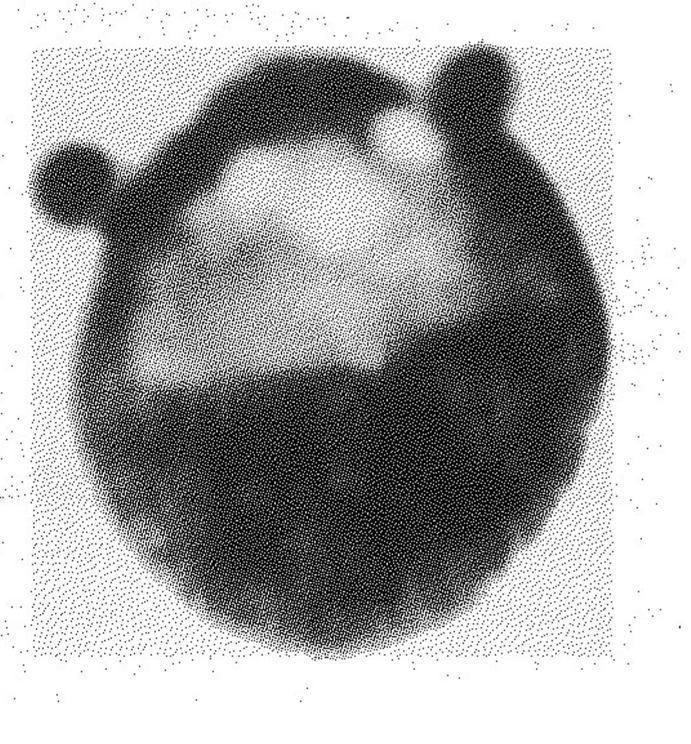
2. Effect of BioBran/MGN-3 on phagocytosis of yeast by MCF-7 cells

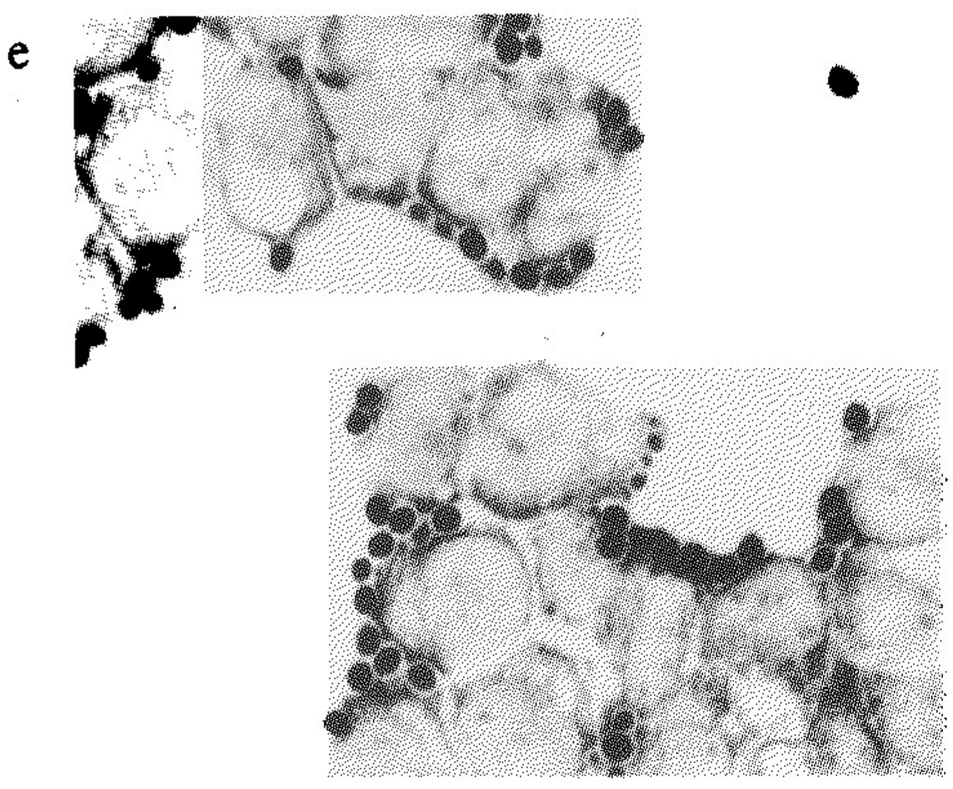
Results in **Figure 2** show that yeast was phagocytized by MCF-7 cells. This was confirmed by flow cytometric analysis which clearly distinguishes cell-bound and internalized yeast (**Figure 2a-c**). Cytospin











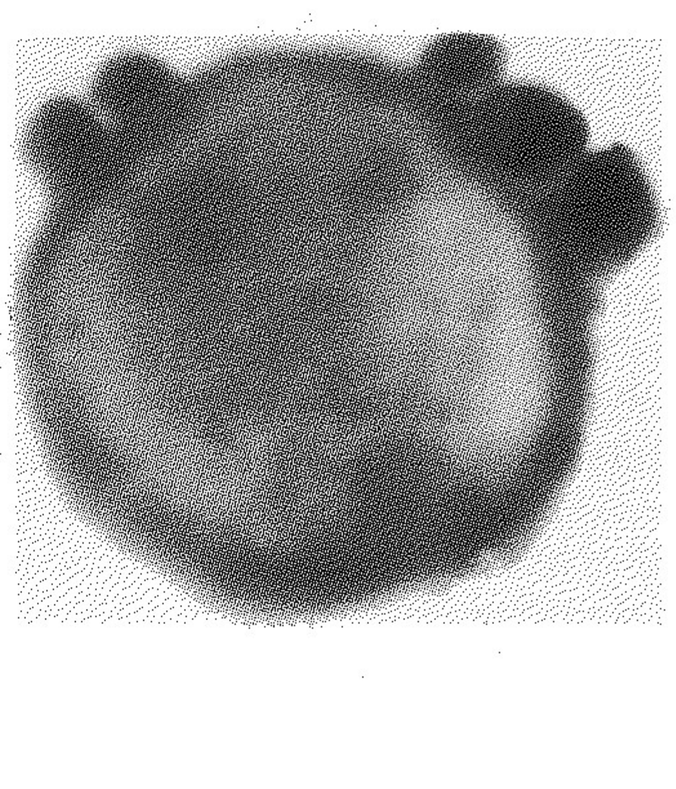
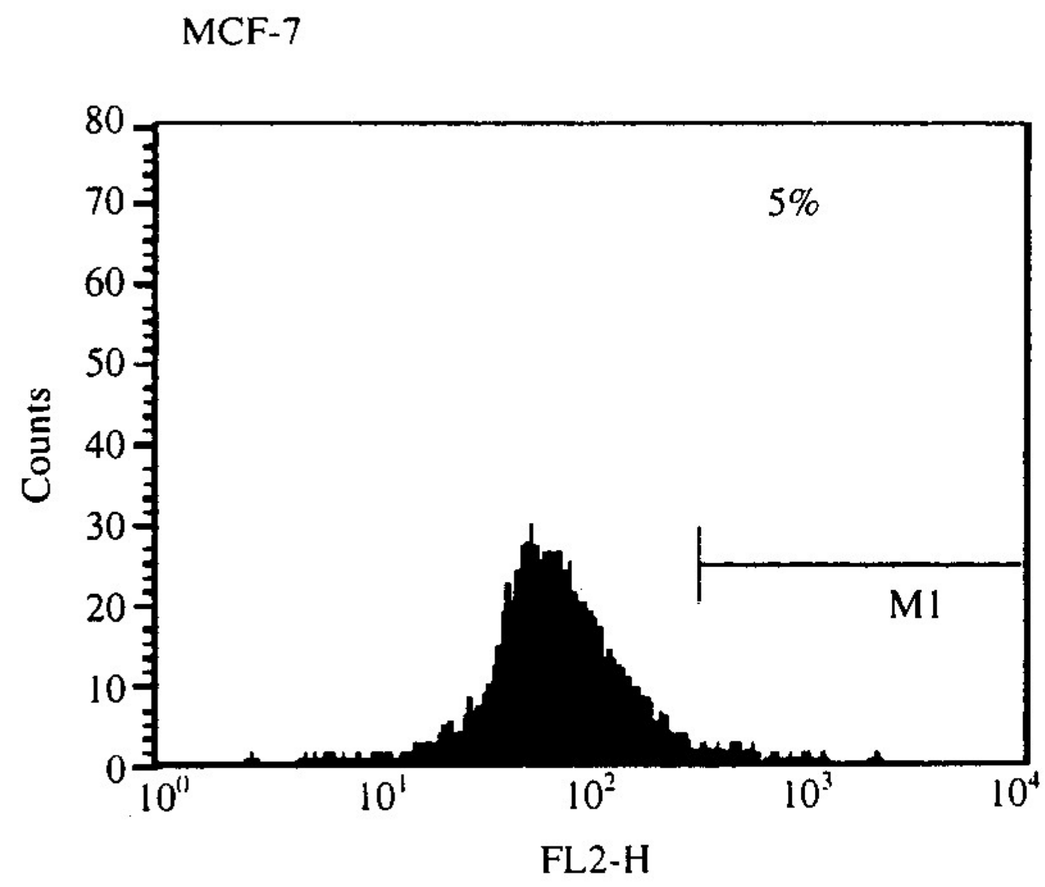


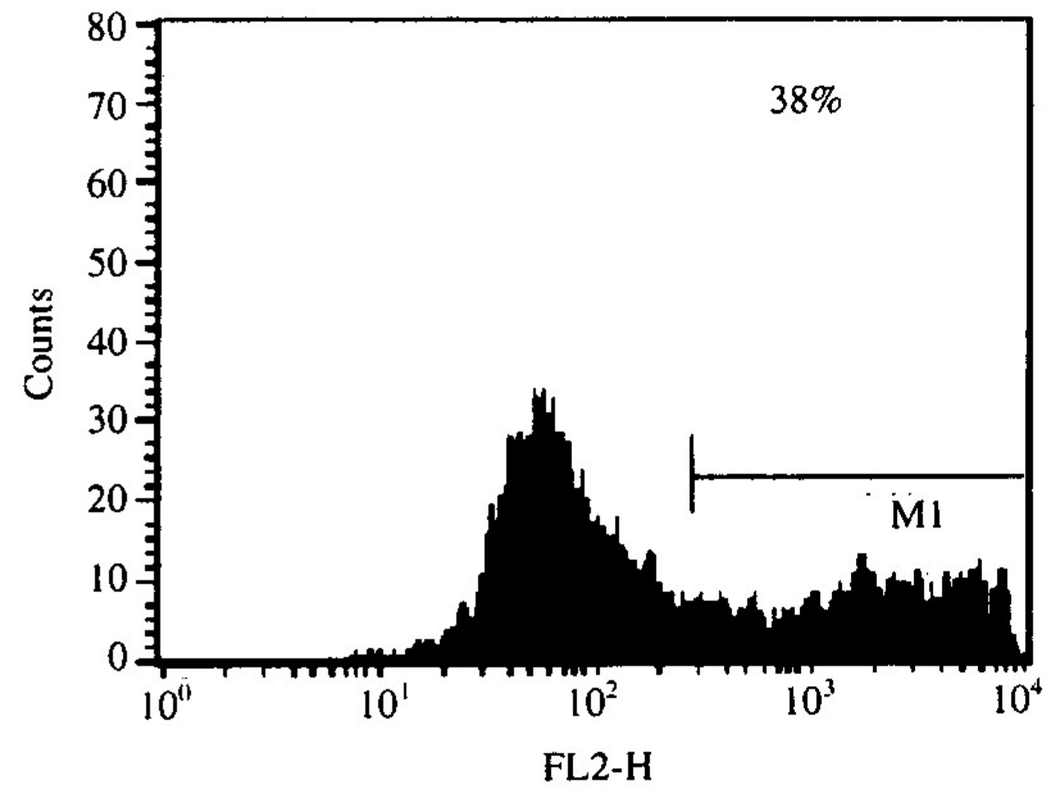
Fig.1 Action of MGN-3 on the attachment of MCF-7 cells to yeast and attachment index.

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.

a) Cells



b) Cells+Yeast



c) Cells+Yeast+Trypanblue

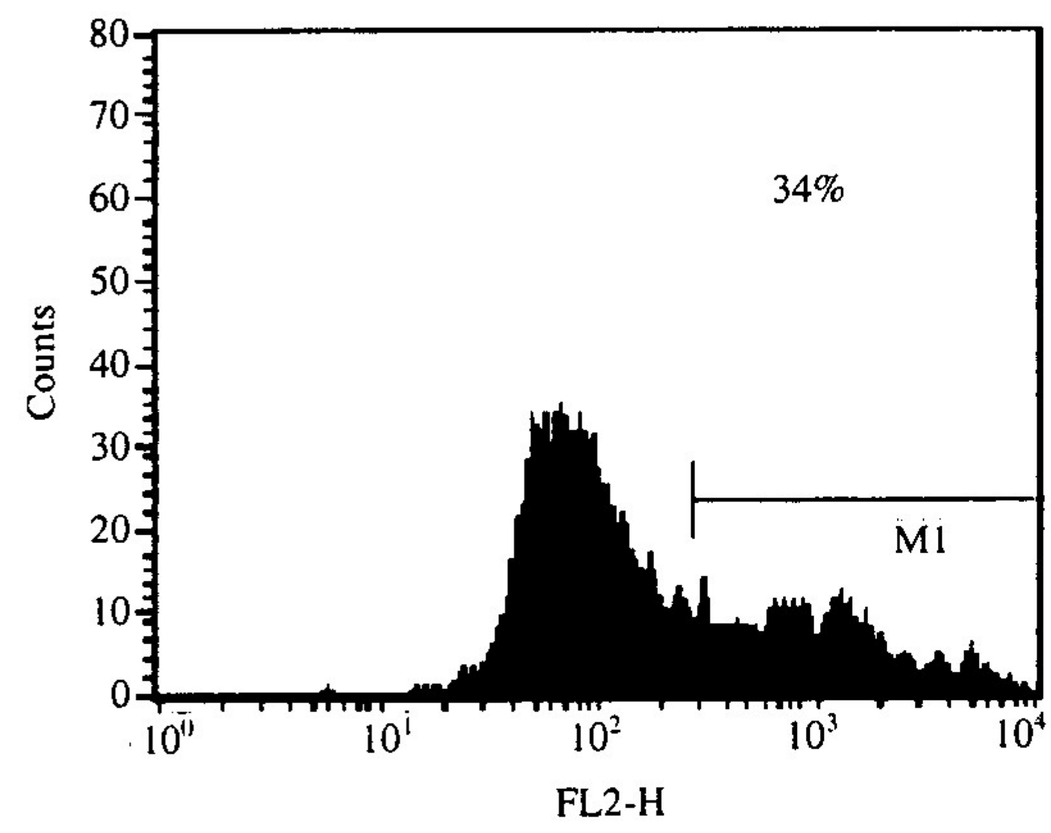


Fig. 2 Phagocytosis of yeast by MCF-7 cells.

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.

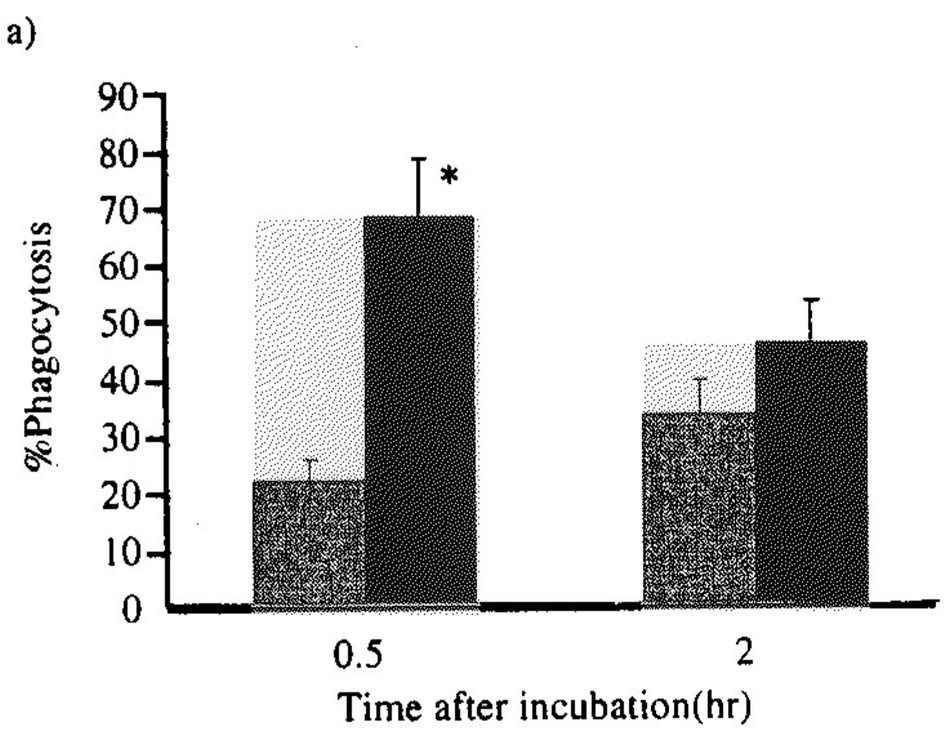


Fig. 3 Effect of MGN-3 on the percent of phagocytosis of yeast by MCF-7 cells and phagocytic index.

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

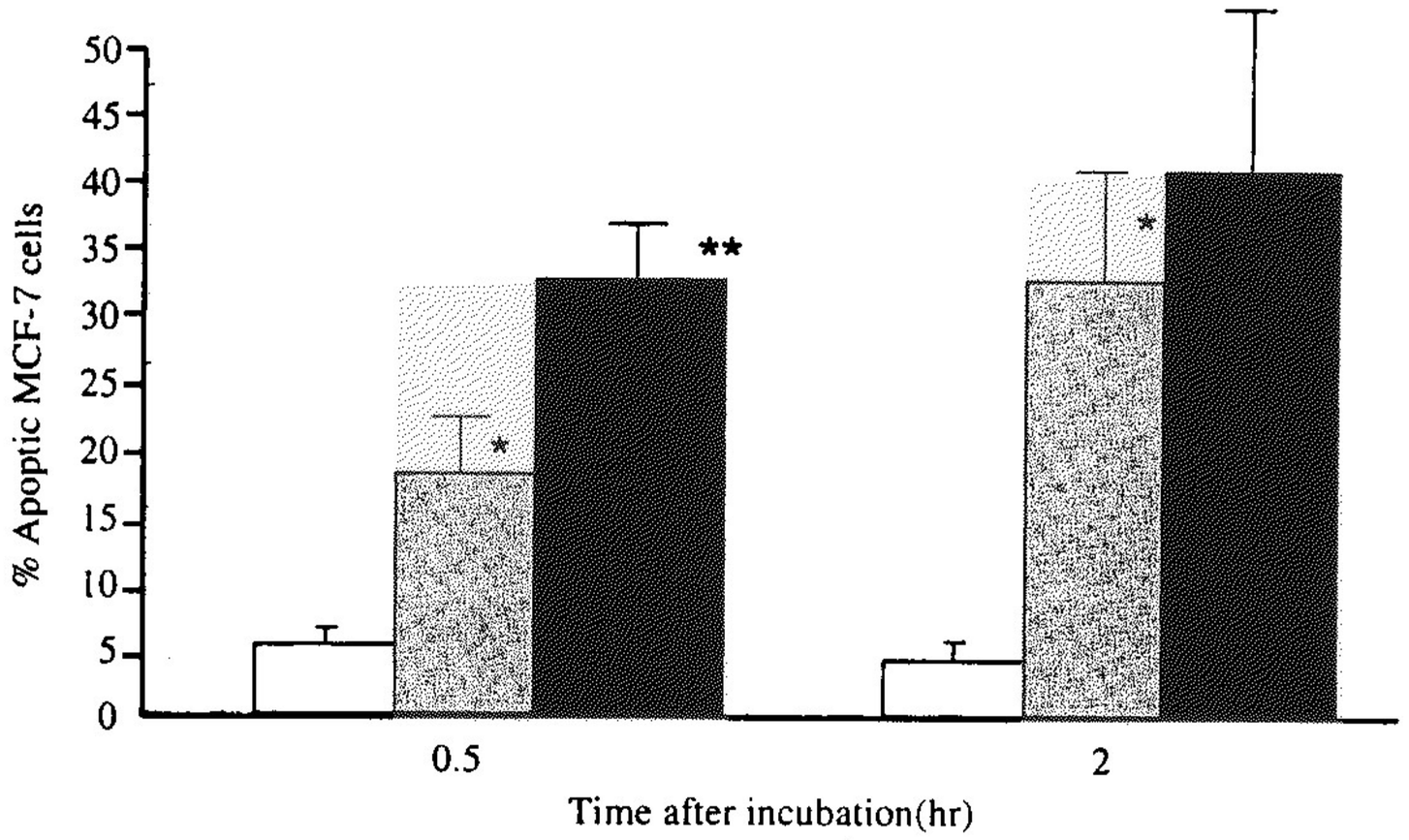


Fig. 4 Effect of MGN-3 on apoptosis of MCF-7 as defined in cytospin preparations.

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-