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MODIFIED ARABINOXYLAN RICE BRAN (MGN-3/BIOBRAN) ENHANCES INTRACELLULAR KILLING OF MICROBES BY HUMAN PHAGOCYTIC CELLS *IN VITRO*

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Phagocytic cells, comprised of neutrophils and moncytyes/macrophages, play a key role in the innate immune response to infection. Our earlier study demonstrated that arabinoxylan rice bran (MGN-3/Biobran) activates murine peritoneal macrophage and macrophage cell lines. In this study, we investigated whether MGN-3 can upregulate the phagocytic activity of human phagocytes in peripheral blood to phagocytize *Escherichia coli* (*E. coli*), trigger the oxidative burst and produce cytokines. Phagocytic cells were pre-labeled with dichlorofluorescin diacetate dye and were incubated with phycoerythrin-labeled *E. coli* in the presence or absence of MGN-3. Phagocytosis and oxidative burst were assessed by flow cytometry. Results showed that treatment with MGN-3 enhanced the phagocytosis of *E. coli* by neutrophils and moncytes. This was associated with an increased oxidative burst. In addition, it caused a significant induction of cytokines (TNF-α, IL-6, IL-8 and IL-10); the effect was detected at 1 µg/ml and increased in a dose-dependent manner ($P \leq 0.01$). Notably, MGN-3 alone had no effect on the growth of 31 strains of bacteria suggesting that MGN-3 modulates phagocytic cellular function. These findings may have applications in the treatment of infections in the elderly and in immunocompromised patients.

Phagocytic cells, such as neutrophils and macrophages, are important components of the innate immune system that are necessary for the successful elimination of an infection. Phagocyte function is based on cellular activation characterized by phagocytosis, activation of the oxidative burst, and production of pro-inflammatory cytokines. Exposure of phagocytic cells to bacteria, or bacterial products, activates these cells which ultimately results in the clearance of pathogens (1). Stimulated macrophages can synthesize and release a large variety of cytokines including pro- and anti-inflammatory cytokines, and these cytokines play an important role in shaping the adaptive immune responses. Abnormalities in phagocytic cell functions are associated with increased susceptibility to infections. Therefore, agents that promote the functional activities of phagocytes are of great value in treating infections.

Many natural products have shown ability to activate macrophages. These include: a) algae like *aphanizomenon flos-aquae* (2); b) plants such as *panax ginseng* (3), shi-ka-ron and Chinese herbs (4), the roots of *Platycodon grandiflorum* (5),

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and fermented papaya preparation (6); c) fungi such as lentinan isolated from *Lentinus edodes* (7), prodigiosin from *Serratia marcescens* (8), and schizophyllan from *Schizophyllium commune* (9); and d) bacteria such as *bacillus Calmette Guerin* (10). Many polysaccharides extracted from these fungi and microbes exhibit immunomodulatory function through ß(1, 3)-glucan. MGN-3/Biobran is a polysaccharide derived from rice bran (11) that has proven to be a novel biological response modifier (BRM). Oral intake of MGN-3 boosts the activity of human NK cells (12-13) and the proliferation of T cells and B cells (11). Since *in vitro* studies have shown that MGN-3 augments the phagocytic activity of mouse macrophages against yeast (14), this study was designed to investigate the ability of MGN-3 to enhance the functions of human phagocytes and to examine the mechanisms underlying its effect. The results show that MGN-3 exerts stimulatory effects on human phagocytic cells and enhances their oxidative burst and cytokine production (TNF-α, IL-6, IL-8, and IL-10).

MATERIALS AND METHODS

Chemicals and bacteria

Levofloxacin laboratory standard powder (as positive drug control); Ficoll-Hypaque (Pharmacia, Uppsala, Sweden); cell permeable dichlorofluorescein diactetae (DCFH-DA) and Dihydrorhodamine 123 (DHR) from Molecular Probes (Eugene, OR); phorbol myristate acetate (PMA), gelatin, glucose and propidum iodide (PI) from Sigma (St Louis, MO); HBSS without phenol red from GIBCO (Grand Island, NY); *Escherichia coli* American Tissue Culture Collection (ATCC) 25922; *Pseudomonas aeruginosa* ATCC 27853; *Staphylococcus aureus* ATCC 29213; and *Enterococcus faecalis* ATCC 29212.

Media

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

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). (Daiwa Pharm., Co., Ltd., Japan).

Culture of MGN-3 with bacteria: cation-supplemented Mueller Hinton broth (25 mg/L Ca^{++} and 12.5 mg/L Mg^{++}) (CSMB) was used. The pH was measured after autoclaving and adjusted to 7.2-7.4 when necessary.

Propidium iodide labeling of bacteria

E. coli grown in peptone broth overnight were washed three times with HBSS without phenol red, fixed and permeabilized with 70% ethanol at 40°C for 30 minutes. The cells were then washed 2 times with phosphate buffered saline containing 1% gelatin and 1% glucose (PBSg) and re-suspended to a final concentration of 10^8 organisms per ml by optical density measurement at 580 nm in a spectrophotometer. The bacteria were then incubated with PI at a final concentration of 50 μg/ml for 30 min. The labeled bacteria were washed and re-suspended in PBSg (10^8/ml).

Phagocytosis

Whole blood (1 ml) from 3 subjects was incubated with PI labeled *E. coli* for 1 hr at 37°C in the presence or absence of MGN-3 (100 μg/ml). The uptake of bacteria by neutrophils and monocytes/macrophages was determined by FACScan. Forward and Side Scatter was used to gate the monocytes and neutrophils. 10,000 cells were acquired and analyzed by the Cellquest Software Programme (BD Biosciences, San Jose, CA).

Assay of oxidative product formation (ROS)

Intracellular production of hydrogen peroxide was measured by oxidation-dependent fluorescence of DCFH-DA. DCFH-DA is a cell-permeable and non-polar molecule. Inside the cell, the acetyl groups are cleaved by the cellular enzymes in the cytoplasm to produce the polar molecule DCFH, and then trapped inside the cells. DCFH-DA is a non-fluorescent molecule but its oxidation yields a fluorescent molecule which is readily detectable by flow cytometry. To load the cells with the dye, whole blood (1 ml) from 5 subjects was incubated with 5 μM of DCFH-DA for 15 minutes at 37°C. The cells were then washed with PBSg and incubated with MGN-3 (100 μg/ml) for 1 hr. DCFH fluorescence was detected by FACScan.

Cytokine production (TNF-α, IL-6, IL-8 and IL-10)

Peripheral blood mononuclear cells (PBMC). PBMC from 3 subjects in CM were cultured at 5 x 10^5 cells/0.6 ml/well of a 24-well plate in the presence of MGN-3 (1-1000 μg/ml). Culture supernatant obtained at 6 hrs after stimulation was assayed for tumor necrosis factor-α (TNF-α), and supernatants which were obtained at 24 hrs for interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-10 (IL-10). Cytokines in the culture supernatant were measured by specific sandwich enzyme-linked immunosorbent assays (ELISAs), according to the

manufacturer's instructions (Endogen, Woburn, MA), by use of matched antibody pairs. Quantification of each cytokine (in ng/ml) was performed based on the standard curve obtained in each assay.

Human macrophage cell line (U937 cells)

U937 cells in CM were pre-cultured with PMA (60 ng/ml) for 3 days in a 24-well culture plate (2.5 x 10^5 cells/0.6 ml/well) to induce differentiation into macrophage-like cells. The adherent cells were cultured with MGN-3 (1-1000 μg/ml) and cytokines in the culture supernatant were measured by ELISA as mentioned above.

In vitro activity of MGN-3 against selected anaerobic and microaerobic bacteria

We performed time-kill studies with MGN-3 using standard procedure (15-17) as described in the Clinical Microbiology Procedure Handbook, Section 5.16.14. MGN-3 concentrations of 64, 256, and 1024 μg/ml were prepared in cation-supplemented Mueller Hinton broth. The pH of the solutions was measured at the starting time and at each sampling time. The MGN-3 and Levofloxacin, used as positive drug control, were added to flasks containing 10 ml of pre-warmed CSMB. The flasks were inoculated with the test strain to approximately 5x10^5 – 1x10^6cfu/ml. A drug-free flask was included as a growth control. Incubation was at 37°C under aerobic conditions for 24 hrs. A small aliquot was removed at the "zero" time, diluted and plated to establish the starting concentration of the strain. All flasks were sampled after 2, 4, 8, and 24 h incubation, diluted 10- or 100-fold, and plated to determine the bacterial counts (cfu/ml). The pH was measured and recorded at each of these times. Levofloxacin was tested at 0.03 μg/ml for *Escherichia coli*, 2 μg/ml for *Pseudomonas aeruginosa*, 0.25 μg/ml for *Staphylococcus aureus*, and 1 μg/ml for *Enterococcus faecalis*.

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

Phagocytosis

A representative FACS histogram of lysed peripheral blood showing the forward and side scatter of different populations of phagocytes; polymorphonuclear leukocytes (PMN), monocytes and lymphocytes are shown in Fig. 1. Whole blood was incubated with PI labeled *E. coli* in the presence or absence of MGN-3 (100 μg/ml) and the uptake of bacteria by phagocytic cells was determined by FACScan. Treatment with MGN-3 significantly enhanced the phagocytosis of *E. coli* by phagocytic cells, PMN 400% (Fig. 2A) and monocytes 110% (Fig. 2B) over the controls (cells + *E. coli*).

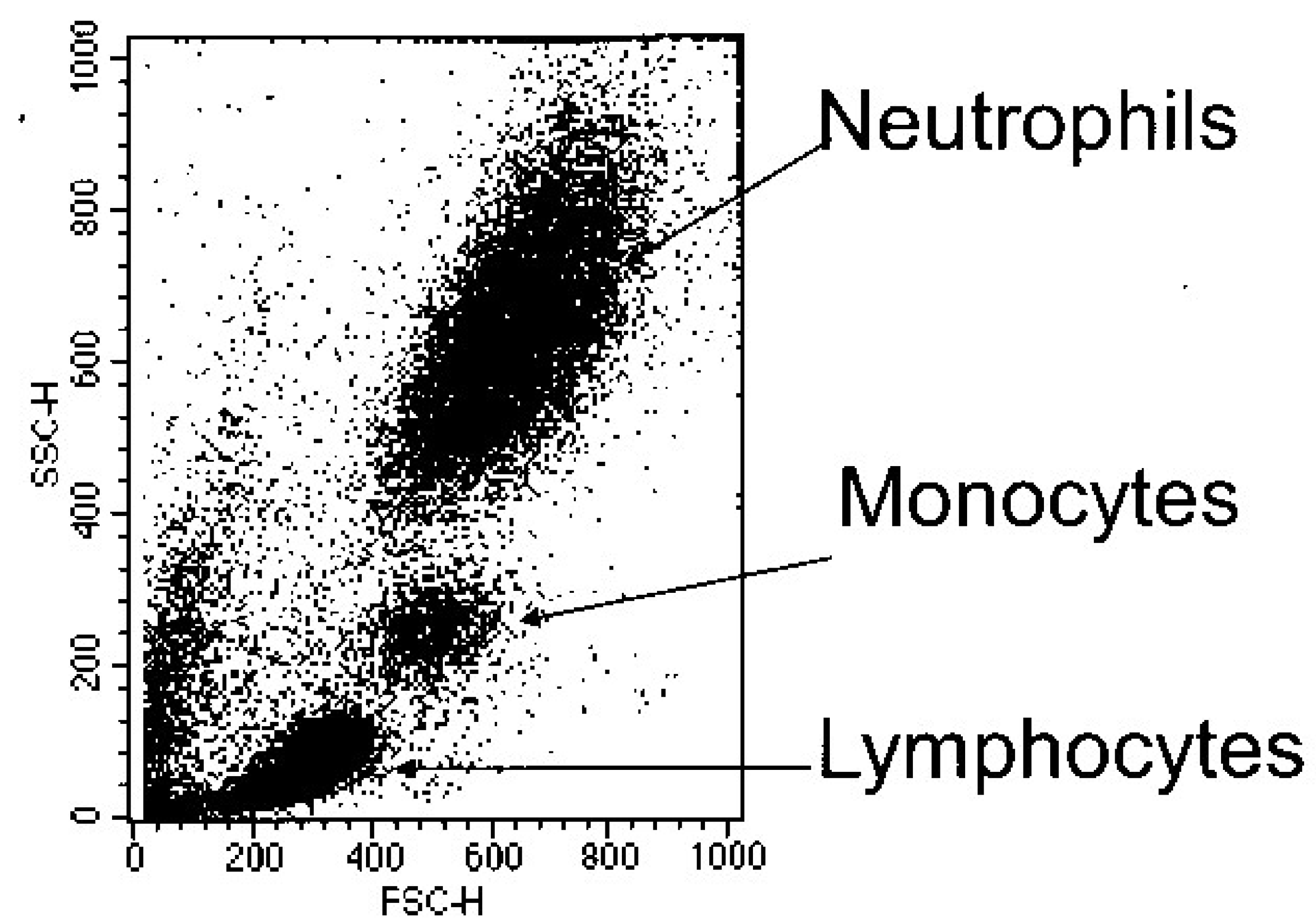


Fig. 1. *A representative FACS histogram of lysed peripheral blood showing the forward and side scatter of different populations of phagocytes; polymorphonuclear leukocytes (PMN) and monocytes.*

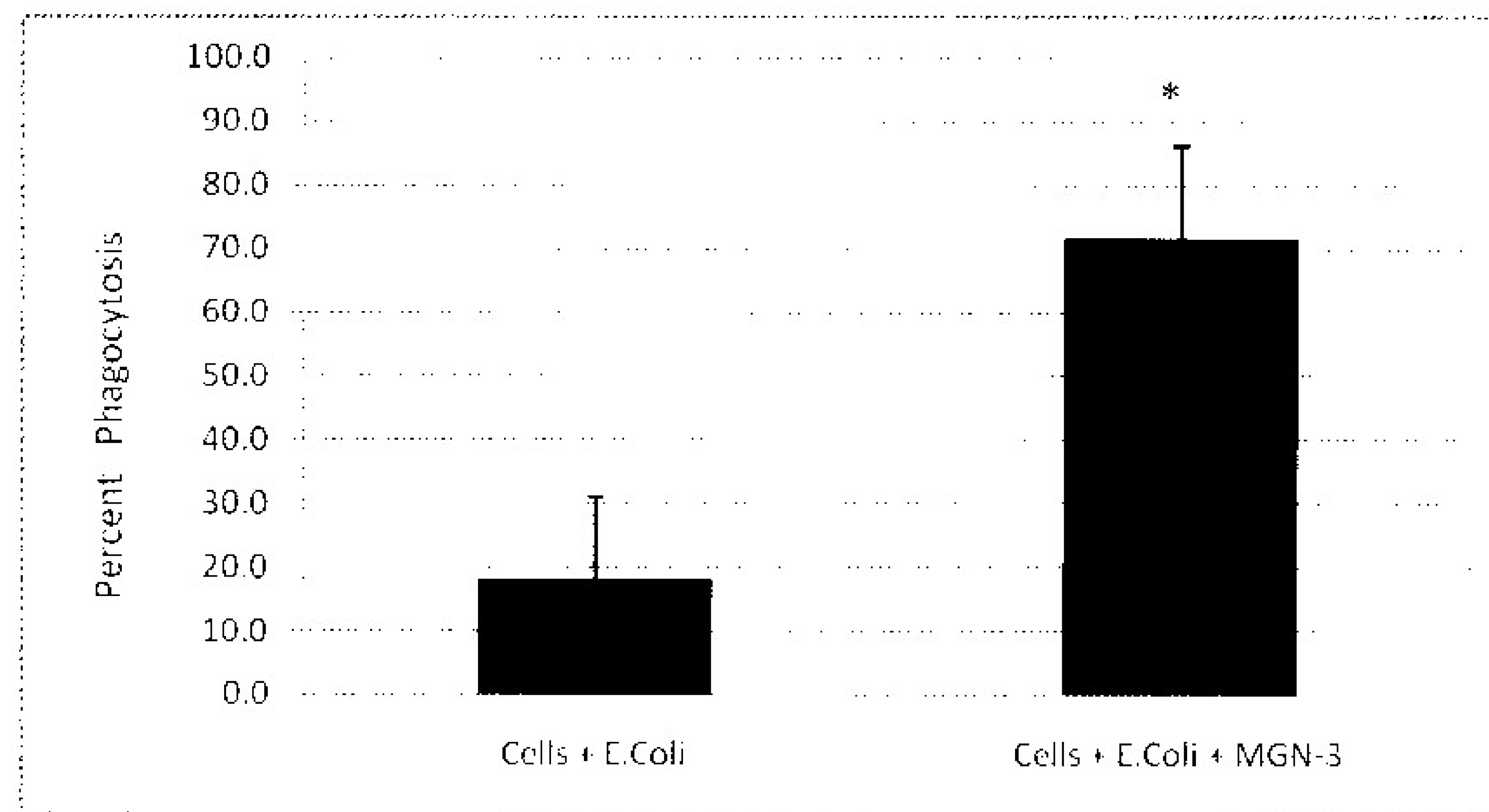
Effect of MGN-3 on oxidative burst

Oxidative burst in neutrophils and monocytes was monitored by measuring the production of hydrogen peroxide using DCFH Dye and FACScan. PMA was used as a positive control to determine the oxidative burst. Fig. 3 shows a representative flow cytograph of oxidative burst in neutrophils and monocytes cultured with *E. coli* in the presence or absence of MGN-3. In the presence of *E. coli*, MGN-3 increased oxidative burst in neutrophils and monocytes. Data in Fig. 4 show that MGN-3 alone had no effect on oxidative burst generation in either of the phagocytic cells.

Effect of MGN-3 on production of cytokines (TNF-α, IL-6, IL-8, and IL-10)

Experiments were carried out to in order to determine the effect of MGN-3 on cytokine production from human peripheral blood mononuclear cells and U937 cells. Cells were co-cultured with MGN-3 (1-1000 μg/ml), cell culture supernatants were obtained at 6 or 24 hrs and were

A. PMN



B. Monocytes

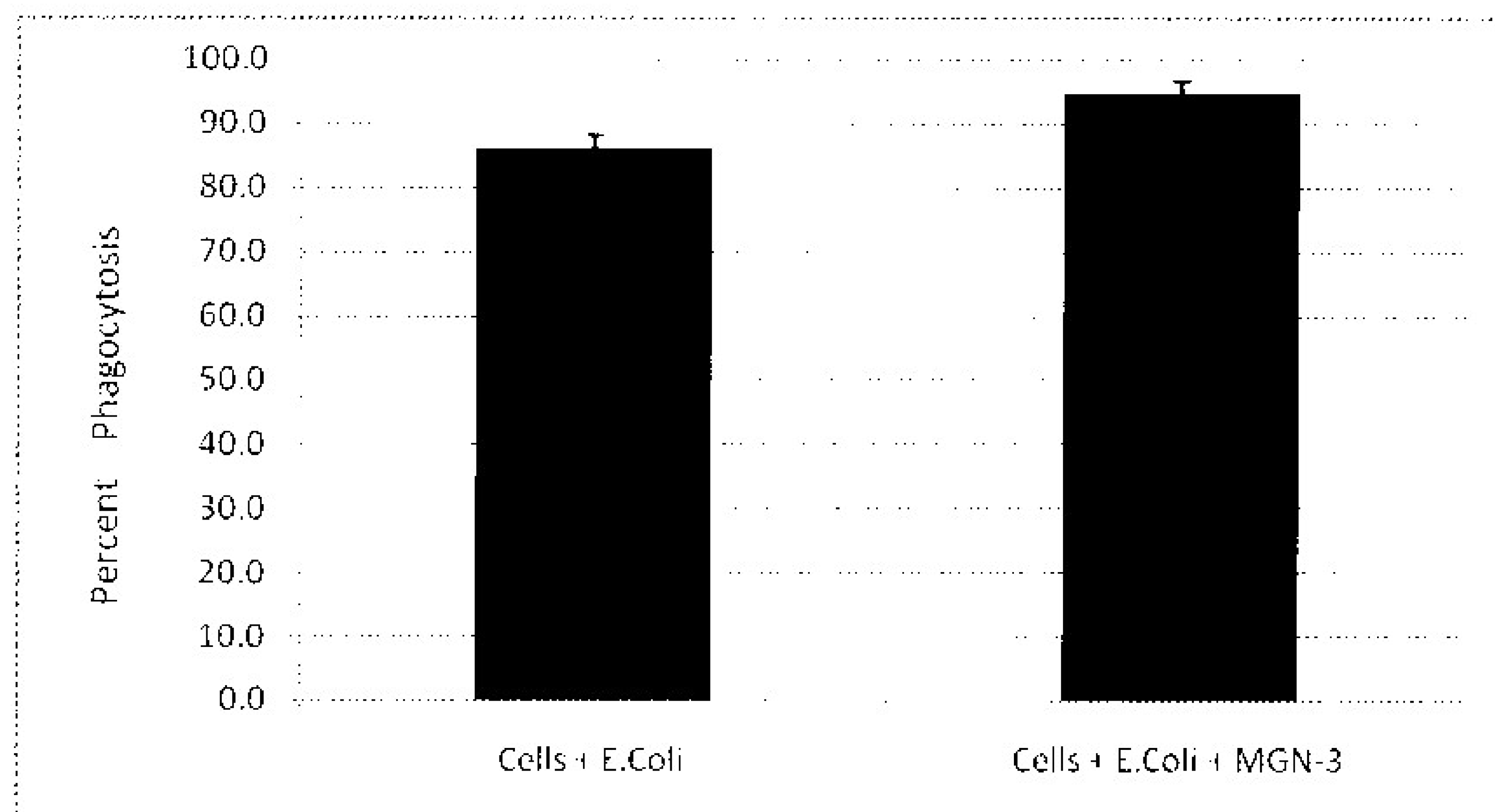


Fig. 2. *Effect of MGN-3 on the phagocytic activity by PMN and monocytes. Whole blood (1 ml) was incubated with PI labeled E. coli for 1 hr at 37°C in the presence or absence of MGN-3 (100 ug/ml). The uptake of bacteria by phagocytic cells was determined by FACscan. Data represent mean±SD of 3 subjects. * P <0.01 as compared to cells + E. coli alone.*

assayed for the production of TNF-**α**, IL-6, IL-8 and IL-10 by ELISA. Data depicted in Fig. 5 show that MGN-3 treatment caused a significant enhancement in the production of these cytokines by PBMC and U937 cells (P≤ 0.01); this was detected at 1 μg/ml and followed a dose dependent fashion, which was significant at concentrations of 10 μg/ml or higher (P< 0.01). In order to exclude a possibility that the increased production of cytokines was due to cell death and release of these cytokines, we examined the effect of MGN-3 on the viability of monocytes by PI technique using FACScan. The data shows that the viability of cells cultured with MGN-3 (87%) was similar to that of control cultures (90%).

In vitro activity of MGN-3 against selected anaerobic and microaerobic bacteria

A compound is usually considered to be bactericidal if the starting inoculum is reduced by 3 $\log_{10}$, i.e. from 10^5 to 10^2 cfu/ml. The results for each

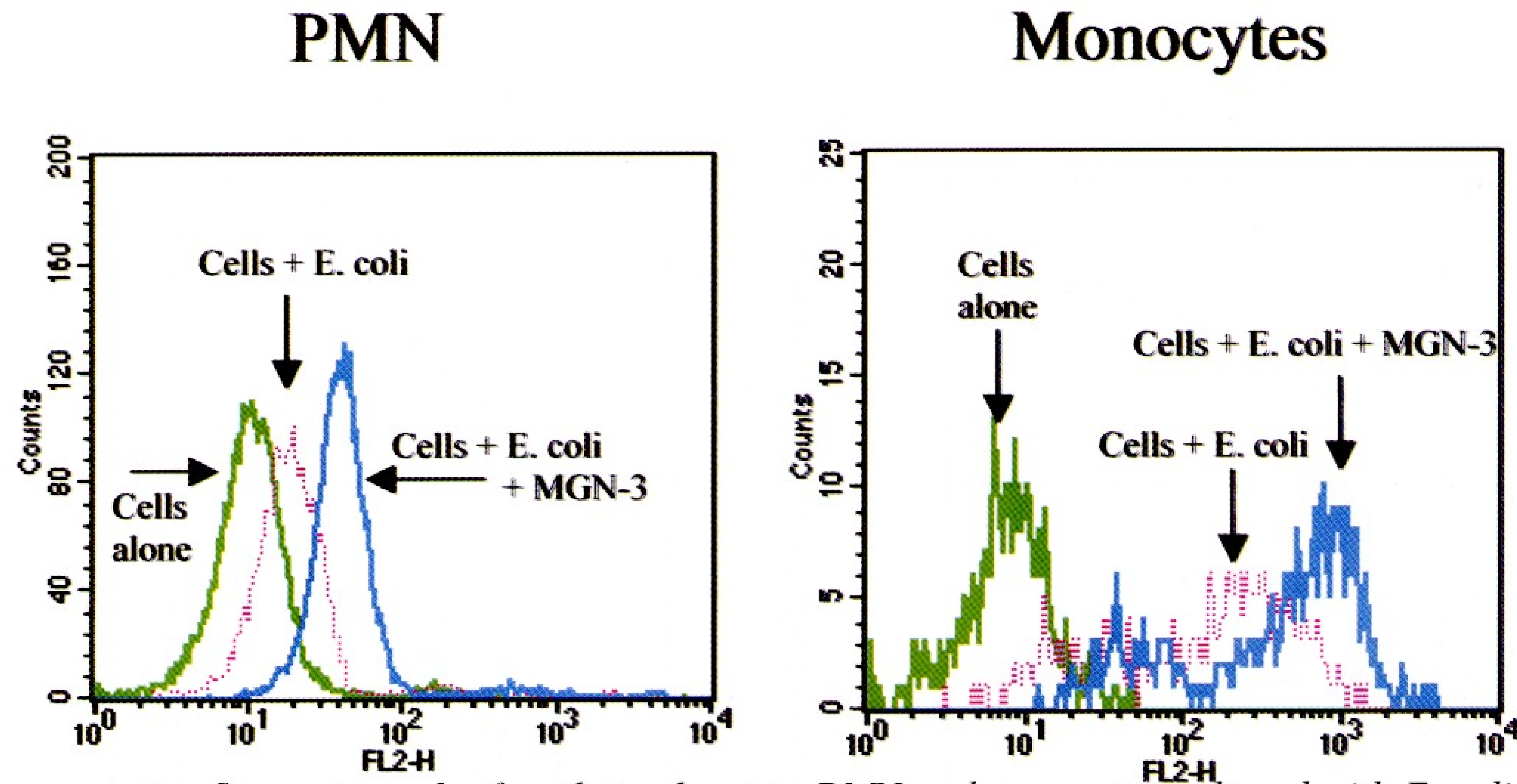


Fig. 3. *A representative flow cytograph of oxidative burst in PMN and monocytes cultured with E. coli in the presence of MGN-3. Oxidative burst in phagocytic cells was monitored by measuring the production of hydrogen peroxide using DCFH Dye and FACScan. Whole blood (1 ml) was incubated with 5 μM of DCFH-DA for 15 minutes and incubated with MGN-3 (100 μg/ml) for 1 hr. DCFH Fluorescence was detected by FACScan. Data representative of 5 separate subjects.*

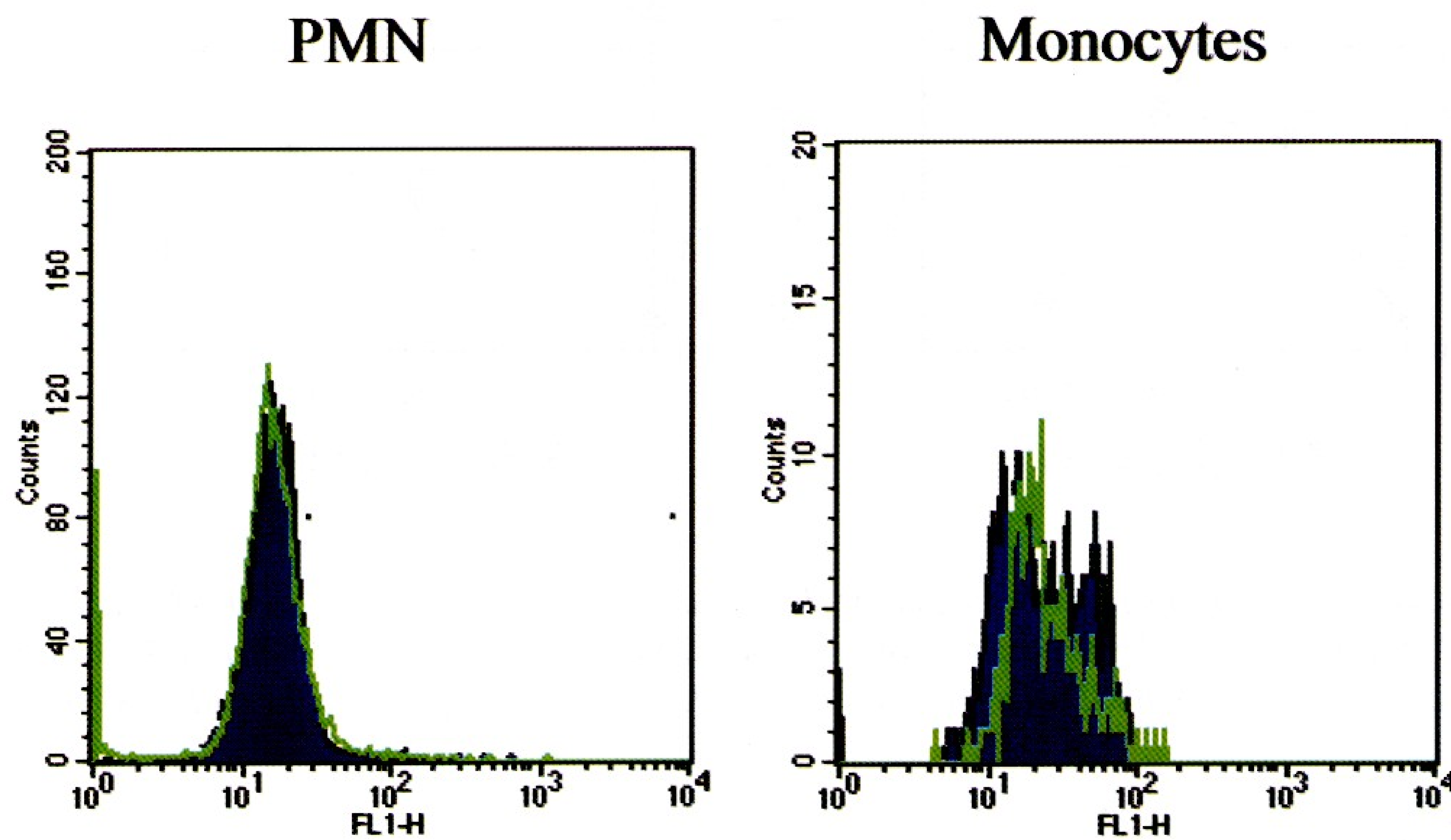


Fig. 4. *Effect of MGN-3 alone on oxidative burst in PMN and monocytes. Oxidative burst in phagocytic cells was monitored by measuring the production of hydrogen peroxide using DCFH Dye and FACScan. Data representative of 4 separate subjects.*

strain at each compound concentration and the growth control were expressed as the cfu/ml at each time tested. A kill curve was constructed (4 strains, each with 3 concentrations of MGN-3; 1 Levofloxacin; 1 drug-free growth control = 5 flasks). Data in Table I show a lack of activity by MGN-3 against a broad spectrum of anaerobic and microaerophilic bacteria as measured by the agar dilution technique.

DISCUSSION

MGN-3/Biobran is a processed hemicellulose that is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from Shiitake mushrooms. The main chemical structure of MGN-3 is an arabinoxylan with a xylose in its main chain and an arabinose polymer

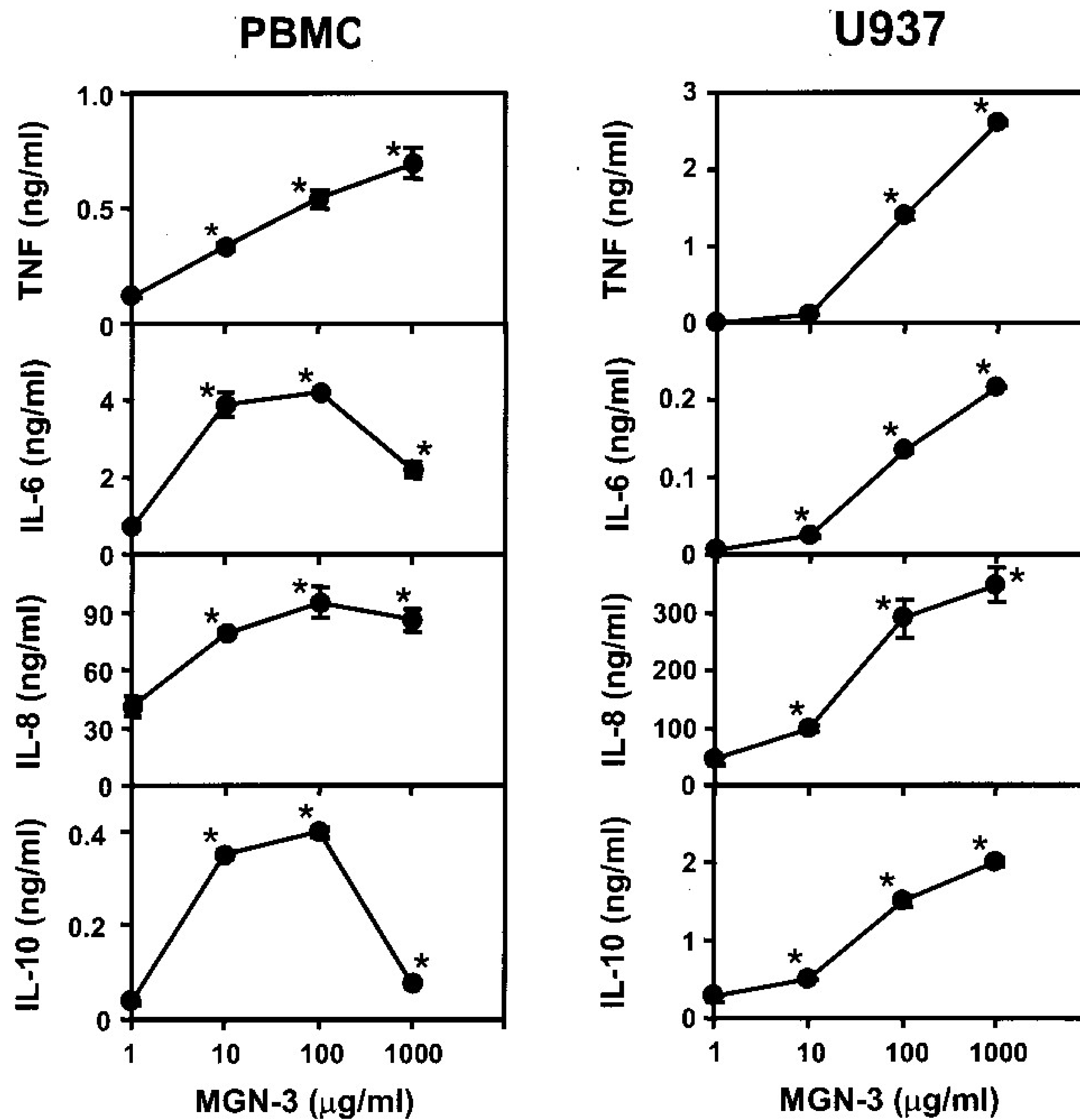


Fig. 5. *Effect of MGN-3 on the production of cytokines by PBMC and U937 cells. PBMC and U937 cells were stimulated with MGN-3 (1-1000 μg/ml) for 24 hr. Production of cytokines, TNF-α, (at 6 hr) IL-6, IL-8 and IL-10, was determined by ELISA. The results represent mean±SD of triplicate samples. *P<0.01 as compared to the culture supernatant without MGN-3.*

in its side chain. (11). MGN-3 possesses anti-tumor activity by sensitizing human leukemia cells to death receptor [CD95]-induced apoptosis (18) and acts synergistically with yeast to induce apoptosis in cancer cells (19). In addition, MGN-3 has proven to be a novel BRM that boosts the activity of NK cells (12-13) and T and B cell proliferation (11). Since *in vitro* studies have shown that MGN-3 augments the phagocytic activity of mouse macrophages against yeast (14), this study was designed to investigate the ability of MGN-3 to enhance the functions of human PMN and monocytes. MGN-3 enhances phagocytosis of *E. coli* by human phagocytic cells and also enhances phagocytosis-induced oxidative burst. In addition, MGN-3 triggers the secretion of pro- and anti-inflammatory cytokines.

Oxidative burst in phagocytosis leads to the generation of reactive oxygen species, such as superoxide anion, which are potent antimicrobial compounds. In this study, we found MGN-3 had no direct killing effect on bacteria; however, it may increase intracellular killing by stimulating ROS. Upon activation with MGN-3, PBMC and U937 monocytic cell line produced several cytokines

Table I. *In vitro activity of MGN-3 against selected anaerobic and microaerobic bacteria.*

Number	Source	Genus	Species	MGN- 3	Clindamycin
				MIC	mcg/ml
ATCC 25285	NCCLS control	Bacteroides	fragilis	> 1024	1
ATCC 29741	NCCLS control	Bacteroides	thetaiotaomicron	> 1024	4
ATCC 43055	NCCLS control	Eubacterium	lentum	> 1024	0.125
13706A	Perit. Fluid	Fusobacterium	nucleatum	"	<=0.03
11971 H	Perit. Fluid	Fusobacterium	nucleatum	"	<=0.03
16551 C	Oral	Fusobacterium	nucleatum	"	0.125
17288 D	Perit. Fluid	Porphyromonas	gingivalis	"	<=0.03
18123 N	Perit. Fluid	Porphyromonas	gingivalis	"	<=0.03
18076 M	Perit. Fluid	Porphyromonas	asaccharolytica	"	<=0.03
17871 D	Perirectal	Prevotella	bivia	"	<=0.03
17928 D	Perinium	Prevotella	bivia	"	<=0.03
18048 H	Perit. Fluid	Prevotella	buccae	"	<=0.03
16523 D	Oral	Prevotella	melaninogenica	"	<=0.03
17287 J	Perit. Fluid	Prevotella	intermedia	"	<=0.03
18120 L	Perit. Fluid	Prevotella	intermedia	"	<=0.03
18164 I	From RML	Peptostreptococcus	micros	"	0.25
17381 D	Perit. Fluid	Peptostreptococcus	micros	"	0.25
12184 G	Perit. Fluid	Peptostreptococcus	asaccharolyticus	"	0.125
17855 A	Appendix	Propionibacterium	acnes	"	0.06
17522 F	Perit. Fluid	Propionibacterium	acnes	"	0.06
17679 I		Actinomyces	israelii	"	1
18076 O	Perit. Fluid	Actinomyces	species	"	0.5
17382 B	Perit. Fluid	Clostridium	perfringens	"	0.25
17679 A	Perit. Fluid	Clostridium	innocuum	"	0.5
17287 G	Perit. Fluid	Clostridium	innocuum	"	0.5
12086 L	Perit. Fluid	Clostridium	ramosum	"	8
CD 191	Stool	Clostridium	difficile	"	>64
17878 E	Sputum	Capnocytophaga	species	"	0.06
16026 E	Perit. Fluid	Eikenella	corrodens	"	>64
17689 A	Perit. Fluid	Streptococcus	sanguis	"	0.125
17873 A	Blood	Streptococcus	sanguis	"	0.25

having a broad variety of biological activities (20-23). These cytokines exhibit either an inflammatory (TNF-α, IL-6 and IL-8) or anti-inflammatory (IL-10) response. Inflammation is a host response to infection to clear the invading organisms. Uncontrolled inflammation leads to tissue damage; therefore, agents that controllably stimulate the production of both types of cytokines are of value in clearing infection rapidly. Our results suggest that MGN-3 may be one such candidate compound. Regarding

the effect of MGN-3 on U937 cell line, there is a change in the results of cytokine production, TNF and IL-6, from an earlier study published (14) which may be attributed to differences in the methods employed: U937 pre-cultured using PMA without dihydroxyvitamin D3, whereas it had been previously used.

The precise mechanism(s) by which MGN-3 stimulates macrophage activities remains to be studied. MGN-3 is a polysaccharide that contains ß-1, 3-glucan, and may function like many other polysaccharides extracted from many natural sources (the cell wall of fungi and certain bacteria), which have ß-1, 3-glucans (7-10) and are known to modulate innate immunity by binding to specific receptors on monocyte/ macrophages (24) and neutrophils (25). Evidence suggests that there are multiple glucan-binding sites on macrophages including Type 3 complement receptor (CR3) (26), non-CR3 glucan-binding sites (27), and lactosylceramide (28). Our findings that MGN-3 alone stimulated cytokine production, but not oxidative burst in phagocytic cells, would suggest that the signaling mechanisms for cytokine production are independent from that of oxidative burst.

We conclude that MGN-3 has an enhancing effect on antibacterial activity by human phagocytic cells. These data may suggest therapeutic properties of MGN-3 as an antimicrobial agent for the elderly.

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