Combination of Glucan, Resveratrol and Vitamin C Demonstrates Strong Anti-tumor Potential

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Abstract. *β-Glucans are naturally occurring carbohydrates found in plants, fungi and some bacterial species, and currently are well-established and powerful immunomodulators with beneficial properties in cancer therapy. Recent studies suggested that some additional bioactive molecules have synergistic effects when combined with glucan. In the current study, we evaluated the anticancer properties of glucan, resveratrol, vitamin C combination. We found that compared to the individual components, the combination was the strongest activator of phagocytosis and antibody formation. Using two different models of cancer treatment, our results demonstrated that the combination strongly suppressed the growth of breast and lung tumors, most likely due to the stimulation of apoptosis.*

Glucans belong to a group of physiologically active compounds sometimes called biological response modifiers and represent highly conserved structural components of cell walls in yeast, fungi, and seaweed. Thbe role of glucan as a biologically active immunomodulator has been well established for over 50 years. Initial interest in the immunomodulatory properties of polysaccharides was raised after experiments showing that a crude yeast cell preparation stimulated macrophages *via* activation of the complement system (1). The best known effects of glucans consist of the direct stimulation of phagocytosis of professional phagocytes, *i.e.*, granulocytes, monocytes, macrophages and dendritic cells, and direct activation of natural killer cells. In this regard, macrophages (2, 3), considered to be the basic effector cells in host defense against bacteria, viruses, parasites and tumor cells, play the most important role. There is evidence that glucan makes a considerable contribution toward the increased production of nitric oxide, one of the most effective reactive nitrogen species, by inducible nitric oxide synthase (iNOS) in macrophages (4). The immunological effects of glucan are manifested through its binding to several specific receptors, most of all Complement receptor-3 (CR3) and Dectin-1 (for review, see 5).

Additional biological effects of glucans include stimulation of infectious immunity, activation of bone marrow cell production, significant anticancer effects and lowering of blood cholesterol (6-9).

Despite the fact that strong immunostimulatory effects of glucan have been well established, recent studies suggested that some additional bioactive molecules have synergistic effects when combined with glucan. First, several scientific studies have confirmed beneficial effects when glucan was given in combination with vitamin C. The main reason why vitamin C has synergistic effects might be the fact that vitamin C stimulates the same types of immune responses as glucan. A mouse study revealed significant healing abilities of a glucan-vitamin C combination in the treatment of infection by *Mesocestoides corti* (10). Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a non-flavonoid polyphenol found in various fruits and vegetables. In addition to various biochemical, biological and pharmacological activities, resveratrol has been found to exhibit numerous immunomodulatory activities, such as suppression of lymphocyte proliferation, changes in cell-mediated cytotoxicity, cytokine production (11) and induction of apoptosis (12). With regard to the effects of resveratrol on cancer, Jang *et al.* showed that resveratrol is able to exert therapeutic effects at all three major stages of carcinogenesis, *i.e.* anti-initiation, anti-promotion, and anti-progression (13). In addition, resveratrol was found to limit negative side-effects of various chemotherapeutic agents, such as cisplatin and doxorubicin (14).

Since we previously found significant synergy between glucan and resveratrol (15, 16), in the current study, we focused on the immunological and anticancer properties of combination of glucan, resveratrol and vitamin C.

Materials and Methods

Animals. Female, 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work

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was carried out according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

Materials. Yeast-derived insoluble glucan #300 was purchased from Transfer Point (Columbia, SC, USA). RPMI-1640 medium, glutamine, antibiotics, Wright stain, and *Limulus* lysate test E-TOXATE, Polymixin B, vitamin C (ascorbic acid), and cyclophosphamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA); fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT, USA); and reseveratrol was purchased from Amax, Inc. (Eugene, OR, USA).

Cells. The Lewis lung carcinoma cells were obtained from Dr. G. Ross (University of Louisville, Louisville, KY, USA) and were cultivated as described in Kogan *et al*. (17). The BALB/c mouse-derived mammary tumor cell line Ptac64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI, USA. These cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

Phagocytosis. The technique employing phagocytosis of synthetic polymeric microspheres is described elsewhere (18). Briefly: peripheral blood cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5×10^8/ml). The test tubes were incubated at 37˚C for 60 min with intermittent shaking. Smears were stained with Wright stain. Cells with three or more HEMA particles were considered positive. Mice were injected with individual substances or their combinations (or PBS as a control). For concentration see Figure legend. All experiments were performed in triplicates. At least 200 cells in 60 high-power fields were examined in each experiment. Experiments were repeated three times,each group held a minimum of five mice.

Apoptosis. Apoptosis experiments used a Human APO-1/Fas/CD95 kit according to manufacturer's instruction (Invitrogen, Camarillo, CA, USA). Experiments were repeated three times, each group held a minimum of five mice.

Antibody formation. Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 100 μg of albumin and the serum was collected 7 days after last injection. Experimental groups received daily *i.p.*. injections of either with individual substances or their combinations. The level of specific antibodies against ovalbumin was detected by ELISA. As positive control, a combination of ovalbumin and Freund's adjuvant was used. Experiments were repeated three times,each group held a minimum of five mice.

Lewis lung carcinoma therapy. Mice were injected *i.m.*. with 1×10^5 of Lewis lung carcinoma cells. Cyclophosphamide (30 mg/kg) was used *i.p.*. at day 8 after tumor application (positive control), individual substances or their combinations were used from day 0 do day 14 after tumor application. The control group of mice (negative control) received *i.p.*. PBS daily. Each group held a minimum of five mice. At the conclusion of the experiment (day 14), mice were euthanized, lungs removed, fixed in 10% formalin and the number of hemotogenic metastases in lung tissue was estimated using a binocular lens at 8× magnification.

Tumor inhibition in vivo. Ptas64 cells in PBS were injected directly into the mouse mammary fat pads at 1×10^6/mouse concentration. The experimental treatment was begun after palpable tumors were found (usually 14 days after injection of cells) and after mice were assigned to experimental groups. Experimental treatment was achieved by *i.p.*. injections of tested samples diluted in PBS (once/day for 14 days). After treatment, the mice were sacrificed, tumors removed and weighed (19). Experiments were repeated three times,each group held a minimum of five mice.

Statistics. Student's *t*-test was used to statistically analyze the data.

Results

First we focused on phagocytosis. We measured the effects of the tested substances on blood neutrophil phagocytosis using a well-established model with synthetic microparticles based on HEMA (Figure 1). The blood was obtained from mice 24 hr after *i.p.*. injection with different doses of glucan, resveratrol, vitamin C or their combinations. From these data, it is clear that both glucan and resveratrol significantly increased phagocytosis, whereas vitamin C had no effect. In combinations, the effects were stronger, with the highest stimulation in the case of all three compounds. The same results were found at all tested doses.

The effects of glucan on humoral immunity are less known. However, the current studies established that glucan also activates the antibody response. In our experiments, neither resveratrol nor vitamin C alone raised meaningful antibody response against ovalbumin. However, combinations showed significant stimulation, with the full combination almost reaching the levels obtained with Freud's adjuvant (Figure 2).

In the next step, we focused on the role of the tested substances in cancer development. First, we used mice challenged with Ptas64 mammary tumors. These experiments were repeated three times with similar results and then again with LPS-free substances (data not shown). The data showed strong lowering of tumor growth by glucan, resveratrol and all tested combinations (Figure 3). Using a model of Lewis lung carcinoma cells, in previous work we showed that cyclophosphamide administered at the used dose caused 70% reduction of the number of lung metastases in comparison to the control group (15). In our current experiments, treatment with glucan alone caused 44% inhibition. Even higher reduction of number of metastases was achieved when tested substances were used in combination - 67% less in case of glucan with resveratrol, 65% less in case of glucan with vitamin C and 86% reduction in case of glucan/resveratrol/ vitamin C combination (Figure 4). The anticancer effects of the combination were further supported by finding that the various combinations caused an increase in apoptosis (Figure 5).

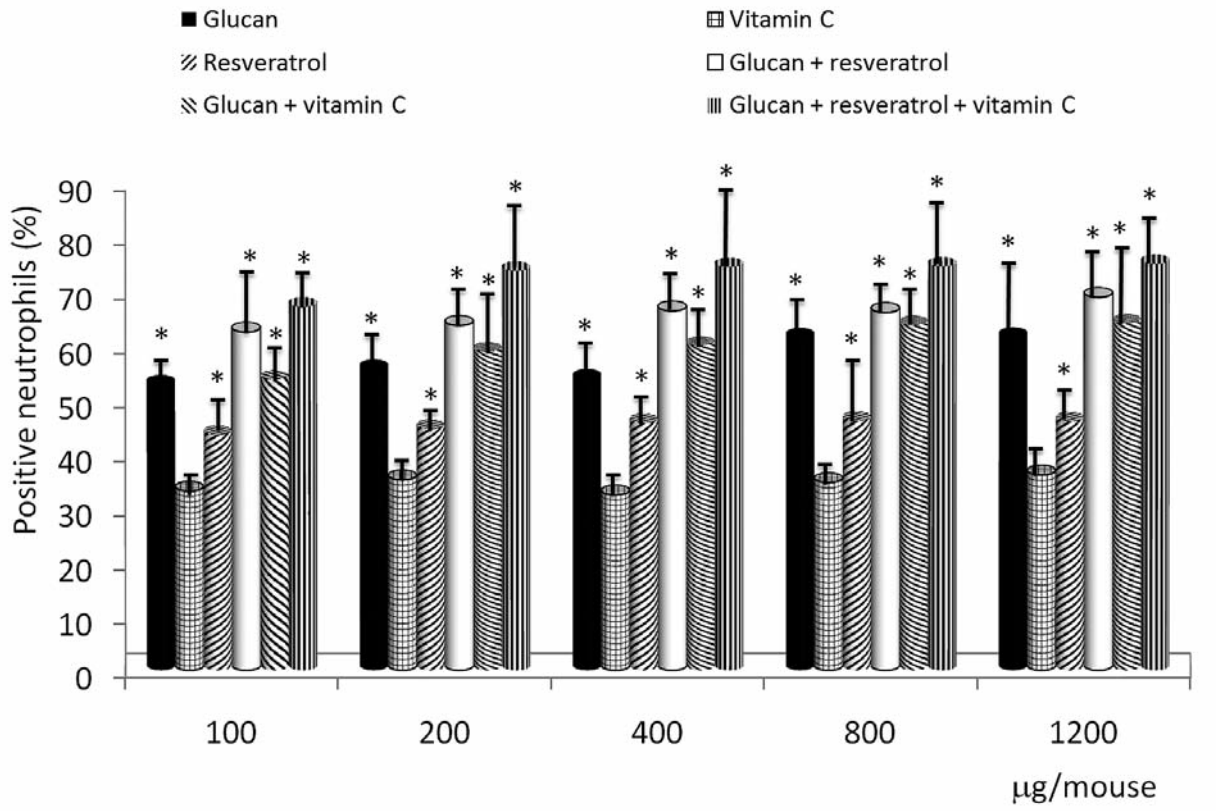

Figure 1. *Effect of an i.p. administration of glucan, vitamin C and/or resveratrol samples on phagocytosis by peripheral blood granulocytes. Each value represents the mean±SD. *Represents significant differences between the control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate. Phagocytosis in the control=30.5%.*

Discussion

Immunomodulators usually offer systemic effects and the mechanisms of their effects are often unknown. This paper focused on the hypothesis that glucan, vitamin C and resveratrol might together offer higher stimulation of immunity that individual molecules. Therefore we decided to monitor their effects on the most important reactions covering both branches of the immune reactions, *i.e.*, both cellular and humoral immunity.

Glucans are well-established immunomodulators with a broad variety of functions, including anti-infection and anticancer activities (for review see 20). At the same time, the activities of glucans were found to increase when used together with various substances such as monoclonal antibodies (21) or vitamin C (10, 22). Our own previous research focused on basic immunostimulating capacity of a combination of glucan and resveratrol and showed the significant synergy of these two compounds (15, 16).

Resveratrol is a non-flavonoid polyphenol with various biochemical, biological and pharmacological activities (23, 24). Vitamin C (ascorbic acid), probably the most popular nutritional supplement, usually acts as a scavenger of free radicals, protecting cells from lethal oxidative stress (25). Recently, both resveratrol (15, 16) and vitamin C (26) gained attention as having possible synergistic addition to glucan.

We tested peripheral blood neutrophils for changes in phagocytosis. Using synthetic microspheres based on 2-hydroxyethyl methacrylate, we found that both glucan and resveratrol caused a significant increase in phagocytosis, whereas vitamin C had no effects. The combinations showed significant synergetic effects. The data shown reflect the effects of a single injection of tested substances. Our preliminary experiments, however, showed that these effects last up to 3 days after treatment (data not shown). These data are in agreement with previously published data using different types of glucan and resveratrol (15, 16).

Glucans are usually considered modulators of the cellular branch of immune reaction and very little attention has been directed to their potential effects on antibody response. Regarding resveratrol and vitamin C, their effects on antibody response have never been tested. We decided to take advantage of our model of evaluating the use of glucan as adjuvant (27). Our results confirmed that

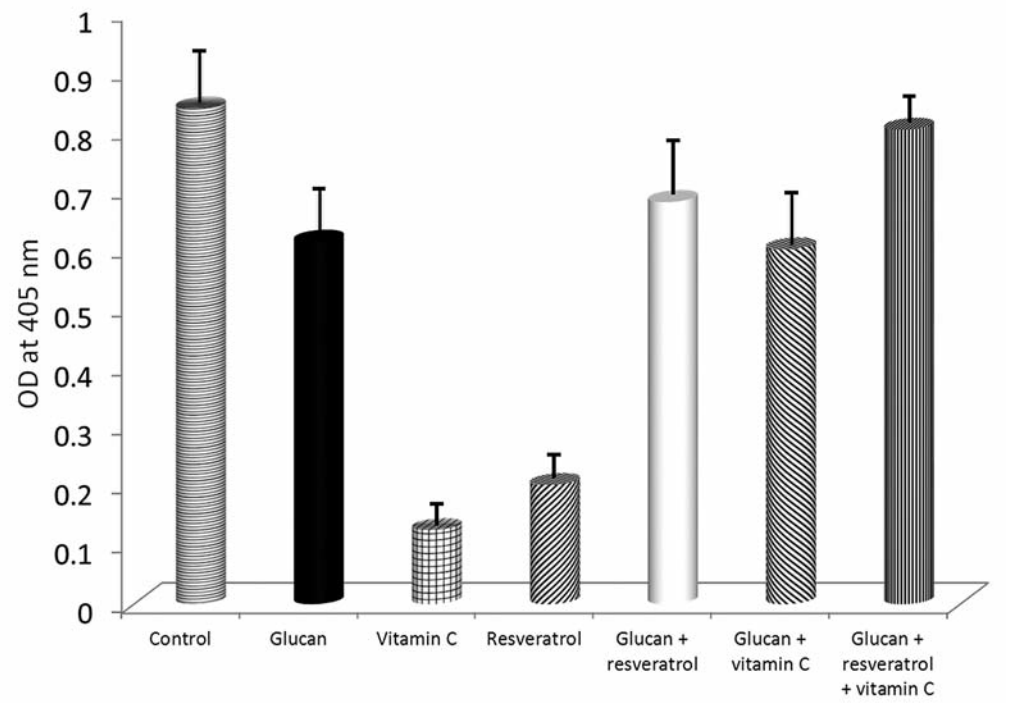

Figure 2. *Effects of two i.p. injections of glucan, vitamin C or resveratrol on formation of antibodies against ovalbumin. Mice were injected twice (two weeks apart) and the serum was collected 7 days after the last injection. The level of specific antibodies against ovalbumin was detected by ELISA. As positive control, Freund's adjuvant was used. *Represents significant differences between the control (ovalbumin alone) and samples at p≤0.05. Individual substances were used at 100 μg/dose. All experiments were performed in triplicate.*

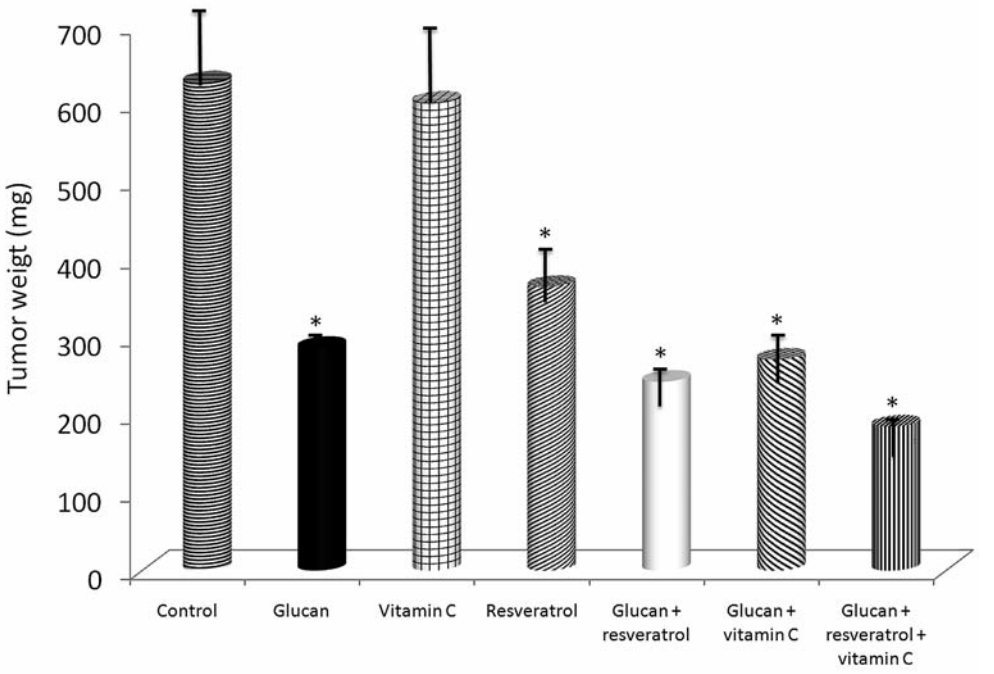

Figure 3. *Therapy of Balb/c mice with Ptas64 mammary carcinoma. Data from three independent experiments are shown. For each experiment, groups of mice were tested for a response to a therapy as indicated by the weight of tumors after two weeks of therapy. For each experiment, individual groups were given daily ip. injections of 100 μg of glucan, resveratrol, vitamin C, or their combination. The control group (659.7±38.5 [mg]) of mice received daily ip. PBS. Each value represents the mean±SD. *Represents significant differences between the control and samples at p≤0.05 level. All experiments were performed in triplicate.*

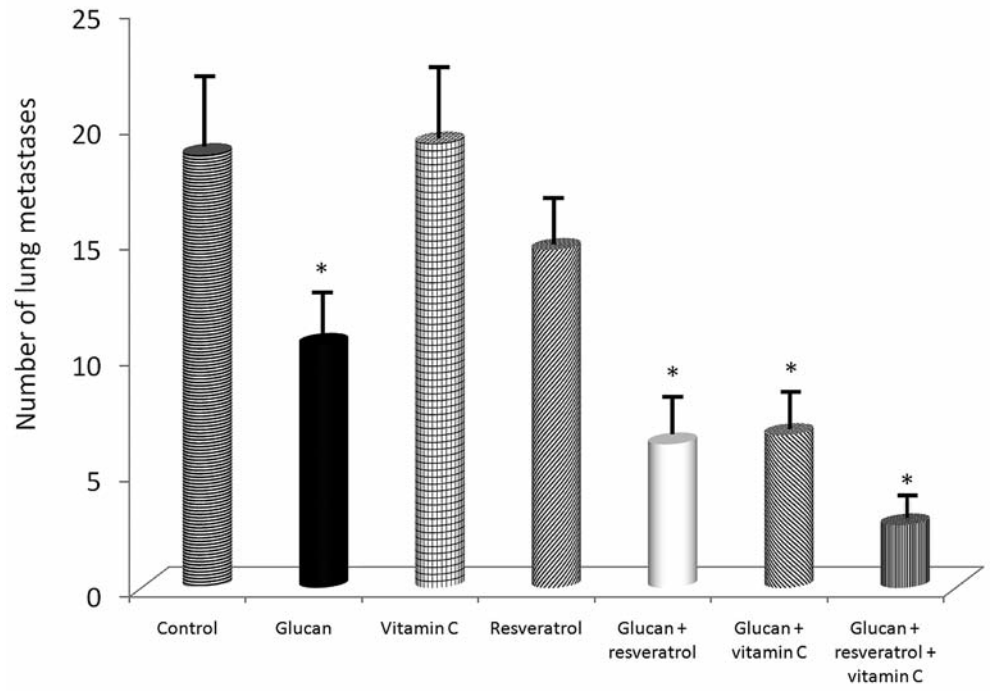

Figure 4. *Effect of tested substances on lung cancer growth in cyclophosphamide (CY)-treated mice. CY (30 mg/kg) was injected into mice on day 8 of the inoculation of 1×10^5 tumor cells. Other experimental groups obtained the daily injections of individual substances over 5 days starting 48 h after injection of CY. Individual substances were used at 100 μg/dose. Each value represents the mean±SD. *Represents significant differences between the control and samples at p≤0.05 level. All experiments were performed in triplicate.*

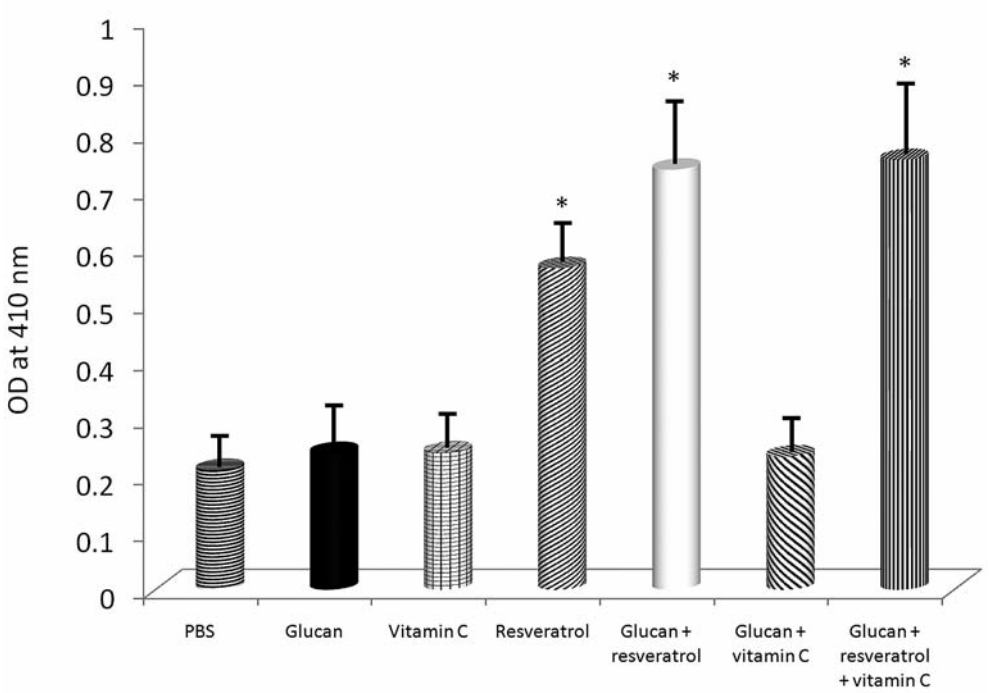

Figure 5. *Effect of an ip. administration of glucan, vitamin C or resveratrol samples on apoptosis. Individual substances were used at 100 μg/dose. Each value represents the mean±SD. *Represents significant differences between control (PBS) and tested samples at p≤0.05 level. All experiments were performed in triplicate.*

glucan elevated the antibody response and that vitamin C and resveratrol alone have no activity. Surprisingly, simultaneous application of both agents showed very strong synergistic effects. The increased response in the glucan-vitamin C group can be ascribed to the presence of glucan.

Our previous studies demonstrated significant *in vitro* and *in vivo* inhibition of mouse and human breast tumor cell growth after glucan treatment (19, 28). Studies performed in numerous laboratories described antitumor activities of glucans in a series of tumor models including hepatic carcinoma, sarcoma and melanoma (for a review see 7). In this study, we used two different models: mouse mammary Ptas64 cells and Lewis lung carcinoma cells, which, in mice can be inhibited by cyclophosphamide treatment, making this model more clinically relevant than sc. injection of tumor cells that is commonly used. In both cases, we found that glucan significantly inhibited cancer formation, reaching 85% of levels obtained with cyclophosphamide (results not shown). This corresponds with the finding of Tsuzuki *et al*. on Sonifilan (29). When the substances were combined, the lowering of cancer cell growth reached 72%, clearly showing synergistic effects of glucan with common chemotherapy. The effects on cancer growth is most probably caused by stimulation of NK cell activity (2, 19, 30). However, based on the observed data, the effects of activation of apoptotic processes cannot be excluded.

Since LPS contamination might mask the effects of any biological response modifier, we checked the LPS contamination of our samples using a 10 μg/ml solution of polymixin B. We prepared LPS-free samples and tested them on their effects on phagocytosis. Because the results were identical to those using regular samples (data not shown), we concluded that LPS is not responsible for data observed in this study.

Lately, the use of several natural immunomodulators combined together is getting more and more attention. In this study, we focused on three modulators – well established glucan and resveratrol, and vitamin C, which role in cancer growth is still controversial. Despite some studies describing the effects of glucan/resveratrol and glucan/vitamin C combination, no study testing all three together exists. To summarize our findings, we can affirm that combined preparation of glucan, resveratrol and vitamin C strongly stimulates both branches of immune reactions and might be useful as a possible alternative approach to cancer therapy. A study attempting to reveal the exact mechanisms of the observed effects is currently in progress.

Conflict of Interests Statement

No conflicts of interest exist for the Authors.

Acknowledgements

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