Evaluation of a Special Combination of Glucan with Organic Selenium Derivative in Different Murine Tumor Models

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Abstract. *β-glucans are well-established immunomodulators with strong effects resulting in slowing or even inhibiting cancer growth. Recent studies have repeatedly suggested that the biological activities of β-glucan can be potentiated by the addition of other bioactive agents. In the current study, we focused on the anticancer effects of a combination of yeast-derived β-glucan and a selenium-linked pseudodisaccharide. Using three different models of murine cancer, we showed that this combination strongly suppressed the growth of all three types of cancers, most likely via the interaction with natural anticancer antibodies.*

Glucans are natural molecules belonging to the cless of biological response modifiers. The most common resources are cell walls of yeast, fungi and seaweed. After original findings of glucan involvement in reduction of infection (1), it was later shown that glucans influence non-specific immunity *via* effects on macrophages, neutrophils and dendritic cells (for review see 2). Later studies demonstrated significant effects on nitric oxide formation (3) and bone marrow restoration (4). Most attention, however, focused on possible glucan-mediated inhibition of cancer. Numerous reports showing positive effects of glucan (5) led up to pre-clinical and clinical trials (6) and, currently, the use of lentinan as a drug in Japanese medicine (7).

With significant immunopotentiating effects of glucan already established, the new search focused on the possibility to further improve already documented biological activities of individual glucans. In fish models, glucan activity was found to be improved by addition of vitamin C (8, 9). Synergistic effects with glucan were also found in cases of trans-3,4',5-trihydroxystilbene (resveratrol), which is a non-flavonoid polyphenol found in various fruits and vegetables (10). Resveratrol has significant anticancer effects alone (11) that were significantly improved by the addition of glucan and vitamin C (12).

Selenium (Se) is a potent micronutrient important for various facets of mammalian health, including optimal immune response. Supplementation with Se increased phagocytosis in sheep (13) and improved activity of natural killer cells in elderly humans (14). In addition, food supplementation with selenium nanoparticles restored blood cells in mice exposed to irradiation (15). Several studies have found that Se-supplementation decreased the risk of colorectal carcinomas (16). Among the suggested mechanisms are antioxidant protection of DNA, stronger carcinogen detoxification, reduction of angiogenesis or stimulation of lymphocyte activity (17, 18).

Interesting findings were obtained when addition of high-Se yeast (commercially produced Se-enriched baker's yeast) was tested on cancer incidence. The National Prevention of Cancer Trial suggested a decrease of the risk of colorectal, lung and prostate cancer (for review see 19). An additional report showed that the predominant form of Se in these products is selenomethionine (20). Together with improvements of glucan effects by the addition of other immunoactive molecules, these facts led us to the study of a possible synergy between glucan and selenium. To achieve this, seleno-pseudodisaccharide derivative (hereinafter referred to as selenide or Se) and yeast-derived β-glucan were used. As this particular glucan was already shown to have significant immunological activity (21), we used three different cancer models to evaluate the possible effects of this combination (hereinafter referred to as Glucan/Se). One model uses mouse breast cancer cell line, the second model evaluates the growth of lung cancer cells. The third experimental model is focused on the role of monoclonal anticancer antibodies.

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Materials and Methods

Animals. Female, 8-week-old BALB/c mice (110 in total) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

Materials. RPMI 1640 medium, glutamine, antibiotics, Limulus lysate test E-TOXATE, polymyxin B and cyclophosphamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA), fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT, USA). Purified $14G_{2a}$ IgG_{2a} anti-G_{D2} mAbs (22) were provided by Dr. Talph A. Reisfeld (Research Institute of Scripps Clinic, La Jolla, CA, USA). Control antibodies of the same isotype were purchased from Sigma. Glucan used in this study is an insoluble b-glucan (62.0% pure) isolated from *Saccharomyces cerevisiae* (Biorigin, São Paulo, Brazil). Insoluble selenium-linked pseudodisaccharide is a 10.65% "organic selenium" from a sugar selenide process synthesis (USP, Brazil). The doses used in this study were 200 μg of glucan and 50 μg of selenide.

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*. (23). The BALB/c mouse-derived mammary tumor cell line Ptas64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI, USA. RMA-S-MUC-1 cells were donated by Drs. O. Finn (Pittburgh Cancer Center Institute, Pittsburgh, PA, USA) and J. Yan (University of Louisville). The human breast cancer cell line ZR-75-1 was purchased from ATCC (Manassas, VA, USA). These cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine and antibiotics.

Lewis lung carcinoma therapy. Mice were injected intramuscularly (*i.m.*) with 5×10^6 of Lewis lung carcinoma cells. Cyclophosphamide (150 mg/kg) was used intraperitoneally (*i.p.*) at day 10 after tumor application. Glucan was used orally from day 0 to day 14 after tumor application. The control group of mice received daily *i.p.* PBS. Each group held a minimum of 5 mice. At the conclusion of the experiment, mice were euthanized and lungs were removed, fixed in 10% formalin and the number of hematogenic metastases in lung tissue was estimated using a binocular lens at ×8 magnification.

Tumor inhibition in vivo. Mice were injected directly into the mammary fat pads with 1×10^6/mouse of Ptas64 cells in PBS. The experimental treatment 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 two weeks of daily oral treatment. After treatment, the mice were sacrificed, tumors removed and weighed (24).

Effect of monoclonal antibodies (mAbs). Evaluation of the effect of Glucan/Se, used simultaneously with anticancer mAbs, was tested as described by Hong *et al*. (25). Mice were implanted subcutaneously (*s.c.*) with RMA-S cells and, after 10 days, treated with the glucan/SE sample given orally and $14.G_{2a}$ anti-G_{D2} ganglioside antibodies. Tumor size and diameters were measured at various intervals (14, 21 and 28 days).

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

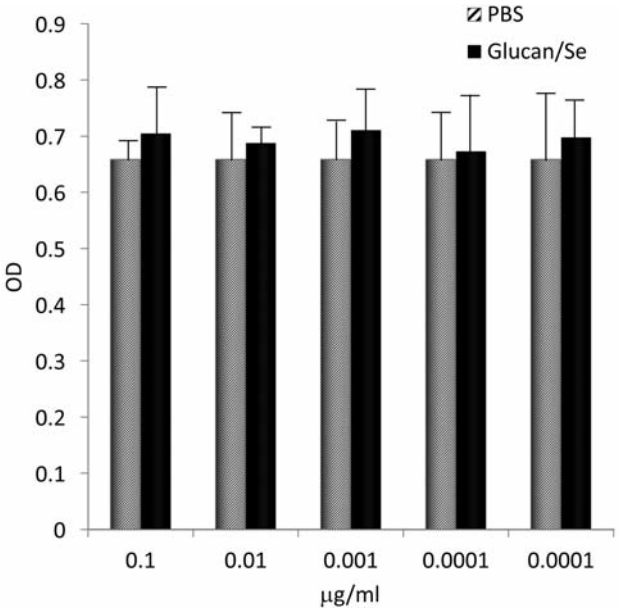

Figure 1. *Effects of addition of different doses of the glucan-selenium-linked pseudodisaccharide combinations to the cultures of ZR-75-1 cell line cultivated in the presence of fetal calf serum. All experiments were performed in triplicates; results are given as mean±SD.*

Results

The effects of the combination of glucan with the selenide have not been yet described. Therefore, we incubated the human cell line ZR-75-1 with different doses of the tested material either in medium enhanced with fetal calf serum or in medium without serum. Data shown in Figure 1 show no stimulating or inhibiting effects of Glucan/Se on the growth of breast cancer cells in the presence of fetal calf serum. When cultured in serum-free conditions, no stimulation of proliferation was observed (Figure 2). In addition, similar experiments were performed with the H209 human lung cancer cell line H209 with identical results.

In the next step, we focused on the role of the tested combination in cancer development. First, we used mice challenged with the Ptas64 mammary cancer cells. These experiments were repeated three times and once with LPS-free material. Our results showed a significant reduction of tumor growth (Figure 3). As the LPS-free experiment showed the same lowering of tumor size (data not shown), we used normal material in all subsequent experiments. Using a model of Lewis lung carcinoma cells, we found a strong 63% reduction of the number of lung metastases in comparison to the control group (Figure 4).

Previous research has shown that the therapeutic effects of glucans are stronger in the presence of naturally-occurring

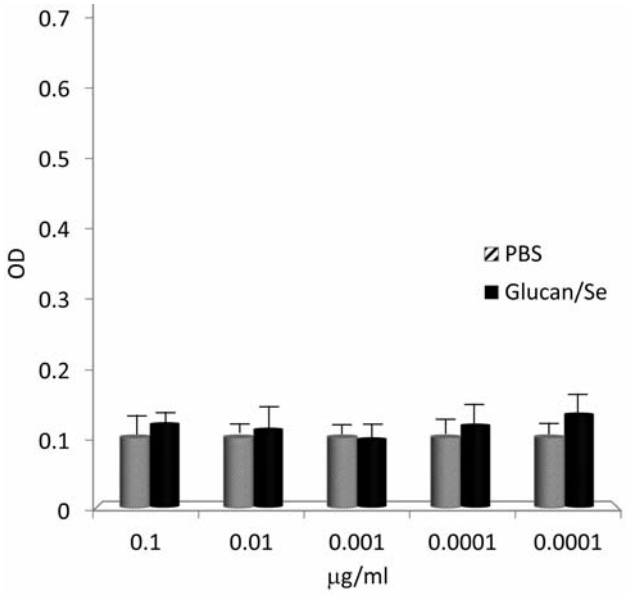

Figure 2. *Effects of addition of different doses of the glucan-selenium-linked pseudodisaccharide combinations to the cultures of ZR-75-1 cell line cultivated in serum-free conditions. All experiments were performed in triplicates. *Each value represents the mean±SD and samples at p≤0.05 level.*

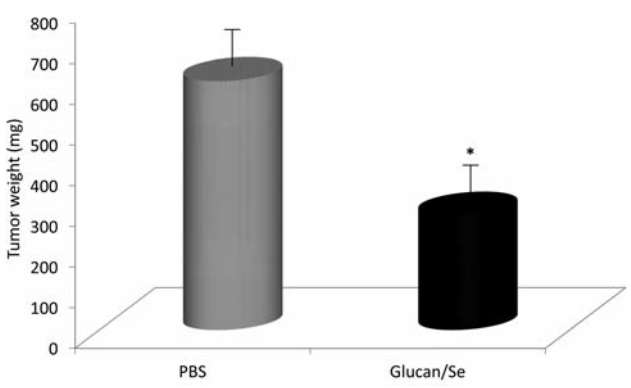

Figure 3. *Therapy of Balb/c mice with Ptas64 mammary carcinoma. Data from three independent experiments are shown. For each experiment, groups of mice were tested for a response to therapy as indicated by the weight of tumors after two weeks of therapy. For each experiment, individual groups were treated orally. The control group (612.7±41.5) of mice received PBS. *Each value represents the mean±SD and samples at p≤0.05 level. All experiments were performed in triplicates.*

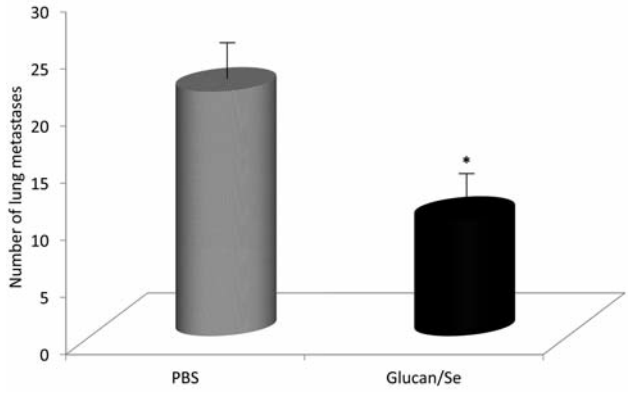

Figure 4. *Effect of tested substances on lung cancer growth in cyclophosphamide-treated mice. Cyclophosphamide (30 mg/kg) was injected into mice on day 8 of the inoculation of 1×10^5 tumor cells, followed by daily oral doses of individual substances starting 48 h after injection of CY. *Each value represents the mean±SD and samples at p≤0.05 level. All experiments were performed in triplicate.*

antitumor antibodies. We decided to test this possibility and used the same experimental design originally developed for yeast-derived glucan (26). The RMA-S-MUC1 cells express high surface density of GD2 ganglioside. As the formation of natural antibodies has been observed after RMA-S-MUC-1 cell implantation (26), it is not surprising that Glucan/Se showed an inhibitory activity even when used alone. However, the combined treatment with Glucan/Se and specific anti-GD2 monoclonal antibody $14.G_{2a}$ was more effective (Figure 5). The antibody alone had no measurable effects, similar to the effects of the control antibody of the same isotype.

Discussion

Biological immunomodulators usually offer systemic effects without clearly defined mechanisms. In this investigation, we focused on the hypothesis that a Glucan/Se combination might offer higher cancer-inhibiting effects than the individual molecules. For our study, we chose three different models of cancer growth – breast cancer, lung cancer, and effects of monoclonal antibodies on cancer growth.

The doses used in this study, 200 µg of glucan and 50 µg of selenide, were based on previously published studies (12, 27). Glucans are not only well-established immunomodulators but, with more than 10,000 published studies, also the most-studied modulators. Their biological activities cover a wide range on biological reactions from cholesterol and blood sugar modulations, stress reduction (28), anti-infectious defense, wound healing support or anticancer effects (29). However, the already substantial activities can be improved by the addition of various biological molecules including vitamin C (8) or humic acid (30).

Selenium supplementation has significant health applications. Its deficiency was observed in numerous

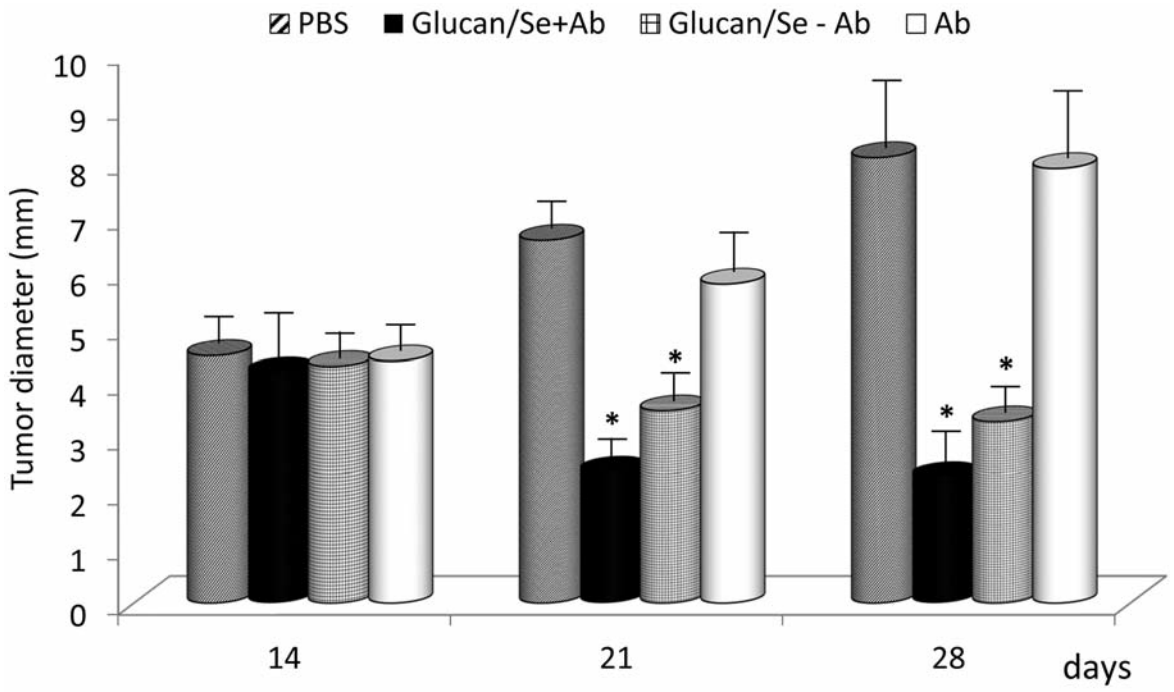

Figure 5. *Combined use of glucan-selenium-linked pseudodisaccharide combination with specific anti-GD2 monoclonal antibodies as treatment of RMA-S-MUC1 cancer cells transplanted into C57Bl/6 mice. *Each value represents the mean±SD and samples at p≤0.05 level. All experiments were performed in triplicate.*

pathological states, which can be changed by addition to food. In addition, its supplementation to food often showed direct beneficial effects. Various versions of selenium-based compounds were found to affect morphology and phagocytic ability of granulocytes (31), ameliorate arthritis in both mouse and rats (27) and reduce splenic immunosuppressive cells (32). In addition, biogenic selenide had immunostimulatory effects in breast cancer studies (33). For our study, we used a well-known glucan combined with an active selenide, which belongs to a category of compounds recently described (34). These kinds of compounds have been reported to have interesting biological properties, such as inhibition of melanin synthesis in melan-A cancer cells (34) and to regulate peroxidase-mediated damage at sites of inflammation (35).

The possibilities of how immunomodulators can influence cancer growth are numerous. Previous studies repeatedly showed both *in vitro* and *in vivo* inhibition of mouse and human breast cancer cell growth after glucan application (24). In order to better evaluate the possible effects of our combination, we used three different models of murine cancer. The first two employed either breast or lung cancer cells. In both cases, the combination of glucan with selenium-linked pseudodisaccharide offered stronger inhibition than individual components. The results are most probably a consequence of the combination of NK cell activation caused by glucan and by induction of IFN-γ and IL-12 secretion caused by the selenide (33).

The third cancer model was based on findings that the therapeutic effects of glucan can be improved by anticancer antibodies. Using a model of RMA-S-MUC1 cells with a high membrane density of G_{D2} ganglioside and corresponding $14.G_{2a}$ monoclonal antibodies, we found a significant tumor regression. We did not only confirm the model introduced by Hong *et al*. (26) but again demonstrated that Glucan/Se caused significantly stronger effects. The requirement for natural anti-cancer antibodies for glucan therapy has been repeatedly described (6, 25).

In conclusion, the present investigation suggests a therapeutic efficacy of glucan in combination with selenide. However, additional studies are required to elucidate the real role of each actively involved compound. In addition, our findings revealed that, when this combination is used together with antitumor antibodies, the tumor regression is stronger. This is lined-up with the current hypothesis suggesting the possibility of using glucan for vaccine preparation where glucan addition would help generate specific anti-tumor antibodies (36). Studies attempting to reveal the exact biological mechanisms of the effects described in our study are currently in progress.

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Conflicts of Interests

No conflicts of interests exist for the authors.

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