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Dossier : Present status of drug from marine origin

Enhancing effects of new biological response modifier β-1,3 glucan sulfate PS3 on immune reactions

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Abstract

Glucans have a long history as non-specific biological modulators; however, but the search for optimal chemical configuration is still on. The objective of this study was to evaluate intraperitoneal application of PS3, a sulfated derivative of a (1−>3)-β-D-glucan isolated from sporophytes of *Laminaria digitata*. PS3 showed significant stimulation of phagocytic activity as well as potentiation of synthesis and release of IL-2 by splenocytes. In addition, PS3 increased NK cell-mediated killing of tumor cells both in vitro and in vivo. When combined, our observations suggest that PS3 is similarly effective as native non-sulfated (1−>3)-β-D-glucan and is generally more active than lentinan.

Keywords: Glucan; Immunity; Defense

1. Introduction

β-1,3 glucans are structurally complex homopolymers of glucose, usually isolated from yeast, fungi, wheat and seaweed. Different physicochemical parameters, such as solubility, primary structure, molecular weight, branching, and polymer charge, influence the biological activities of β-glucans. β-1,3 glucans have been studied extensively for their immunological and pharmacological effects. More than 2000 papers describing their biological activities exist. Thus far, strong immunostimulating effects of β-1,3 glucans have been demonstrated in all tested animal species including earthworms, shrimp, fish, mice, rats, rabbits, guinea pigs, sheep, pigs, cattle and humans [1], making glucan one of the most conserved defense molecules.

Despite decades of research on β-glucan, the controversy between the notion that water-insoluble glucans show only little bioactivity, whereas soluble glucans are highly active (e.g., ref. [2]) and the numerous papers showing that the question of bioactivity is more a question of purity and chemicophysical properties than solubility [3,4]. In addition, detailed studies established that glucans are taken up by macrophages, transported into the immune organs and subsequently degraded into small fragments [5], making the question of solubility irrelevant. This hypothesis, originally suggested by DiLuzio more than 35 years ago, was clearly forgotten [6].

Despite the fact that glucans are recently attracting much attention both in the field of nutritional supplements and new drugs, the search for optimal chemical configuration continues. Some studies suggested that sulfation might increase the biological activities or even potentiate new effects [2]. As naturally sulfated polysaccharides generally have antiviral properties [7], it is not surprising that experimentally sulfated glucans were also successfully used for their antiviral activity [8,9].

Besides the ability to mimic the effects of heparin on smooth muscle cells [10], very little is known about the effects of laminarine sulfate. The follow up study performed by the same group later found inhibition of tumor metastatis by

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laminaran sulfate [11]. Semisynthetic sulfated glucans were also found to be promising anti-inflammatory molecules [12]. In addition, curdlan sulphate was shown to inhibit *Plasmodium falciparum* [13].

The immunostimulating effects of Phycarine (laminarin), (1−>3)-β-D-glucan with a single β-glucose branch at position 6, isolated from Laminaria digitata, are well established both in animals [14,15] and plants [16,17].

(1−>3)-β-D-glucans represent highly conserved structural components of cell walls in yeast, fungi, and seaweed. However, how they mediate their effects remains unknown. The aim of this study was to evaluate intraperitoneal application of sulfated seaweed-derived β-1,3-glucan PS3, since no quality study on immunological effects of sulfation of glucans exist in the literature. We used a sulfated version of Phycarine with a degree of sulfation of 2.4. Previous studies on plant defense showed stronger effects than regular Phycarine [17,18], suggesting that chemical sulfation of Phycarine might increase its elicitor activity. Our observations suggest that PS3 is similarly effective as native Phycarine and is generally more active than lentinan.

2. Materials and methods

2.1. Animals

Female, 6−10 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

2.2. Materials

RPMI 1640 medium, sodium citrate, antibiotics, sodium azide, bovine serum albumine (BSA), obalbumin, Wright stain, Freund's adjuvant, polymixin B and Concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO), fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

2.3. Antibodies

For fluorescence staining, the following antibodies have been employed: anti-mouse CD4, CD8, CD11b and CD19, conjugated with FITC were purchased from Biosource (Camarillo, CA), anti-mouse CD71 and CD122, also conjugated with FITC, were purchased from Pharmingen (San Diego, CA).

2.4. Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12 × 75-mm glass tubes using standard techniques for 30 min on ice. After washing with cold PBS, the cells were resuspended in PBS containing 1% BSA and 10 mM sodium azide. Flow cytometry was performed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer and the data from over 10,000 cell/sample were analyzed.

2.5. Cell lines

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. Murine tumor cell line YAC-1 was provided by Dr. Julie Djeu of the Moffitt Cancer Research Center, Tampa, FL. Each line was maintained in RMPI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

2.6. β-1,3 glucans

The soluble mushroom-derived β−glucan, lentinan (MW app. 1000 kD), was obtained from the Developmental Therapeutic Program, Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

2.7. Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [19]. Briefly: peritoneal cells were incubated 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. The cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types. The same technique was used for evaluation of phagocytosis of peripheral blood cells [19].

2.8. In vitro cytotoxicity assay

Spleen cells were isolated from spleen of mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-s incubation in distilled water, and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion-only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6/ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with Phycarine (2 μg/ml) for 30 min at 37 °C and then washed three times with RPMI 1640 medium. After washing, 50 μl of target cell line YAC-1 (three different concentrations of target cells were used so the final effector-target ratio was 10:1, 50:1 and 100:1). After spinning the plates at 250 × *g* for 5 min, the plates were incubated for 4 h at 37 °C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 10 μl of lysis solution was added into appropriate control wells 45 min before the end of incubation. The next step was to spin the plates at 250 × *g* for 5 min, followed by transferring 50 μl of supernatant into flat-bottomed, 96-well microplates. After 50 μl of reconstituted substrate was added into each well, plates were covered and incubated for 30 min at

room temperature at dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:Percent-specific killing (% cytotoxicity) = 100 × [(OD_{492} experimental − OD_{492} spontaneous) divided (OD_{492} maximum − OD_{492} spontaneous)] as described in manufacturer's instructions, where spontaneous release was target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

2.9. Evaluation of IL-2 production

Purified spleen cells (2×10^6/ml in RPMI 1640 medium with 5% FCS) were added into wells of a 24-well tissue culture plate. After addition of 1 μg of Concanavalin A into positive control wells, cells were incubated for 72 h in a humidified incubator (37 °C, 5% CO_2). At the endpoint of incubation, supernatants were collected, filtered through 0.45 μm filters and tested for the presence of IL-2 [20]. Levels of the IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

2.10. 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 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 daily intraperitoneal injections of Phycarine diluted in PBS (two individual doses). After two weeks of treatment, the mice were sacrificed and the tumors removed and weighed.

2.11. Statistics

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

3. Results

The effects of various glucans on macrophages are well established. However, in order to demonstrate that a new type of glucan really exhibits an immunomodulatory characteristic, an evaluation of phagocytosis is necessary. We measured the effects of one dose of PS3 on phagocytosis of synthetic HEMA microspheres by peripheral blood monocytes (Fig. 1) and neutrophils (Fig. 2). Our results showed that the internalization of synthetic particles by monocytes was more influenced by PS3 application, due to the stimulation of phagocytosis being long lasting (days 2–4), whereas the phagocytosis by neutrophils was stimulated only 24 h after injection. Lentinan showed higher effects on neutrophils.

We then evaluated the effects of PS3 on expression of several membrane markers. At three different time intervals (24, 48, 72 and 96 h, respectively) after an i.p. injection of PS3, spleen cells were isolated and the surface expression of CD4, CD8, CD11b, CD19, CD71 and CD122 was evaluated.

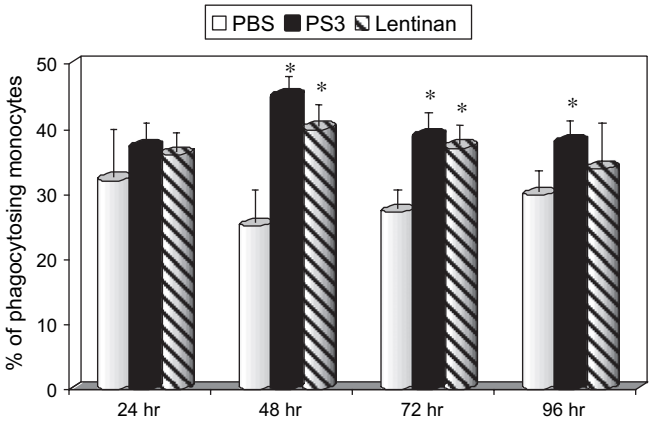

Fig. 1. Effect of i.p. injection of 100 μg PS3 or lentinan on phagocytosis of synthetic microspheres by peripheral blood monocytes. The results represent the mean of three independent experiments ± SD. *Represents significant differences between PBS and glucan at $P \leq 0.05$ level.

The results summarized in Fig. 3 show that PS3 has effect only on splenic lymphocytes, whereas splenic macrophages (CD11b-positive cells) did not show any changes. At the same time, number of CD4-, CD8-, CD71- and CD122-positive cells showed the most increase days 1 and 2, the changes in numbers of CD19-positive cells (B lymphocytes) were significantly increased on days 3 and 4, whereas the increase in positive cells was seen only later.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Fig. 4). The production of IL-2 was measured after a 72 h in vitro incubation of spleen cells isolated from control and glucan-treated mice. PS3 has almost twice the intensive stimulation of IL-2 production than either lentinan or Concanavalin A used as a positive control.

For evaluation of PS effects on NK cells, murine YAC-1 cells were incubated with mouse spleen cells stimulated by either by PS3 or lentinan (Fig. 5). A brief 30-min treatment of PS3 was adequate to cause significant enhancement of

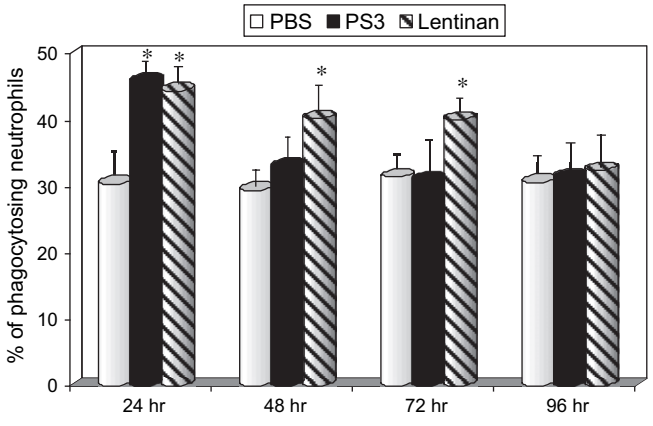

Fig. 2. Effect of i.p. injection of 100 μg PS3 or lentinan on phagocytosis of synthetic microspheres by peripheral blood neutrophils. The results represent the mean of three independent experiments ± SD. *Represents significant differences between PBS and glucan at $P \leq 0.05$ level.

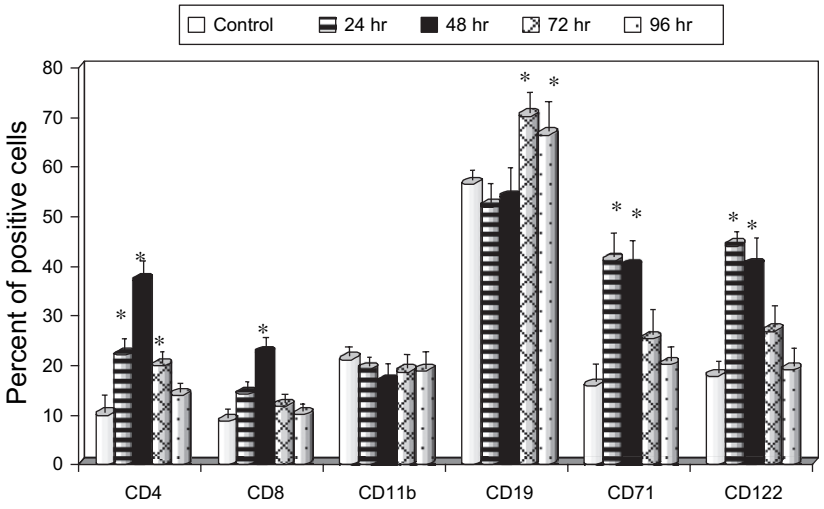

Fig. 3. Effect of i.p. injection of 100 μg PS3 or lentinan on the expression of CD4, CD8, CD11b, CD19, CD71 and CD122 markers by spleen cells. The cells from three donors at each time interval were examined and the results given represent the means ± SD. *Represents significant differences between PS3 and lentinan at $P < 0.05$ level.

cytotoxicity at the higher ER ratio (from 55.2% killed cells vs. 42.1% at ET 50:1 to 65.1% killed cells vs. 48.2% at ET 100:1).

In the final step, mice challenged with Ptas64 mammary tumors were tested for a therapeutic response to daily intraperitoneal injections of PS3 or lentinan (Fig. 6). This experiment was repeated five times (three mice/experimental group) with similar results and was then repeated twice with LPS-free PS3 (data not shown). Our data showed the strong inhibition of tumor growth by both doses of PS3. The inhibition was always significantly higher than that caused by lentinan. In addition, the lower dose (100 μg) of PS3 was almost as effective as much higher dose of 250 μg.

4. Discussion and conclusion

High numbers of individual glucans have been described in the current literature. Due to the huge differences in activities among various glucans isolated from numerous sources, it is important to evaluate its biological properties before any suggestions for use of a particular glucan in clinical practice can be made.

This investigation focused on the biological activities of a sulfated seaweed-derived (1−>3)-β-D-glucan PS3. Originally, interests in seaweed-derived glucans (laminarin) all but disappeared after publishing a study by Baba et al. which stated only moderate effects. However, detailed analysis of this controversial paper revealed that the 62% inhibition of S180 sarcoma cells was mistakenly omitted [21]. Since then, laminarin is used almost entirely in the research of defense reactions of invertebrates [22]. Recently, we demonstrated Phycarine (parental glucan of the PS3 used in this study) to be one of the most biologically active glucans [14,23]. As some studies on plant defense showed stronger effects than regular Phycarine [17,18] and suggested that chemical sulfation of

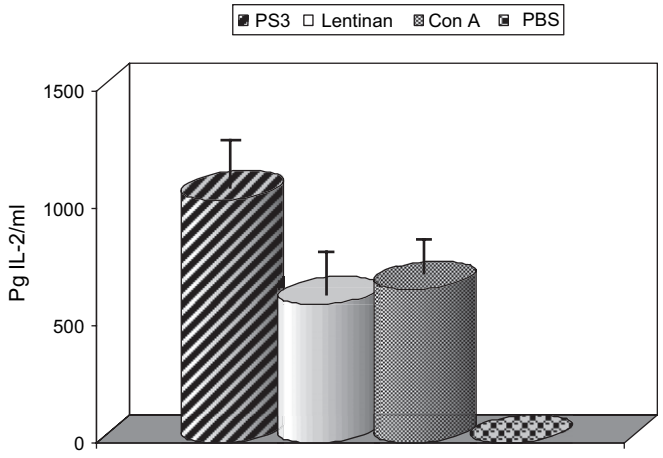

Fig. 4. Effects of glucans on Con A-stimulated secretion of IL-2 by spleen cells. *Represents significant differences between control (PBS) and samples at $P \leq 0.05$ level.

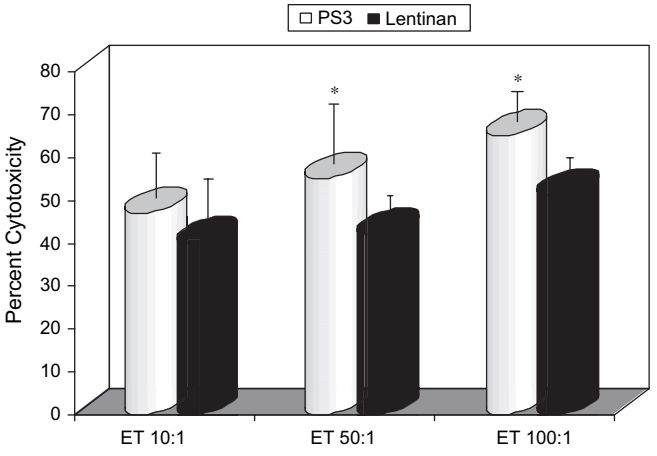

Fig. 5. Different ratios of NK cells to YAC-1 cells were tested for cytotoxicity in the presence or absence of β-glucans for 30 min at 37 °C. The data points shown are mean values ± SD from three experiments. *Differences significant at $P < 0.05$ level at all three effector to target cell ratios.

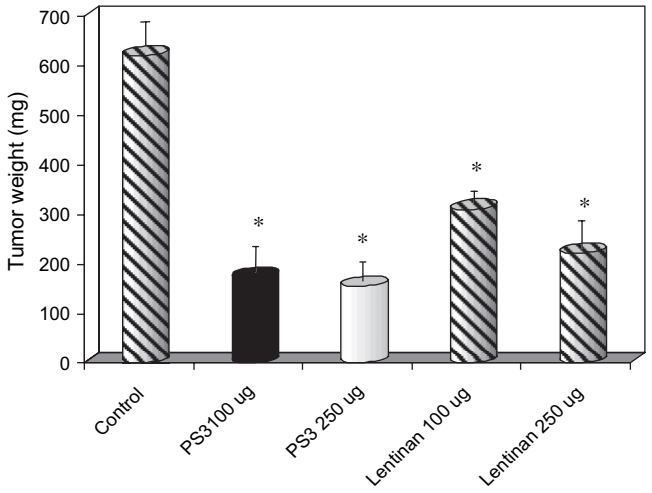

Fig. 6. PS3 or lentinan therapy of Balb/c mice with Ptas64 mammary carcinoma. Data from five experiments are shown. For each experiment, three groups of mice were tested for a response to PS3 or lentinan as indicated by the weight of tumors after two weeks of therapy. For each experiment, individual groups were given daily i.p. injections of 100 or 250 μg of PS3 or lentinan, respectively. The control group of mice received daily i.p. PBS. Each value represents the mean ± SD. *Represents significant differences between PS3 and lentinan treatment at $P < 0.05$ level.

Phycarine might increase its activity, we evaluated the biological activities of PS3.

Various glucans are well known to stimulate phagocytosis [24–26], therefore one of the first tests of the immunological characteristics of any particular glucan is the phagocytosis. We used synthetic microspheres based on 2-hydroxyethyl methacrylate. These microparticles have a slight negative charge and therefore do not specifically adhere to the cell surface, which guarantees that only actively phagocytosing cells will internalize these inert particles [27]. Our data showed that PS3 caused a long lasting stimulation of monocytes phagocytosis. Surprisingly, this stimulation was slower that that of Phycarine [14]. In all intervals, PS3 was more active than lentinan. A different situation has been found in neutrophils-sulphated glucan was active only 24 h after application, whereas lentinan stimulated neutrophils till 72 h after application.

The evaluation of the effects of PS3 on expression of cell surface markers was not easy to interpret. We observed a significant increase in numbers of CD4, CD8, CD71 and CD122 splenocytes. This increase lasted up to 72 h after application. Phycarine showed similar effects but the effects lasted for a shorter period [14]. A similar increase in the number of CD4-positive cells after glucan application has been described by Arinaga's group [28]. Increase of CD19 positive cells (B lymphocytes) occurred later—72 and 96 h after injection. As transferrin receptor (CD71) is present on activated lymphocytes and macrophages, IL-2 receptor (CD122) is present mostly on NK cells. The numbers of positive cells clearly reached over one-hundred percent, indicating that at least some of the tested markers were present on the same population of cells. The double and triple staining cell fluorometry experiments are currently in progress.

In addition to the direct effect on various cells of the immune system, the immunostimulating action of β-glucans is caused by potentiation of a synthesis and release of several cytokines such as TNFα, IFNγ, IL-1 and IL-2. This cytokine stimulating activity is dependent on the triple helix conformation [29]. The only glucan without a trace of pro-inflammatory cytokine stimulation is PGG-glucan [30]. We focused on the stimulation of IL-2 production by spleen cells in vitro and found that PS3 stimulated not only a higher release of IL-2 than lentinan but also than Concanavalin A.

Next we focused on the effects of PS3 on natural killer cells. As numerous papers described glucan-mediated stimulation of NK cells [14,31], it was not surprising that PS3 is also able to stimulate killing of YAC-1 cells by splenic NK cells. In addition, the PS3-mediated killing of tumor targets was significantly stronger than in case of lentinan.

However, the in vitro situation does not always correspond to the situation in vivo. We therefore decided to test the possible effects of PS3 on mouse breast tumor cell line Ptas64. Our previous work demonstrated that there is a high similarity of mouse and human CR3 in response to glucans. This makes the mouse tumor models suitable for investigation of glucans [32]. We used the same experimental design as published before using yeast-derived glucan [33] and PS3 (in two different doses, 100 μg and 250 μg, respectively) had even higher effects (over 70% inhibition of cancer growth) as yeast-derived β-glucan.

LPS contamination might mask the real effects of any glucan preparation. Therefore we checked the LPS contamination of PS3 solutions. In addition, we functionally depleted LPS from PS3 by treatment with 10 μg/ml of polymixin B. We found identical results in all cases. The similarity between results obtained with regular and LPS-free PS3 indicated that minor LPS presence is not responsible for elevation of immunological activities and/or antitumor response.

To summarize our data, our report suggests that PS3 is similarly effective as native Phycarine and is generally more active than lentinan. Conversely, at least on mammalian model, no increase in bioactivity was found.

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