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Effects of marine β−1,3 glucan on immune reactions

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Abstract

Glucans have a long history as nonspecific biological modulators. A novel glucan—Phycarine—was isolated from sporophytes of *Laminaria digitata*. Phycarine showed significant stimulation of phagocytic activity as well as potentiation of synthesis and release of IL-1, IL-6 and TNF-α. In addition, Phycarine increased NK cell-mediated killing of tumor cells both in vitro and in vivo while acting via complement receptor type 3 (CR3) receptors.

Keywords: Glucan; Receptors; Stimulation; CR3; Lentinan

1. Introduction

Polysaccharides, particularly glucans, have a long history as immunomodulators. Interest in glucans increased after experiments showed that zymosan stimulates the macrophages via the activation of complement system [1].

β-1,3 Glucans are structurally complex homopolymers of glucose, usually isolated from yeast and fungi. The isolation from various types of mushrooms was a logical follow-up of the folk remedy use of mushrooms in numerous countries. The number of individual glucans is almost as great as the number of sources used for isolation. Different physicochemical parameters, such as solubility, primary structure, molecular weight, branching, and polymer charge, influence the biological activities of β-glucans.

A variety of β-1,3 glucans have been shown to bind to glucan receptors on monocytes, macrophages, neutrophils and NK cells [2,3]. Despite the progress, it is not clear if there is a separate receptor for glucan [2], only CR3 (CD11b/CD18) receptor [3], dectin-1 receptor [4], or a combination of all these receptors. As biological effects of glucans appear to be multifactorial, it is not surprising that glucans also influence the production and secretion of cytokines.

β-1,3 Glucans have been studied extensively for their immunological and pharmacological effects. More than 600 papers describing the biological activities of glucans exist. Thus far, strong immunostimulating effects of β-1,3 glucans have been demonstrated in all tested animal species including earthworms [5], shrimp [6], fish [7], mice, rats [8], rabbits,

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guinea pigs [9], sheep, pigs [10], cattle [11] and humans.

Various types of glucans have been isolated from almost every species of yeast and numerous fungi. This investigation focused on Phycarine—a seaweed-derived β-1,3 glucan corresponding to previously described laminarin. Laminarin was originally shown to increase phagocytosis [12] and defend against some infections [13]. However, after some papers describing rather confusing data [14], attention was directed to sulphated laminarin [15] that focused on different types of glucan. Only recently, did the existence of a highly purified linear β-1,3 glucan—Phycarine—and subsequent study showing that Phycarine induced a broad range of defensive reactions in tobacco cells [16], return laminarin into the family of biologically active glucans.

2. Materials and methods

2.1. Animals

Female, 6- to 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 Louisvile IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

2.2. Materials

RPMI 1640 medium, socium citrate, dextran, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumine (BSA), Wright stain, and *Limulus* lysate test E-TOXATE were obtained from Sigma (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, USA). The OKM1 hybridoma secreting anti-CR3 α-chain (C-terminal domain)-specific mAb [17] was obtained from the ATCC (Manassas, VA).

2.4. Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12 × 75-mm glass tubes using standard techniques. For test of staining by FITC-labeled Phycarine, pellets of 5×10^5 cells were incubated with 10 μl of Phycarine-FITC (1 to 20 μg/ml in PBS) for 30 min on ice. After washing with cold PBS, the cells were resuspended in PBS containing 1% BSA and 10 mM sodium azide. To measure blockage of monoclonal antibodies [3], cells were incubated in PBS containing Phycarine and then stained by incubation with an OKM-1 monoclonal antibody conjugated with FITC. 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. Hybridomas secreting anti-CR3 (CD11b) antibodies were obtained from the ATCC. Each were maintained in RMPI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

2.6. Blood cells

Blood from healthy volunteers was drawn into sodium citrate anticoagulant. After sedimentation of erythrocytes with 3% dextran, mononuclear cells and neutrophils were separated by centrifugation on a two-step ($d = 1.08$ and 1.105) density gradient of Ficoll Hypaque. The neutrophil cell fraction was washed five times in RPMI 1640 medium and maintained on an ice bath until used. Sterile buffers and aseptic conditions were used in each step. All media and buffers were tested for endotoxin contaminations and shown to contain 0.1 ng/ml of endotoxin using the *Limulus* lysate test (E-Toxate).

2.7. β-1,3 glucans

Phycarine was extracted and purified from the marine brown alga *Laminaria digitata* as described by Klarzynski et al. [16]. Briefly: *L. digitata* sporophytes, harvested in late summer, were extracted with hot water for 2 h. The water extracts were fractionated by two ultrafiltrations, first with a cutoff of 300 kD and second with a cutoff of 1 kD. Resulting retentate was desalted and lyophilized. Molecular weight of laminarin was 5300 kD, as measured by molecular size chromatography coupled with a refractometric detector. Purity, size and structure were further analyzed by ^{13}C NMR spectroscopy and HPAEC-PAD. Using the *Limulus* lysate test, we determined the LPS contamination to be below 0.005 U/ml.

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

2.8. Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [18]. 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 and more HEMA particles were considered positive. The same smears were also used for evaluation of cell types. For phagocytosis of *Candida albicans* (ATCC 18804), the peritoneal cells were incubated with 2×10^8 *C. albicans* spores. After incubation at 37 °C for 60 min with intermittent shaking, the cells were centrifuged. Smears were stained with Wright stain. The cells with three or more *C. albicans* spores were considered positive. *C. albicans* was maintained as described [19].

2.9. 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 $250 \times g$ 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 (Madison, WI, USA) 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 $250 \times g$ 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) = $100 \times$ [(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.

2.10. Cytokine evaluation

BALB/c mice were intraperitoneally injected with various doses (50, 100, and 250 μg) of Phycarine. Control mice obtained PBS only. After various time intervals (10, 30, 60, and 90 min, respectively), the mice were sacrificed and blood was collected in Eppendorf tubes. Subsequently, the serum was prepared, collected and stored at −80 °C for no more than 1 week.

Table 1
CR3 specificity of neutrophil fluorescence staining by FITC-labeled Phycarine

% Inhibition of specific fluorescence by	
Phycarine (50 μg/ml)	OKM-1 anti-CR3 (50 μg/ml)
99.9	76.9

To examine the specificity of Phycarine staining, the cells were incubated for 30 min before staining with either 100-fold excess of unlabeled Phycarine or anti-CR3 antibody OKM-1.

The levels of TNF-α, IL-6 and IL-1 in serum samples were evaluated using a commercial kit OptEIA Mouse TNF-α (Mono/Mono), OptEIA Mouse IL-6 and OptEIA Mouse IL-1 Set (Pharmingen) according to the manufacturer's instructions. The optical density was determined using a STL ELISA reader (Tecan U.S.) at 450 nm with a correction at 570 nm. Data shown in Fig. 2 were calculated from the standard curve prepared by the automated data reduction using linear regression analysis. A standard curve was run with each assay.

2.11. 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 2 weeks of treatment, the mice were sacrificed, tumors removed and weighed.

2.12. Statistics

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

3. Results

Structural analysis performed by ^{13}C NMR spectroscopy and HPAEC-PAD confirmed that Phycarine (laminarin) is, essentially, a linear glucan composed of cca 33 glucopyranose units joined by acetalic β-(1,3) linkages with molecular weight of 5000 Da. The molecule had a β-D-glucopyranose structure comprising 1,3 linkages and a low level of 1,6 branching. The terminal units of the main chain consisted of glucose or mannitol, thus providing two types of molecules, respectively called G or M, in a ratio 25:75. HPLC Dionex evaluation showed purity higher than 97%.

Our previous work demonstrated that normal blood neutrophils were stained in a similar manner by either FITC-glucan or various anti-CR3-FITC antibodies and that it was possible to block the binding of one

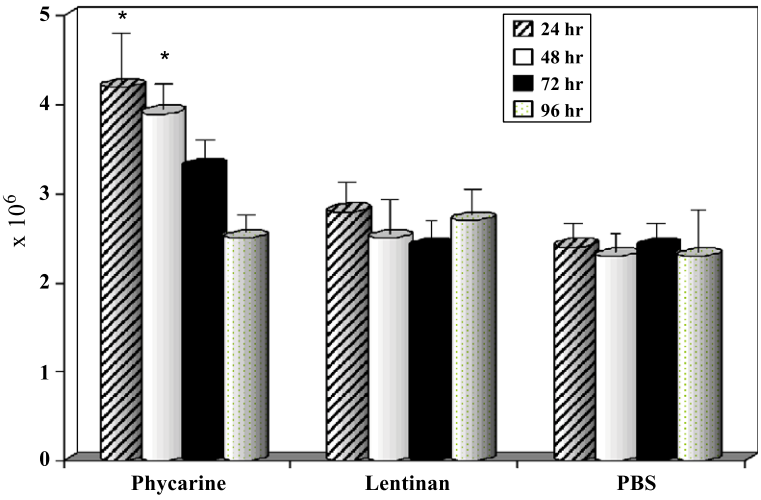

Fig. 1. Effect of i.p. injection of 100 μg Phycarine or lentinan on number of peritoneal cells. The results represent the mean of three independent experiments ± SD. *Represents significant differences between Phycarine and lentinan at $P<0.05$ level.

substance by preincubation of cells with the other [3]. Therefore, we tested the specificity of Phycarine binding to the human neutrophils by preincubation with either 100-fold excess of unlabeled Phycarine or anti-CR3 mAb (Table 1). The data (99.9% inhibition by unlabeled Phycarine and 76.9% inhibition by anti-CR3 mAb) were similar to that found earlier using different type of glucan.

We then tested whether intraperitoneal application of Phycarine has any effect on changes of cellularity in the peritoneal cavity of mice. The cell counts were performed at various time intervals following an i.p. application of 100 μg/mouse of Phycarine or lentinan. The number of cells showed a major increase 24 h after the treatment (Fig. 1), but the significant increase was observed after as long as 48 h. When compared to PBS, the differences were significant even at day 3. The changes were mainly caused by an influx of macrophages, as the numbers of lymphocytes were almost the same.

We then evaluated the effects of Phycarine on expression of several membrane markers. At several time intervals after an i.p. injection of either Phycarine or lentinan, spleen cells were isolated and the surface expression of CD4, CD8, CD11b, CD19, CD71 and CD122 was evaluated. The results summarized in Fig. 2 show that the effects of Phycarine on splenic lymphocytes are 1- to 2-days delayed compared to

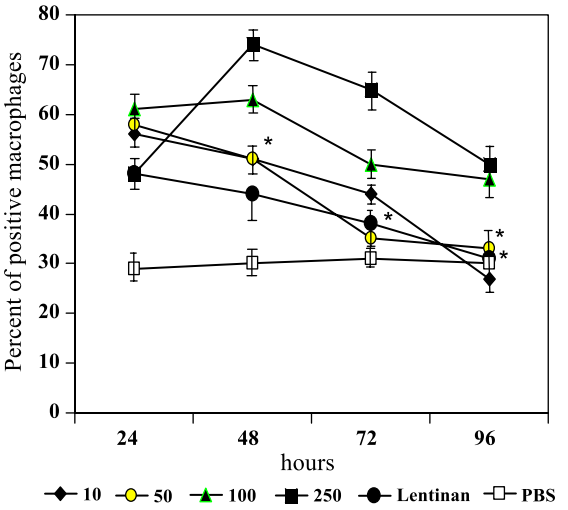

Fig. 3. Potentiation of phagocytosis of synthetic microspheres (HEMA particles) by different doses of i.p. injected Phycarine or lentinan. Peritoneal macrophages with three and more HEMA particles were considered positive. Each value represents the mean ± S.D. All differences were significant at $P<0.05$ level except when marked as *.

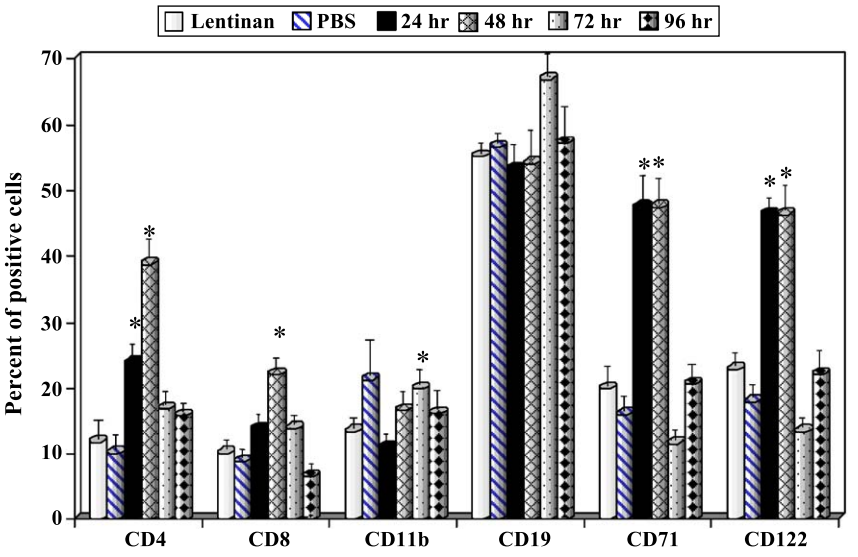

Fig. 2. Effect of i.p. injection of 100 μg Phycarine or lentinan on the expression of CD4, CD8, CD11b, CD19, CD71 and CD122 markers by spleen cells. The cells from three donors at each time interval were examined and the results given represent the means ± SD. *Represents significant differences between Phycarine and lentinan at $P<0.05$ level. The data on lentinan show only 24 h, but these levels were the highest of all four intervals tested. There were no differences between lentinan and PBS.

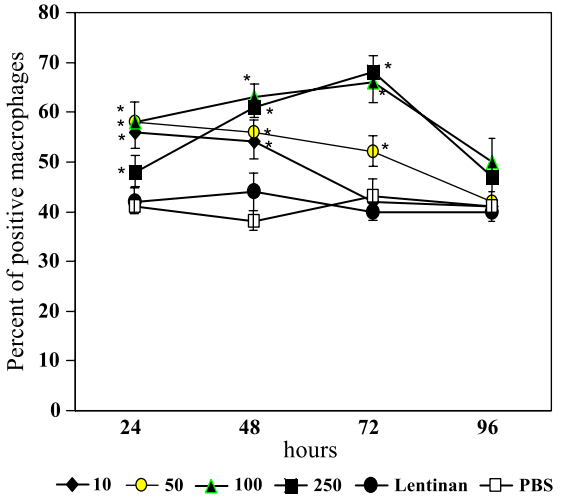

Fig. 4. Potentiation of phagocytosis of *C. albicans* particles by different doses of i.p. injected Phycarine or lentinan. Peritoneal macrophages with three and more *C. albians* particles were considered positive. Each value represents the mean ± S.D. *Represents significant differences at $P<0.05$ level. The data on lentinan show only 250 μg, but these levels were the highest of all four concentration tested. There were no differences between lentinan and PBS.

effects on splenic macrophages (CD11b) as well as peritoneal cells. CD19, the marker of B lymphocytes, was the only marker that did not show any change. The data on lentinan show only 24 h, but these levels were the highest of all four time intervals tested and did not differ from PBS control.

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 different doses of Phycarine on phagocytosis of synthetic HEMA microspheres (Fig. 3) and *C. albicans* (Fig. 4). The internalization of synthetic par-

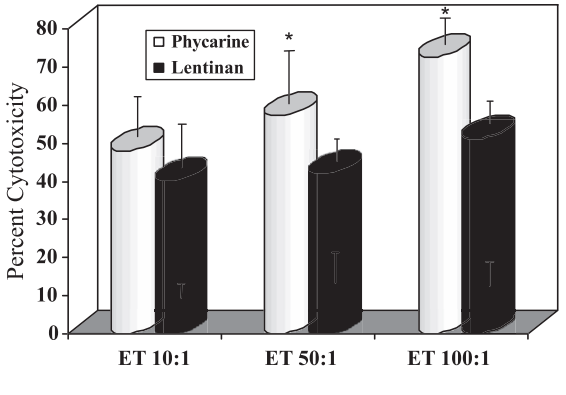

Fig. 5. Phycarine enhances NK cell cytotoxicity of YAC-1 cells. Different ratios of NK cells to YAC-1 cells were tested for cytotoxicity in the presence or absence of β-glucans for 30 min at 37 °C. The data points shown are mean values from three experiments. The differences were significant at $P<0.05$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

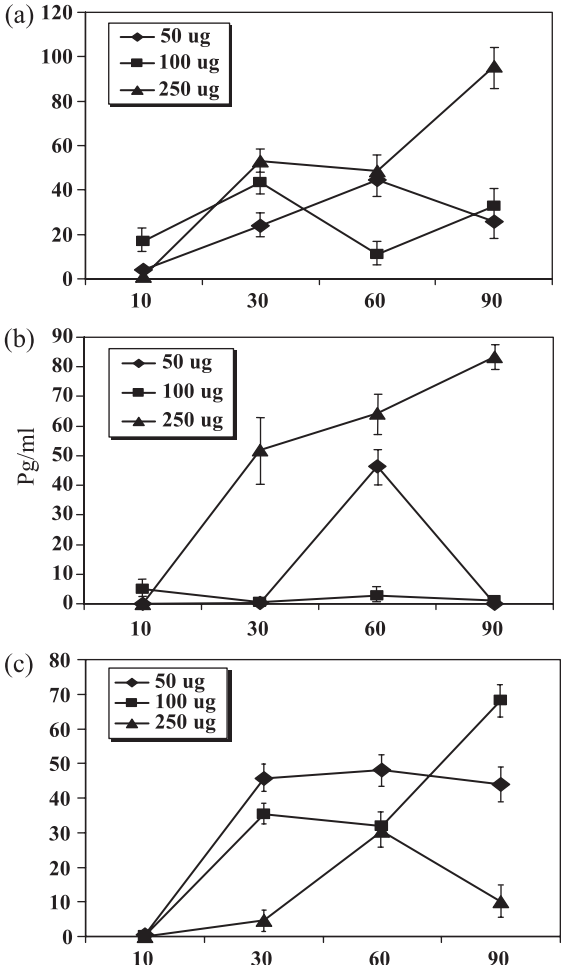

Fig. 6. Effect of different doses of injected Phycarine on levels of IL-6 (a), TNF-α (b), and IL-1 β (c) in peripheral blood. For details, see Materials and methods. Each value represents mean ± S.D. As the control values (PBS) were always zero, each value represents significant differences at $P<0.05$ level.

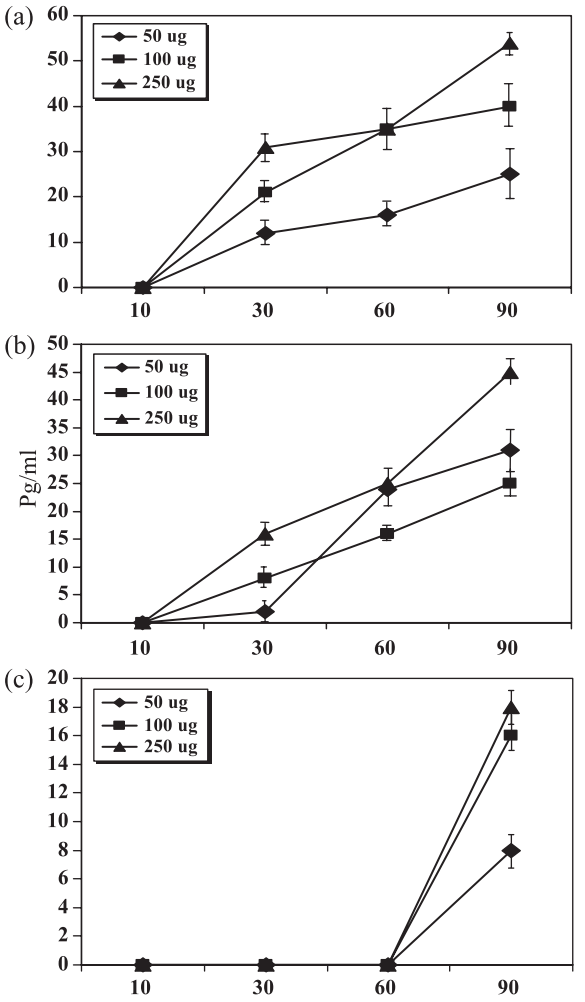

Fig. 7. Effect of different doses of injected lentinan on levels of IL-6 (a), TNF-α (b), and IL-1 β (c) in peripheral blood. For details, see Materials and methods. Each value represents mean ± S.D. As the control values (PBS) were always zero, each value represents significant differences at $P<0.05$ level.

ticles was more influenced by Phycarine application, as higher doses (100 and 250 μg, resp.) caused significant elevation even 4 days after injection. All doses of both glucans showed significant differences compared to PBS at days 1 and 2 only. In the case of phagocytosis of *C. albicans*, the increase was significant up to day 3. The data on lentinan show only 250 μg, but these levels were the highest of all four concentration tested. There were no differences between lentinan and PBS control.

For evaluation of Phycarine effects on NK cells, murine YAC-1 cells were incubated with mouse spleen cells stimulated by either by Phycarine or lentinan (Fig. 5). A brief 30-min treatment of Phycarine was adequate to cause significant enhancement of cytotoxicity at the higher ER ratio (from 57.3% killed cells vs. 42% at ET 50:1 to 72.3% killed cells vs. 53.2% at ET 100:1).

We have compared the effects of a single intraperitoneal injection of three different doses of Phycarine and lentinan on systemic in vivo release of three cytokines, IL-1 , IL-6 and TNF-α. Peripheral blood was isolated at four different intervals after the injection and the serum obtained was stored at − 80 °C for no more than 1 week. The data summarized in Figs. 6 and 7 show significant elevation in levels of IL-6 after every tested dose. These elevated levels were demonstrated as long as 90-min after application of Phycarine. Generally, lentinan-caused increase is lower and more time-dependent. A different situation was observed in the case of TNF-α, where only the highest

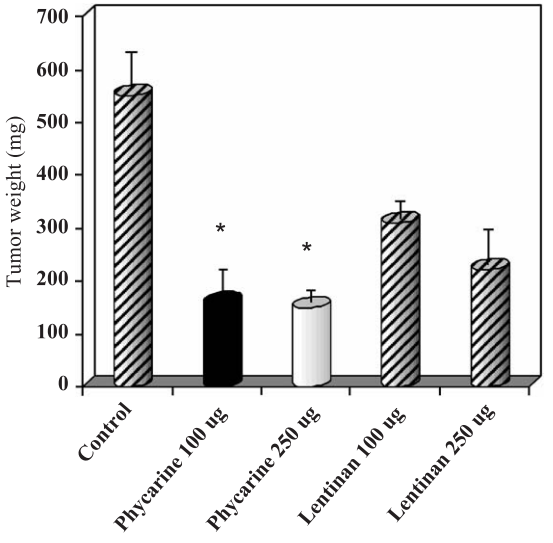

Fig. 8. Phycarine or lentinan therapy of Balb/c mice with Ptas64 mammary carcinoma. Data from five experiments are shown. For each experiment, three groups of mice were tested for a response to Phycarine or lentinan as indicated by the weight of tumors after 2 weeks of therapy. For each experiment, individual groups were given daily i.p. injections of 100 or 250 μg of Phycarine or lentinan, resp. The control group of mice received daily i.p. PBS. Each value represents the mean ± S.D. *Represents significant differences between Phycarine and lentinan treatment at $P<0.05$ level.

dose of Phycarine had significant and long lasting effects. The 40 pg/ml found that 60-min after the administration of the lowest dose cannot be accurately explained. Again, lentinan caused a steady time-related increase, but the levels of TNF-α were always significantly lower and TNF-α appeared later (zero after 10 min). In the case of IL-1β, all three doses caused an elevated production of the cytokines and with a 100-μg dose, there was a clear time-dependent increase in IL-1β. In the case of lentinan, IL-1 was demonstrated only 90 min after application.

In the final step, mice challenged with Ptas64 mammary tumors were tested for a therapeutic response to daily intraperitoneal injections of Phycarine (Fig. 8). This experiment was repeated five times with similar results and then was repeated with LPS-free Phycarine (data not shown). This data showed the strong inhibition of tumor growth by both doses of Phycarine, and the inhibition was always significantly higher than that caused by lentinan.

4. Discussion

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

This investigation focused on the biological activities of a seaweed-derived 1,3β glucan Phycarine. The initial interest in laminarin all but disappeared after publishing a study by Baba et al. [14] which stated only moderate effects. However, detailed analysis of this controversial paper revealed that the 62% inhibition of S180 sarcoma cells was mistakenly omitted. Since then, laminarin is used almost entirely in research of the defense reactions of invertebrates [20].

The present investigation shows that soluble β-1,3 glucan Phycarine is functionally similar to other polysaccharides in current use for therapy. As previously demonstrated, these glucans act via binding to the complement receptor type 3 (CR3) receptors present on NK cells, macrophages and neutrophils [21]. Later, these data were validated further by the use of cells from CR3-deficient mice, which were resistant to the glucan effects [22]. After our data confirmed that Phycarine acts via the same mechanism, we focused on testing its biological effects.

Our group demonstrated previously that normal blood neutrophils, as wells as NK cells and monocytes, are stained in a similar manner by either FITC-glucan or various anti-CR3-FITC antibodies and that it is therefore possible to test the specificity of this binding by using excess of the unlabeled material [3]. When we tested the specificity of Phycarine binding to the human neutrophils by preincubation, with either 100-fold excess of unlabeled Phycarine or anti-CR3 mAb, we found virtually identical results as when yeast-derived glucan [3] was used.

As various glucans are well known to stimulate phagocytosis [23–25], one of the first tests of the immunological characteristics of any particular glucan is the phagocytosis. We used two different and independent experimental design—one using yeast particles and the second using synthetic microspheres based on 2-hydroxyethyl methacrylate. In contract to yeast particles, the HEMA particles have only slight negative charge and therefore do not specifically adhere to the cell surface. This guarantees that only actively phagocytosing cells will internalize these inert particles (for review, see Ref. [26]). In both cases, we found a significant increase of number of phagocytosing peritoneal macrophages after all tested doses of Phycarine. This finding, together with significant 3-day lasting influx of macrophages into the peritoneal cavity, clearly shows the activation of macrophages by Phycarine.

The evaluation of the effects of Phycarine on expression of cell surface markers was not easy to interpret. The only significant changes (in case of CD4, CD8, CD71 and CD122) were shown 24 and 48 h after application of Phycarine. The value later returned to normal. Similar increase in number of CD4-positive cells after glucan application has been described by Arinaga's group [27]. 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 100%, 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, and IL-1. This cytokine stimulating activity is dependent on the triple helix conformation [28]. The only glucan without a trace of cytokine stimulation is PGG-glucan [29].

Most glucans, including oligomer prepared from laminarin [30], have been shown to stimulate TNF-α both in vivo and in vitro [31,32]. The production of TNF-α results in a protective effects against infections [33]. It is hypothesized that glucans enhance leukocyte functions through increased TNF-α secretion, particularly during early stages of infection. In some cases, however, the stimulation of TNF-α was found to be secondary effect of a massive release of IL-1 [34]. The elevated levels of TNF-α and IL-1 after Phycarine injection correlated with findings of effects of lentinan in treatment of human cancer [35] and stimulation caused by Paramylon [36].

As Phycarine binds to the CD11b/CD18 receptor (CR3), it is not surprising that it is also able to stimulate killing of YAC-1 cells by splenic NK cells. However, the in vitro situation does not always correspond to the situation in vivo.

We therefore decided to test the possible effects of Phycarine on mouse breast tumor cell line. Our previous work demonstrated that there is a high similarity of mouse and human CR3 in response to glucans, which makes the mouse tumor models suitable for investigation of glucans [22]. We used the same experimental design as published before using yeast-derived glucan [37] and Phycarine (in two different doses, 100 and 250 μg, resp.) had identical results (60% 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 Phycarine solutions and also functionally depleted LPS from Phycarine by addition of 10 μg/ml of polymixin B. We found identical results in all cases. The similarity between results obtained with regular and LPS-free Phycarine indicated that minor LPS presence is not responsible for elevation of immunological activities and/or antitumor response in treated animals.

The recent isolation and description of a seaweed-derived, highly purified linear β-1,3, glucan named Phycarine [16] stimulated our interests in this currently less common glucan. Our current paper clearly demonstrates that Phycarine acts via the same mechanisms as yeast-derived glucans and that it is, in many cases, even more biologically active. When compared to lentinan, Phycarine showed higher stimulation of defense parameters. These characteristics, together with the fact that it can be easily isolated in sufficient batches and high purity, make Phycarine a prime candidate for commercial use.

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