Orally administered marine (1 → 3)-β-D-glucan Phycarine stimulates both humoral and cellular immunity

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Abstract

(1 → 3)-β-D-Glucans represent highly conserved structural components of cell walls in yeast, fungi, or seaweed. However, it is still unknown how they mediate their effects. The aim of this study was to evaluate both intraperitoneal and oral application of seaweed-derived (1 → 3)-β-D-glucan Phycarine. Phycarine showed significant stimulation of phagocytosis by peripheral blood cells. In addition, the efficiency of chemotherapy of Lewis lung carcinoma with cyclophosphamide was potentiated by Phycarine administration. Phycarine also strongly shortened the recovery of leucopenia caused either by chemotherapy or irradiation. Besides the role in stimulation of cellular immunity, we also found a significant increase of antibody formation. Using a suckling rat model for evaluation of the absorption and tissues distribution of enterally administered 125I-Phycarine, we found that the majority of Phycarine was detected in the stomach and duodenum 5 min after the administration. This amount sharply decreased during first 30 min. A significant amount of Phycarine entered proximal intestine in a shortly after the gavage. Its transit through proximal intestine was decreasing with time and simultaneously increasing in the ileum. Systemic blood levels were very low (less than 0.5%). Taken together, these observations suggest that Phycarine is similarly effective both after i.p. and oral application, has very strong stimulating effects on three types of experimentally induced leucopenia and stimulates both humoral and cellular branch of immune reactions. The majority of Phycarine can be detected throughout the gastrointestinal tract, supporting the feasibility of enteral administration of Phycarine in the treatment of gastrointestinal diseases.

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1. Introduction

Natural products, useful in treating and/or preventing various diseases, have been sought through the history of man. Most of these natural products are plagued with a common problem, i.e., the fact that they often represent a complex mixture of individual ingredients, each of which can contribute to their biological activity. Natural (1 → 3)-β-D-glucans from yeast and mushrooms are well-established biological response modifiers [1,2].

The (1 → 3)-β-D-Glucans have been extensively studied for their immunological and pharmacological effects. More than 2000 papers describing the biological activities of glucans exist. So far, strong immunostimulating effects of (1 → 3)-β-D-glucans have been demonstrated in all tested animal species including earthworms [3,4], shrimps [5], fish [6], mice, rats [7], rabbits, guinea pigs [8], sheep, pigs [9], cattle [10] and humans [11].

Numerous types of glucans have been isolated from almost every species of yeast and fungi. Recently, the existence of a highly purified linear (1 → 3)-β-D-glucan named Phycarine and subsequent study showing that Phycarine induced a broad range of defense reactions in tobacco cells [12], brought new attention to seaweed-derived glucans [13–15]. Our subsequent study showed that Phycarine significantly stimulated phagocytosis,
2. Material and methods

2.1. Animals

Female, 6-10-week-old BALB/c and C57Bl/6J 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 CO2 asphyxiation.

Phycarine absorption studies were performed in 15-day-old Sprague-Dawley rats (Charles River Labs, Portage, MI).

2.2. Materials

RPMI 1640 medium, sodium citrate, dextran, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumin (BSA), gentamycin, Wright stain, and Limulus lysate test E-TOXATE, cyclophosphamide, and 5-fluorouracil were obtained from Sigma Chemical Co. (St. Louis, MO), fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

2.3. Glucan

Phycarine was extracted and purified from the marine brown alga Laminaria digitata as described in Klarzynski et al. [12]. 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 kDa and second with a cutoff of 1 kDa. Resulting retentate was desalted and freeze-dried. Molecular weight of laminarin was 5300 Da, as measured by molecular size chromatography coupled with a refractometric detector. Purity, size and structure were further analyzed by 13C NMR spectroscopy and HPAEC-PAD. Using the Limulus lysate test, we determined the LPS contamination to be below 0.005 U/ml.

2.4. Antibody formation assay

Mice were fed with Phycarine in drinking water (250 μg/mouse) for 28 days. Five days before the end of experiment, mice were immunized i.p. with 0.5 ml of sheep erythrocytes in PBS. The antibody formation by splenocytes was evaluated by modified Jerne plaque assay [21].

2.5. Phycarine iodination

One mg of IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma) was dissolved in 5 ml of chloroform and 50 μl of this solution was transferred into glass tubes. Chloroform was evaporated and a thin film of IODO-GEN was deposited on the walls of the glass tubes. Tubes were stored at 0°C until use. One hundred micrograms of Phycarine dissolved in PBS were incubated with 11.1 MBq of NaI125 (Amersham Biosciences, Piscataway, NJ) at 25°C for 1 h. Following the incubation, the mixture was purified by gel filtration on a PD 10 column (Sephadex G25M, Sigma) using 0.1 M phosphate buffer (pH 7.2), as a mobile phase.

2.6. Cell lines

The Lewis lung carcinoma cells were obtained from Dr. G. Ross (University of Louisville, Louisville, KY) and were cultivated as described in Kogan et al. [22]. YAC-1 cells were obtained from ATCC (Manassas, VA) and cultivated in RPMI 1640 medium supplemented with 10% FCS.

2.7. Irradiation

Mice were irradiated by the 500 cGy sub-lethal doses (Department of Radiology, University of Louisville). At different intervals, mice were fed orally with Phycarine in water. Subsequently, individual mice were sacrificed and the cellularity in peripheral blood, bone marrow, spleen and thymus was evaluated. The number of cells in blood has been evaluated as follows: one drop of blood from the orbital plexus was mixed with 95 μl of Turk’s solution and incubated for 5 min at room temperature. One drop of this solution has been dropped into hemocytometer and counted under an optical microscope.

The numbers of cells in bone marrow were evaluated as follows: mice were killed by cervical dislocation, the legs were separated from the body at the hip joint and the feet were removed. The legs were placed in a Petri dish obtaining RPMI 1640 medium. All muscle tissue from the femurs and tibia was removed and the bones were separated (only femurs were used). The epiphyses were cut off on both ends, the bone end was...
punctured with a 23G needle and flushed out with 3 ml of warm (22°C) RPMI 1640 medium. The large debris and cell clumps were removed by layering the cell suspension over 3 ml of heat-inactivated FCS for 10 min on ice. The cells collected from the top of FCS were washed once by centrifugation at 300 x g for 10 min at 4°C and kept in RPMI 1640 medium containing 5% FCS. One drop of the final solution was dropped into hemocytometer and counted under an optical microscope.

2.11. 5-Fluorouracil

Mice were injected i.m. with 5 x 10^6 of Lewis lung carcinoma cells. Cyclophosphamide (150 mg/kg) was used i.p. at day 10 after tumor application, Phycarine (250 µg/mouse) was used either i.p. or orally from days 0 to 14 after tumor application. The control group of mice received daily i.p. PBS. Each group held a minimum of five mice. At the conclusion of the experiment, mice were euthanized, lungs removed, fixed in 10% formalin and the number of hematogenic metastases in lung tissue was estimated using a binocular lens at 8 x magnification.

2.12. Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [24]. Briefly non-stimulated peritoneal cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5 x 10^8 ml^-1). 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.

2.13. Apoptosis

Six Balb/c mice from the control group (drinking water) and six mice from the glucan group (drinking Phycarine) were sacrificed by cervical dislocation. Splenes were disintegrated in glass homogenizer in of HEMEmd medium (Sebac Co., Germany) and the splenocyte suspension was washed. Cells were pipetted into 96 U-bottom microtiter plates (0.75 x 10^6 per well) and then 2 x washed in FACS-PBS (PBS, 0.1% gelatine, 0.02% sodium azide). To avoid non-specific binding of monoclonal antibodies, washed cells were blocked with 10% heat-inactivated murine serum (10 µl per well) for 20 min on ice and stained by mAb CD19-biotine (Becton-Dickinson, Franklin Lakes, USA), diluted 1:2000 (10 µl per well) for 30 min on ice. After being washed 3 x, PE-Cy7-labelled streptavidin (Caltag, CA), diluted 1:200, was added to bind to biotinylated CD19 antibody (10 µl per well) for 30 min on ice. After streptavidin binding, the cells were 2 x washed by FACS-PBS and 1 x washed in Annexin V binding buffer (Exbio, Prague, Czech Republic) and then were stained by of FITC-labeled Annexin V, diluted 1:100 (10 µl per well) for 15 min on ice. Finally, cells in each well were resuspended in 20 µl of Annexin V binding buffer. Ten minutes before measuring, 10 µl of Hoechst 33258 dye (Molecular Probes, OR), final dilution 0.1 µg/ml, was added to all samples to exclude dead cells and to stain phases of apoptosis (necrosis and late phase). Cells in the early phase of apoptosis show Annexin V positivity and Hoechst 33258 negativity [25]. FACS analyses were performed on LSRII Instrument (Becton-Dickinson, Franklin Lakes, FL). Collected data were analyzed by cytometric data analysis software Flowjo (Tree Star, OR).

2.14. Absorption studies

Forty pups, both male and female originating from four different litters, were used in these studies. Pups were fasted for 20 h before the beginning of experiment. Fasted pups were kept in plastic cages, which were placed with their bottom half on an electric heating pad to help the pups regulate their normal body temperature. At the beginning of experiment, pups were gavaged with 200 µl of 0.9% saline containing approximately 12,000 cpm of 125I Phycarine.

At selected time points (5, 10, 30, 60, and 120 min after Phycarine administration), the pups were anesthesitized and decapitated. A 100 µl of systemic blood was collected. Then, the stomach, duodenum, the small intestine, liver, and kidney were quickly removed. The small intestine was divided into two halves (proximal and distal) and all collected samples were counted on a Packard Cobra II gamma counter (Packard BioScience, Downers Grove, IL). Results were expressed as a percentile of the original dosage. To evaluate the fate of administered Phycarine, total recovery of labeled Phycarine was calculated as a percentile of administered dose.

2.15. Statistics

Student's t-test was used to statistically analyze the data.
recovery of thymocytes occurred later (ca. day 10).

Cyclophosphamide was injected either alone or in combination with Phy­

carine. Peripheral blood neutrophils 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 the lowest dose at 24 h.

3. Results

Structural analysis of the (1 → 3)-β-D-glucan used in this study was performed by 13C NMR spectroscopy and HPAEC-PAD and confirmed that Phy­
carine is a linear laminarin composed of ca. 33 β-1,3-linked Glc residues with molecular weight of 5000 Da.

Our previous work confirmed the well-established effects of glucans on phagocytic activity of peritoneal macrophages. To be sure that Phy­
carine is also affecting blood cells, evaluation of phagocytosis of peripheral blood cells was necessary. We measured the time-dependent effect of different doses of Phy­
carine on phagocytosis of synthetic HEMA microspheres (Fig. 1). All four tested doses of Phy­
carine caused a significant increase of internalization of synthetic particles, and, with exception of 10 μg/mouse dose, 48 h was necessary to see the stimulation. However, this increase was significant up to day 4 after the treatment.

Using a model of Lewis lung carcinoma cells, we showed that cyclophosphamide administered in the used dose caused 70% inhibition of the number of lung metastases in comparison to the control group. Treatment with Phy­
carine (either applied orally or intraperitoneally) caused 80% and 76% inhibition, respectively. Even higher inhibition was achieved when both substances were used in combination—92% inhibition in both cases (Fig. 2). The suppression of cancer growth might be caused by significant stimulation of natural killer cells shown in Fig. 3—the increase at effector-target ratio 64:1 was not significant, but lower ratio proved this trend.

Based on earlier studies showing that glucan can repair the bone marrow damage caused by irradiation [26], we tested if Phy­
carine can reverse the leucopenia caused by irradiation. Data summarized in Fig. 4 show the fast cellular recovery in bone marrow, blood, and spleen under all experimental conditions, recovery of thymocytes occurred later (ca. day 10).

Similarly, we tested cyclophosphamide-induced leucopenia in peripheral blood and bone marrow. On day 0, cyclophosphamide was injected either alone or in combination with Phy­
carine, and cellularity of peripheral blood and bone marrow was evaluated daily. The results summarized in Fig. 5 show that the blood cell counts were significantly suppressed even 10 days after cyclophosphamide treatment, but Phy­
carine-treated animals already had a normal blood cell counts. These data were even more profound in bone marrow-cyclophosphamide-treated mice were again leucopenic even at day 10. However, Phy­
carine-treated animals showed normal bone marrow cell counts as early as day 7. The faster recovery in peripheral blood than in bone marrow probably represents either demargination of the white cells from the endothelium or changes in homing of cells into individual lymphatic organs.

Similarly to cyclophosphamide, cancerostatic drug 5-fluorouracil is well known for significant depression of immune system [27]. We evaluated the effects of orally given Phy­
carine on fluorouracile-induced leucopenia. The data showed that Phy­
carine increase the recovery of bone marrow in all tested time intervals with the fastest recovery being when used prophylactically 14 days before injection of 5-FU (Fig. 6). In blood, only

Fig. 1. Potentiation of phagocytosis of synthetic microspheres (HEMA parti­
cles) by different doses of i.p. injected Phy­
carine. Peripheral blood neutrophils 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 the lowest dose at 24 h.

Fig. 2. Phy­
carine therapy of C57Bl/6 mice with Lewis lung carcinoma. Data from three experiments are shown. For each experiment, mice were tested for a response to Phy­
carine and/or cyclophosphamide (CPA) as indicated. CPA was used at day 10 after tumor application. Phy­
carine was used either i.p. or orally from days 0 to 14 after tumor application. The control group of mice received daily i.p. PBS. Each value represents the mean ± S.D. All differences were significant at P < 0.05 level.

Fig. 3. Oral feeding with Phy­
carine increased natural killer cell activity. For each experiment, four wells per sample were evaluated, each experiment was repeated three times. Control group of mice was fed with PBS. * Not significant, ** significant at P < 0.005 level.
Phycarine, used 7 and 14 days prior 5-FU treatment, showed any effects; however a return to a 55% original cellularity was found as soon as 4 days after 5-FU. In thymus, all time intervals were similar. A different situation has been found in spleen, where all intervals showed fastened recovery, but the longest Phycarine treatment once again showed a sharp improvement as early as 4 days (Fig. 6).

The results of evaluating the effects of oral administration of Phycarine on antibody response are shown in Fig. 7. Mice were fed for 28 days and showed significant increase in number of B lymphocytes forming both IgM and IgG antibodies.

The protective effects of Phycarine were further supported by finding that feeding with Phycarine caused decrease on relative portion of early phases of apoptosis in spleen cells (Fig. 8).

As expected, the majority of Phycarine was detected in the stomach and duodenum 5 min after the administration. This amount was sharply decreasing during first 30 min (from 76% to 24%), then slower decreasing trend continued up to 120 min (Fig. 9). A significant amount of iodine-labeled Phycarine (approx. 18%) entered proximal intestine in a very short time after the gavage. Phycarine transit through proximal intestine was decreasing with time and simultaneously increasing in the ileum. This suggested that a significant portion of Phycarine passed throughout the proximal intestine into ileum (Fig. 9). At 30, 60 and 120 min, about 25–29% of administered Phycarine was detected in the ileum. The amount of Phycarine detected in the liver was approximately 0.5–1.5% (Fig. 6) and similar results were detected in kidney (0.2–1%; Fig. 5). Levels of Phycarine in systemic blood levels were very low (less than 0.5%). In the

Fig. 4. Phycarine-induced cell recovery in bone marrow (A), spleen (B), thymus (C), and peripheral blood (D) after irradiation. At different intervals (i.e. at days 0, 7 or 14 days before irradiation), mice were daily fed orally with Phycarine in water. Subsequently, individual mice were sacrificed and the cellularity in blood, bone marrow, spleen and thymus was evaluated. The results represent mean of 10 mice in three independent experiments.

Fig. 5. Mice were injected on day 0 with either cyclophosphamide or with cyclophosphamide and Phycarine (250 μg/mouse). On each subsequent day, mice were sacrificed and assayed for peripheral blood (A) and bone marrow and (B) cellularity. The results represent mean of 10 mice in three independent experiments.
Fig. 6. Phycarine-induced cell recovery in bone marrow (A), spleen (B), thymus (C), and peripheral blood (D) after 5-fluouracil-induced leucopenia. At different intervals, mice were daily fed orally with Phycarine in water. Subsequently, individual mice were sacrificed and the cellularity in blood, bone marrow, spleen and thymus was evaluated. The results represent mean of 10 mice in three independent experiments.

Fig. 7. Absolute number of mouse splenocytes forming hemolytic plaques/10⁶ splenocytes. Mice were fed with Phycarine for 28 days, control group was fed with PBS. * Significant at <0.05 level, ** significant at <0.005 level.

initial time point of this study (5 min), the majority of labeled Phycarine (95%) is localized in the stomach and proximal small intestine. The total recovery of administered Phycarine (Fig. 10) was gradually decreasing with time, reaching approx. 60% after 120 min.

4. Discussion

Our previous paper focused on basic immunostimulating capacities of Phycarine and explained that the mechanism of action is based on interaction of Phycarine with the CR3 receptor [16]. This investigation showed that Phycarine has a strong ability to augment hematopoesis as documented by causing fast recovery after several types of experimental leucopenia. In addition, these effects were similarly pronounced when Phycarine was applied via intraperitoneal or oral administration. The mechanisms of glucan transfer through the gastrointestinal track were evaluated and discussed.

Various types of glucans are known to stimulate phagocytosis [19,28]. Therefore detailed evaluation of this basic type of immune reaction is important for determination of effectiveness of glucan. After establishing the effects of Phycarine on macrophages, we tested also the peripheral blood leukocytes for changes in phagocytosis. Using synthetic microspheres based on 2-hydroxyethyl methacrylate, we found that all tested doses of Phycarine caused significant increase in phagocytosis of these synthetic particles. In all cases (with exception of dose 10 μg/mouse), there was a 24 h lack period before any signifi-

Fig. 8. Relative percentage of early phase of apoptosis in splenocytes isolated from mice fed either with Phycarine or PBS. The differences are significant at P < 0.005 level.
significant changes could be observed. Conversely, the effects of a single injection of Phycarine were long-lasting with significant changes found even 4 days after treatment. These data were in agreement with stimulation of peritoneal macrophages [16].

Our previous studies demonstrated significant in vitro and in vivo inhibition of mouse and human breast tumor cell growth after (1→3)-β-D-glucan treatment [16,29]. Studies performed in numerous laboratories described anti-tumor activities of glucans in a series of tumor models including hepatic carcinoma, sarcoma and melanoma (for a review see Ref. [30]). In this study, we used a model of Lewis lung carcinoma cells, which in mice can be inhibited by cyclophosphamide treatment, making this model more clinically relevant than s.c. injection of tumor cells that is commonly used. Our data showed that both oral and intraperitoneal delivery of Phycarine was at least as effective as treatment of mice with cyclophosphamide. This corresponds with the finding of Tsuzuki et al. on sonifilan [23]. When both substances were combined, the inhibition of cancer cell growth reached 92%, clearly showing synergistic effects of (1→3)-β-D-glucan with common chemotherapy. The inhibition of cancer growth is most probably caused by stimulation of NK cell activity [16,31].

The major side effects of both traditional chemotherapy and/or irradiation are leucopenia and significant suppression of the immune system. Leukopenia caused by either chemotherapy or irradiation is a significant problem in most cancer patients. Both of these negative effects limit the dosage and frequency of treatment [32]. The decision to use 5-fluorouracil and cyclophosphamide as models for measuring the effects of glucan-based recovery was based on two facts—effects of fluorouracil on cellularity of lymphoid organs is well-documented and cyclophosphamide was previously used, allowing the comparison of different glucans. The effects of injected (1→3)-β-D-glucan on enhanced recovery after experimental leucopenia have been documented [33], in the cyclophosphamide-induced leucopenia model there usually was a strong peak around day 3 followed by slow recovery [23,34]. Similar data were found after irradiation. Phycarine exhibits a preferred type of recovery without the short-term overstimulation of myelopoiesis.

Currently, glucans are getting closer to clinical practice. In addition, orally effective lentinan is already being used in Japan [11]. It was therefore important to know if Phycarine can similarly improve the cell recovery when used orally. Using a 5-fluorouracil model with well-established destruction of...
immune cells [27], we found a strong prophylactic effect of orally given Phycarine. These effects are most probably caused by CR3-mediated effects of glucan on hematopoietic progenitor cells [35]. A limited number of studies evaluating the effects of (1 → 3)-β-D-glucan on apoptosis yielded conflicting results ranging from no effects [36] to inhibition [37] or even direct induction [38]. Based on our data, we can conclude that at least some glucans offer protection against apoptosis.

Glucans are usually considered as immunomodulators affecting only the cellular branch of immune reactions, and their potential to increase antibody response was demonstrated only when used as an adjuvant [39]. Our results showing a strong stimulation of both IgM and IgG formation by four weeks of oral administration of Phycarine show that glucan can modulate and stimulate both humoral and cellular immunity and further support its possible clinical applications.

However, very little is known about the gastrointestinal absorption of glucans. Rice’s group showed significant differences among various glucans in plasma concentration and binding of glucan by gastrointestinal epithelial and GALT cells [40]. These studies were performed in the adult rats with fully developed digestive and immune systems. Our focus was on the absorption of (1 → 3)-β-D-glucan during the suckling period when the intestinal barrier function and transport function are not fully established. Immature intestine undergoes dramatic developmental changes of the surface area influenced by rapid growth of intestinal mucosa [41]. All these changes often lead to the increased susceptibility of developing organism and compromised immune response. Thus, clarification of the absorption of (1 → 3)-β-D-glucan in neonatal and pediatric patients. Results from our studies suggest that only very limited amount of glucan is absorbed by the gut and transferred into systemic blood. In sucking rats, the majority of radioactivity was detected in the gastrointestinal tract and the liver. Thus, we speculate that the gastrointestinal epithelium, GALT cells, and Kupffer cells are likely the most affected systems by orally administered (1 → 3)-β-D-glucan. The enteral administration of (1 → 3)-β-D-glucan during the suckling period could very well be an effective approach in treating gastrointestinal diseases and disorders.

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References