PROTECTIVE EFFECT OF *DIXONIELLA GRISEA* (RHODOPHYTA) POLYSACCHARIDE AGAINST MYELOID GRAFFI TUMOR IN HAMSTERS

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Summary

Immunomodulators of natural origin have raised a considerable interest in red microalga as a producer of high molecular weight polysaccharides because these biopolymers often show advantages over the polysaccharides that are currently in use. We investigated the protective effect of *Dixoniella grisea* polysaccharide (DgPSH) on Graffi myeloid tumor in hamsters (GTBH). Various doses and ways of application of polysaccharide administered before and after tumor transplantation were studied. The mean survival time, inhibition of tumor growth, mortality percent and transplantability were determined. We further investigated the in vitro effect of DgPSH on the functional state of peritoneal macrophages by macrophage spreading and phagocytosis. The obtained experimental data demonstrate the protective effect of *Dixoniella grisea* polysaccharide (DgPSH) on the survivability of GTBH as expressed by decrease of the tumor transplantability, inhibition of tumor growth, elongation of mean survival time and reduced mortality percentage. The DgPSH increases both spreading and phagocytic ability of peritoneal macrophages in healthy and GTBH. The stimulation followed a dose-dependent pattern. The observed protective effect at the beginning stage of tumor progression can be partly explained by the temporary immunostimulating and immunorestorating action of the polysaccharide on the immune cells (macrophages, lymphocytes, NK-cells) or by direct cytotoxicity on Graffi myeloid tumor. Studies aimed at elucidation of immunomodulating activities of red microalgal polysaccharides in Graffi tumor bearing hamsters are currently in progress.

Key words: Dixoniella grisea, polysaccharide, Graffi myeloid tumor, macrophages.

Introduction

Algal polysaccharides have been extensively studied because of their diverse biological activities since they are potent anticoagulant, anti-inflammatory, antioxidant, antitumor and antiviral agents (10,11,13,20,30,36,41). The sulfated PSH of Porphyridium species exhibited antiviral activity against Herpes simplex virus types 1 and 2 both in vitro and in vivo (10). Red microalgal PSHs significantly inhibited the production of murine leukemia virus (MuLV) and cell transformation by murine sarcoma virus (MuSV-124) in culture (36). The most effective inhibition of cell transformation and virus production was established when the PSH was added 2 hours before or at the time of infection. The biological activities of algal PSHs have attracted more attention because of their immunomodulatory and antitumor effects (2,4,8,28,42). The enhancement of host defense mechanisms is a possible means of inhibiting tumor growth without harming the host. The PSHs may exert their immunostimulating effects through activation of various effector cell types - macrophages, T cells and etc. (4,34,42,43,44). Most studies support that sulfated PSHs can enhance the innate immune response by promoting tumorocidal activities of macrophages and NK-cells (23,42). Antigen-presenting cells migrate into and out of tumor tissue to present tumor antigen to T-helper cells, as well as to produce cytokines (IL-1β and TNF-α) that stimulate T-helper cells. As a result, T-helper cells promote the activity of cytotoxic T-cells, which has the strong cytotoxic effect on tumor cells (23,34,42,44).

The purpose of this study was to investigate the antitumor effect of Rhodella PSH against Graffi myeloid tumor in hamsters. As markers for protective effect we determined percentage of transplantablity and mortality, mean survival time (MST) and inhibition of tumor growth in hamsters transplanted with Graffi tumor. We analyzed not only tumor growth but also parameters of functional activity of peritoneal macrophages, determining the in vitro effect of DgPSH on macrophage spreading and phagocytosis.

Materials and methods

Algal strain: The microalgal strain *Dixoniella grisea* (Geitler) (31) former Rhodella reticulata (Rhodophyta) strain UTEXZB2320, from

the algal collection of the department of Botany, University of Austin, Texas, USA was grown intensively for 5 days with aeration (air + 1% CO_2) described by Georgiev (7) on the medium of Pekarkova (26), pH 7.2; light intensity 260 $\mu Em^{-2}s^{-1}$ and temperature 30^oC. Algal growth was followed by cell count and the cells were harvested at late exponential growth phase. The accumulated extracellular polysaccharide was assessed after preparation according to the method of Simon (32). The viscosity was measured on a Viscosimeter B3 and liophylisation performed.

Isolation and purification of Polysaccharide from Dixoniella grisea (DgPSH): The extracellular PSH was isolated from the cell-free culture medium by dialization for 2 days of the medium against distilled water, precipitation with ethanol (1:2, v/v), dissolving of gel-like precipitate in deionized water and sterilization of the solution through a bacterial filter (0.2μ). The polysaccharide content ($mg.ml^{-1}$) was determined by the phenol-sulfuric acid method using glucose as the standard. The total protein content was estimated by the method of Lowry (19) with bovine serum albumin as the standard.

The sugar components of the polysaccharide complex were hydrolyzed with 2N H_2SO_4 for 3 hours at 100^oC in a sealed glass tube. After cooling, the solution was neutralized with $BaCO_3$. The mixture was filtered, and the solution was passed through an exchange column (Amberlite IR-120-H^+ resin), and then concentrated.

Thin layer chromatography (TLC) was used to analyze the neutral sugar content. Thin-layer chromatografic (TLC) study was carried out on Kieselgel 60 F_{254} (0.24 mm thick, Merck) plates, using the solvent systems EtOH-NH_3-H_2O (80:4:16). The spots were visualized by spraying with anisidin/hydrogen phthalate reagent followed by heating at 110^oC for 3-5 min.

Acid hydrolysis: The sample (100 mg) was macerated with 0.5 ml 70% H_2SO_4 for 18h at room temperature. After that the mixture was diluted with H_2O to 5% H_2SO_4 and was heated 24 h at 100^oC. The product of hydrolysis was neutralized with $BaCO_3$, filtered, concentrated and tested by co-TLC with authentic samples.

Experimental animals: Hamsters weighing about 80-100g, two months of age, from both sexes were used. Treatment of animals - the DgPSH was diluted in PBS (pH-7.2). Doses of 2.0, 5.0 and 10 mg/kg were applied in animals. Two other groups of hamsters were injected with tumor cells preliminary coincubated in vitro with DgPSH. They were divided into eight groups. Experimental Groups: 1 - hamsters treated s.c. with 2.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 2 - hamsters treated s.c. with 5.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 3 - hamsters treated s.c. with 10.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 4 - hamsters treated s.c. with 2.0 mg/kg PSH simultaneously with transplantation of 5.10^4 tumor cells; 5 - hamsters treated twice with 5.0 mg/kg PSH 2 hours before and 24 hours after transplantation of 5.10^4 tumor cells; 6 - hamsters treated with 5.10^4 tumor cells incubated 2 hours in the presence of 30 μg/ml PSH; 7 - hamsters treated with 5.10^4 tumor cells incubated 2 hours in the presence of 1000 μg/ml PSH; 8 - hamsters treated only with 5.10^4 viable tumor cells

Experimental Graffi myeloid tumor in hamsters: Transplantable Graffi myeloid tumor was induced in newborn hamsters by murine leukemia Graffi virus and was maintained as solid tumor by montly s.c. injection of $1\text{-}2.10^6$ viable trypan blue excluded tumor cells (39) in the interscapular area of hamsters. In the present experiment the animals were transplanted with 5.10^4 viable tumor cells. This amount of tumor cells induced 100% transplantability and 100% mortality in control TBH. Spontaneous regression of Graffi tumor was not observed.

In vitro incubation of tumor cell with PSH: Isolation of tumor cells was conducted under aseptic conditions and the single cell suspension was prepared by pressing tumor pieces mechanically. The cell suspension was passed through a 100-gauge stainless steel sieve and then cells were washed twice in cold PBS. The viability of Graffi tumor cells determined by the trypan blue exclusion test was >90%. Tumor cells (5.10^6/ml) were cultured in Petri dishes in the presence of respective amount of DgPSH for two hours at 37^oC in 5% CO_2 humidified incubator. The hamsters were challenged by s.c. injection of $5.10.^4$ tumor cells in 0.5 ml PBS coincubated for two hours with DgPSH 30 μg/ml or 1000 μg/ml.(group 6 and group 7 respectively).

Tumor protection in vivo: As markers for antitumor activity we determined biometric parameters such: Transplantability (T%) and mortality (M%) percentage, inhibition of tumor growth (ITG) and mean survival time (MST) in hamsters transplanted with Graffi myeloid tumor.

Tumor growth was followed by measuring the length and width of tumors on odd numbered days after challenge. Transplantability was calculated as animals developed tumors from all transplanted hamsters. Mortality percent and mean survival time were also determined.

Determination of functional activity of peritoneal macrophages

Isolation of peritoneal macrophages: Healthy and Graffi tumor bearing hamsters were killed under deep ether anesthesia, the peritoneal cells were aseptically collected by washing the peritoneal cavity with 10 ml sterile ice-cold PBS (pH=7.2-7.4). The peritoneal lavage fluid was individually collected and placed in plastic tubes (10 ml) kept in an ice bath. For total cell determination nine volumes of peritoneal cells were added to one volume of Trypan blue, and counts were performed using Turk hemocytometer. Differential cell counts were determined by cytospin preparations stained with Romanovski-Giemsa. This procedure yielded peritoneal cells with more than 98% being macrophages; only suspensions with 95% or more viability determining by trypan blue exclusion test were used.

Assessment of spreading of macrophages was determined by the spreading technique adapted by Rabinovitch (29) with modifications introduced by Passeti (25). Thus, 0.2 ml (2.10^6 cells/ml) of the cellular suspension obtained in the peritoneal cavity was placed over glass cover slips, that were kept on multi-well (24x13 mm) tissue culture plates at room temperature for 20 min. The non-adherent cells were removed by washing with cold (4^oC) phosphate buffer saline (PBS) and the adherent cells were incubated in culture medium RPMI-1640 without FBS containing different doses of DgPSH (50 μg/ml or 100 μg/ml) at 37°C for 2 h. Following this the culture medium was removed and the cells were fixed with 2.5% glutaraldehyde. Then the cells were stained with Giemsa and examined under microscopy where the percentage of spread cells was determined under 40 x magnification.

Spread cells were those that presented cytoplamic elongation, while the non-spread cells were rounded. Two hundred cells were examined and classified as either round or spread and index of macrophage spreading was calculated as follows: SI% = (number of spreading macrophages x100)/200 adherent cells.

Phagocytosis of peritoneal macrophages: Macrophage phagocytosis was performed using the same method described above. But before two hours of incubation of peritoneal macrophage monolayers with DgPSH, a total of 1mg of a zymosan solution (5mg/ml) was added to each well. Using the same microscope, objective and methods as described above, an index of phagocytosis (PI) was then calculated as follows: PI% = (number of macrophages with phagocytic activity x100)/200 adherent cells counted, i.e. macrophages with zymosan particles phagocytized.

Ethycal aspects: The animal test was conducted in accordance with the principles for laboratory animal use and care as found in European Community guidelines.

All of the studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as proposed by the Committee on Care of Laboratory Animal resources Commission on Life Sciences, National Research Council. Hamsters were housed in the animal care facilities of the IEPP Institute, which are fully accredited for Laboratory Animal Care.

Results

Thin layer chromatography (TLC) was used to analyze the sugar content of DgPSH. A TLC method was designed for quantitative analysis of the component monosaccharides of an polysaccharide isolated from red microalga *Dixoniella grisea*. After acid hydrolysis by co-TLC with authentic samples D-xylose and L-arabinose were identified to be the main component monosaccharides, followed by glucose.

PROTECTIVE EFFECT OF POLYSACCHARIDE FROM *DIXONIELLA GRISEA*

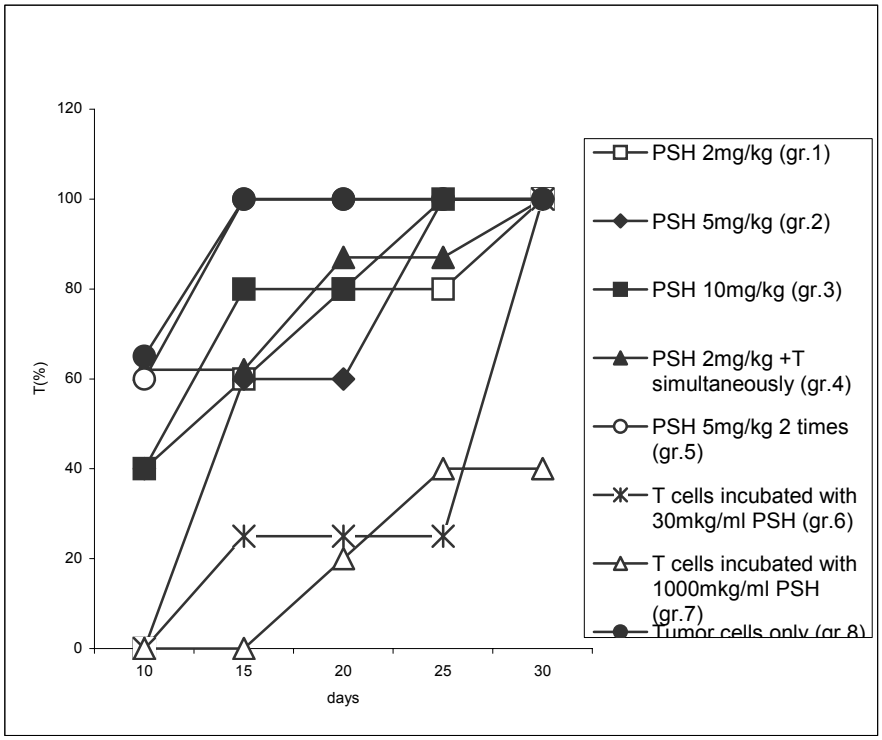

Fig.1. Transplantability (%) of Graffi myeloid tumor after treatment with DgPSH. Experimental groups: 1 - hamsters treated s.c. with 2.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 2 - hamsters treated s.c. with 5.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 3 - hamsters treated s.c. with 10.0 mg/kg PSH 2 hours before transplantation of 5.10^4 tumor cells; 4 - hamsters treated s.c. with 2.0 mg/kg PSH simultaneously with transplantation of 5.10^4 tumor cells; 5 - hamsters treated twice with 5.0 mg/kg PSH 2 hours before and 24 hours after transplantation of 5.10^4 tumor cells; 6 - hamsters treated with 5.10^4 tumor cells incubated 2 hours in the presence of 30 μg/ml PSH; 7 - hamsters treated with 5.10^4 tumor cells incubated 2 hours in the presence of 1000 μg/ml PSH; 8 - hamsters treated only with 5.10^4 viable tumor cells.

The effect of DgPSH on tumor transplantability was assessed at day 10, 15, 20, 25 and 30 after tumor transplantation. After treatment with DgPSH the T(%) in experimental groups decreased significantly between 0-40%, 65% control respectively (Fig.1). The T(%) (on day 10) in the group 1, 6 and 7 was 0, as compared with untreated group 8. The T(%) of group 7 was also decreased in day 15 and remained lower till 30 day of examination (Fig.1). Hamsters that received either 2 mg + tumor cells simultaneously (group 4) or 5 mg/kg - twice (group 5) failed to show any evidence of tumor protection as compared with untreated controls. The T(%) were 62% and 60% respectively and control being 65% (Fig.1). By day 15, 100% of untreated (control group) and hamsters from group 5 developed tumors, whereas hamsters of group 6 and 7 had 25% and 0% tumors respectively. After 30 days all of the control and groups of DgPSH - treated hamsters (group 6) had developed tumors. Two month after tumor injection, 60% of hamsters injected with DgPSH-treated tumor cells had still not developed tumors (group 7) (Fig.1).

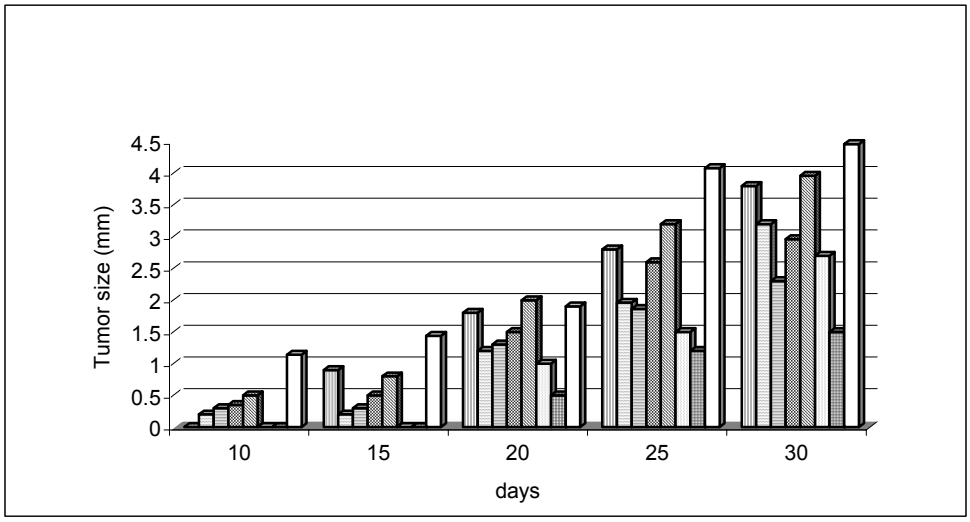

Fig.2. Tumor growth (mm) of Graffi tumor in hamsters, treated with DgPSH. Experimental groups: as noted in Fig.1

Antitumor effect of DgPSH was demonstrated on locally implanted Graffi myeloid tumor in hamsters by the tumor growth. DgPSH (in all doses used) inhibited growth of Graffi tumor by 56% - 100%; 37.5% - 100% on day 10 and 15 respectively (Fig.2).

Mean tumor size in hamsters injected with DgPSH-treated tumor cells were also significantly lower (100% inhibition of tumor growth on 10^{th} and 15^{th} day) than in untreated control (Fig.2). The most impressive was the inhibition of tumor growth in groups 6 and 7 at all stage of investigation (between 39% and 66.3% inhibition on day 30 of experiments.

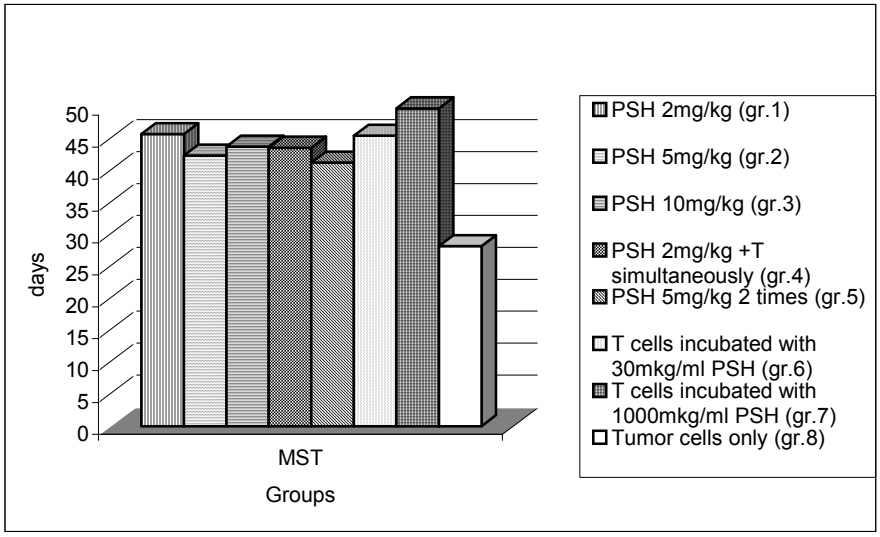

Fig.3. Mean survival time of GTBH, treated with DgPSH. Experimental groups: as noted in Fig.1

The effect of PSH on inhibition of tumor growth was further examined by determining Mean survival time (MST). A significant increase of MST was seen in all groups of DgPSH- treated animals that had been exposed to DgPSH, when compared with untreated hamsters (Fig.3). The MST of control hamsters treated only with tumor cells was 28 ± 1.8 days and it was increased to 41.3 - 49.8 in DgPSH - treated hamsters (Fig.3). The highest survival time - 45.5 and 49.8 days (group 6 and 7 respectively) was found for animals transplanted with tumor cells coincubated with 30 μg or 1000 μg DgPSH in vitro.

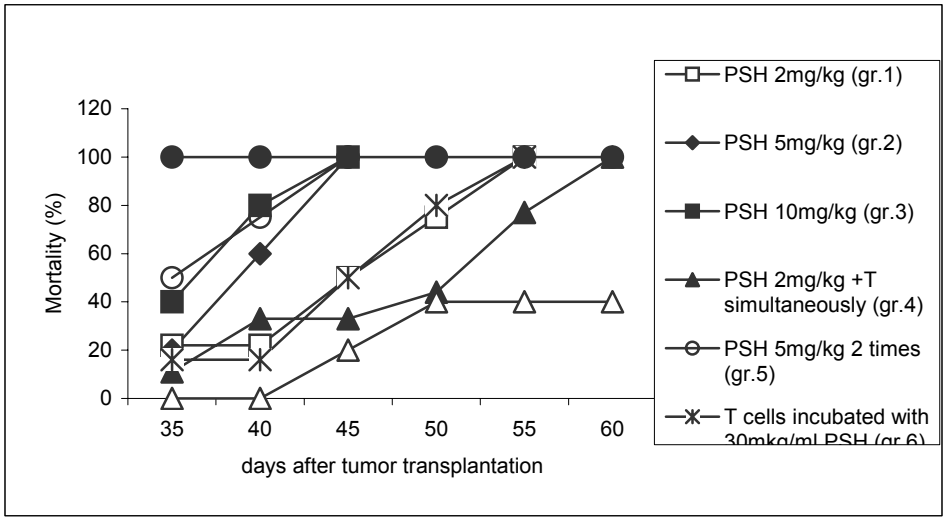

Fig.4. Mortality (%) of GTBH treated with DgPSH. Experimental groups: as pointed in Fig. 1

We investigated the mortality percentage in GTBH. As can been seen in Fig.4 the M(%) was significantly decreased by DgPSH in all experimental groups. When hamsters were grafted with tumor cells treated with 30 μg/ml DgPSH, the M(%) decrease significantly ($p<0.5$) till 50 days (Fig.4). When compared with treatment with 1000 μg/ml DgPSH, were found to have higher inhibiting effect (only 40% M till 60 days of observation).

Determination of the percentage of spreading of peritoneal macrophages: The results obtained for the variable percentage of spreading of macrophages harvested in the peritoneal cavity of the animals are shown in Fig. 5. The treatment with DgPSH showed an increase in the functional activity of macrophages of healthy and Graffi TBH animals. These results when compared with the control group (without PSH) were statistically significant. The presence of tumor in hamsters decrease the percentage of macrophage spreading. This fact can be observed in the tumor control group (incubation without PSH) which differ from the healthy control group (Fig.5). It should also be pointed out that for the tumor-bearing groups treated with DgPSH (0, 10, 30, 50 and 100 μg/ml) there was an increase in the median value of spreading percentage accompanying the increase

in the PSH dose (Fig.6), and the difference among such values and control one was statistically significant.

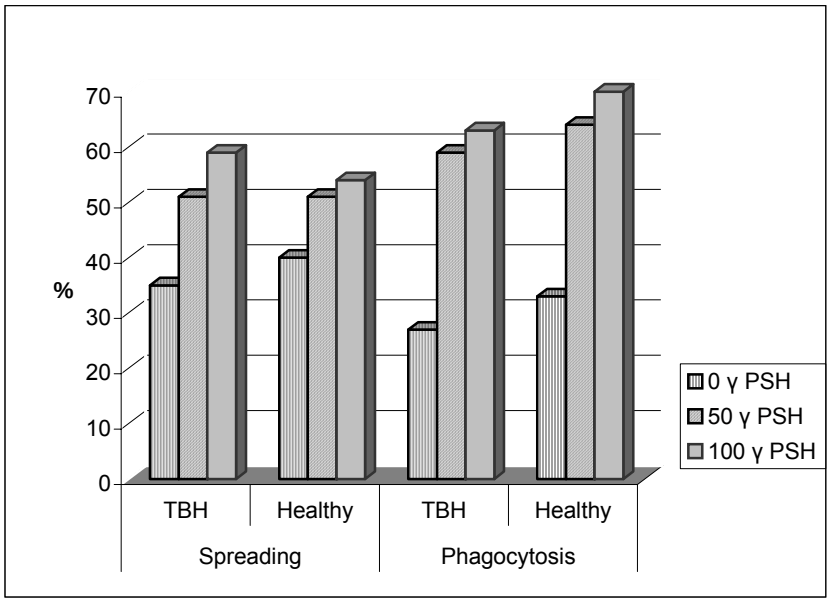

Fig.5. Spreading and phagocytosis of peritoneal macrophages from healthy and GTBH incubated in vitro with different doses of DgPSH.

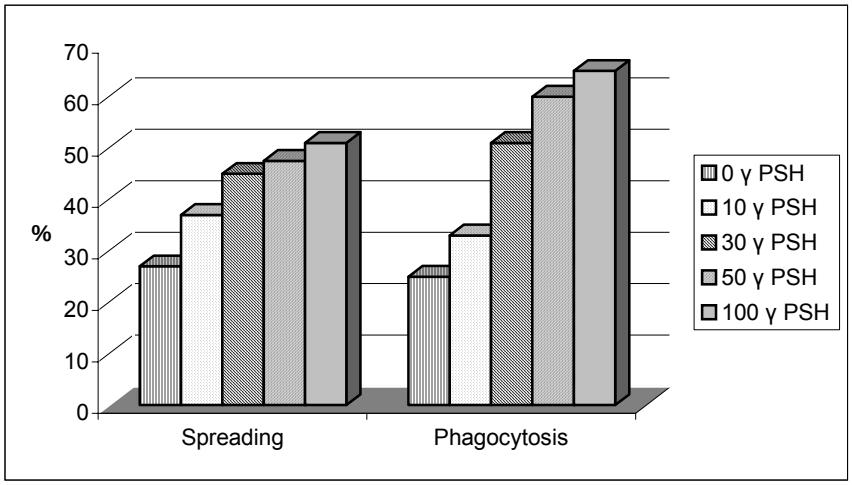

Fig.6. Spreading and phagocytosis of peritoneal macrophages from GTBH incubated in vitro with different doses of DgPSH.

Determination of the percentage of phagocytosis of peritoneal macrophages: The effect of DgPSH on the function of macrophages was further examined by determining phagocytic ability. Figures 5 and 6 show that the incubation of peritoneal macrophages with various doses (10-100 μg/ml) of DgPSH significantly increased the phagocytosis of zymosan particles by macrophages, suggesting that phagocytic activity was increased by PSH. The phagocytic activities of peritoneal macrophages increased in a dose-dependent manner (Figs. 5 and 6).

Discussion

The objective of this study was to investigate the protective effect of PSH, isolated from red microalga *Dixoniella grisea* on tumor growth in hamsters and to determine its action on functional activity of peritoneal macrophages.

Our results demonstrate that DgPSH have antitumor activity as expressed by biometric parameters - elongation of MST, ITG, decreased mortality and transplantability percentage. Clinical trials have indicated that sulfated polysaccharides influenced the survival in animals and patients with advanced malignancy (17,18). The antitumor and anti-metastatic effects of sulfated PSHs are attributed by their heparanase-inhibiting activity and by inhibition of VEGF - and FGF-mediated angiogenesis (15,24,27,33). Unexpected results was obtained when hamsters were grafted with tumor cells incubated in vitro with 30 μg/ml or 1000 μg/ml. Tumors not appeared in 60% of hamsters till two month after tumor transplantation, while 100% of control hamsters (treated only with tumor cells, incubated without the PSH) developed tumors within 15 days after tumor transplantation (Fig.1, group 7). The direct cytotoxic activity of the PSH on Graffi tumor cells was not excluded. Sulfated PSHs directly bind to growth factors to inhibit the growth of tumors (14). PI-88, a sulfated oligosaccharide, induces apoptosis of pancreatic islet carcinoma (15). Sulfated heteroPSH isolated from the red alga Schizymenia dybui can induce the terminal maturation of non-small-cell bronchopulmonary carcinoma cells (NSCLC-N6) and arrest cells in the G1 phase (2). Application of sulfoethylated (1→3)-beta-D-glucan (SEG) leads to inhibition of tumor growth in mice and potentiates therapeutic action of cyclophosphamide (16).

Therapeutic action of SEG on the tumor growth in mice is realized predominantly through induction of apoptosis and is accompanied by a substantial increase of the activity of cysteine ptoteases catepsin B and L in tumor tissues.

A large number of PSHs have been shown to possess nonspecific stimulating activities on cell-mediated responses by promoting the tumorocidal activities of macrophages and natural killer cells (23,42). Nascimento (21,22) demonstrated that activated macrophages can inhibit the growth of variety of tumor cells and microorganisms due to an increase in spreading and phagocytic ability, nitric oxide (NO), hydrogen peroxide (H_2O_2) and cytokine production.

Further experiments were performed to measure the functional activity of peritoneal macrophages. In this study spreading and phagocytic activities in peritoneal macrophages, treated in vitro with DgPSh were significantly higher ($p<0.05$) than those in the control (Fig.5 and 6). The stimulation demonstrated a dose-dependent pattern. Spreading is an active process that represents one of alterations found during macrophage activation. Bacteria and products proceeding from tumor cells induce the spreading (9,45). According to Tatefuji (37) macrophage spreading is the preliminary step which precedes macrophage infiltration into tissues and considered an important marker of macrophage activation. The phagocytosis, on the other hand, is a process closely related to spreading, including the activation of several macrophage membrane receptors. Macrophage phagocytosis can be modulated in vitro and in vivo to different states by bacterial and tumoral challenges (5,6,12). The mechanisms mediating the recognition and phagocytosis of zymosan are complex: non-opsonic recognition mediated by type 2 membrane receptor Dectin-1, expressed on macrophages, dendritic cells and neutrophils (1,3,38).

Macrophages are known to play an essential role in host defense against microbial agents and neoplasia (40). Since phagocytes acts as a regulatory and effector cells in the immune system, the enhancement of phagocyte function has potential therapeutic efficacy against infections and cancer. It is well known that the phagocytosis action of macrophages is a key process in nonspecific immune function (35).

A hetero-polysaccharide, isolated from the green seaweed *Ulva lactuca,* exhibited an immunostimulating effect on the functions of macrophages and PMNs from healthy mice and restored the suppressed functions of peritoneal macrophages, as well as the proliferating ability of spleen lymphocytes from hamsters with transplanted Graffi tumors.

The mechanisms involved in antitumor action of PSHs are complex . One possibility is that simple molecules (monosaccharides) derived from the complex carbohydrates exert their biological effect. The other possibility is the enhancement of host defense mechanisms. The antioxidant activity of PSHs may be another mechanism underlying its antitumor effect. Anti-angiogenesis properties may represent also an important mechanism of antitumor activity.

The in vivo tumor suppression properties of DgPSH might be due to direct toxicity of the polysaccharide fractions, tumor-sensitive toxic metabolic derivatives of the polysaccharide fractions, or immunostimulatory properties of the polysaccharide. In conclusion, treatment with PSH induced protective effect in TBH and stimulated functional activity of peritoneal macrophages. Studies aimed at elucidation of immunomodulating activities of red microalga polysaccharides in Graffi tumor bearing hamsters are currently in progress.

Conclusions

1. The results clearly demonstrated the protective effect of PSH from *Dixoniella grisea* in experimental Graffi myeloid tumor in hamsters.
2. PSH-*Dixoniela* treatment significantly increased MST with 10-20 days; reduced the mortality rate and inhibited tumor growth. The transplantation of Graffi tumor was markedly depressed on day 10 of examination.
3. DgPSH stimulated the functional activity (spreading and phagocytosis) of peritoneal macrophages from healthy and GTBH in a dose-dependent manner. Thus the antitumor effect observed might be due to an increase of peritoneal macrophage activity.

4. Based on these results we could suggest that the tested algal DgPSH is promising candidate as antitumor agent. Further studies are needed to elucidate its biological effect and immunopotentiating activity in experimental animals.

Acknowledgments

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