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Anti-viral activity of red microalgal polysaccharides against retroviruses

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Abstract

Red microalgal polysaccharides significantly inhibited the production of retroviruses (murine leukemia virus- MuLV) and cell transformation by murine sarcoma virus(MuSV-124) in cell culture. The most effective inhibitory effect of these polysaccharides against both cell transformation and virus production was obtained when the polysaccharide was added 2 h before or at the time of infection. Although, addition of the polysaccharide post-infection significantly reduced the number of transformed cells, but its effect was less marked than that obtained when the polysaccharide was added before or at the time of infection.The finding that the inhibition of cell transformation by MuSV-124 was reversible after removal of the polysaccharide suggested that microalgal polysaccharides inhibited a late step after provirus integration into the host genome. In conclusion, our findings could support the possibility that the polysaccharide may affect early steps in the virus replication cycle, such as virus absorption into the host cells, in addition to its effect on a late step after provirus integration.

Background

Retroviruses [1–3] – viruses that contain reverse transcriptase, an RNA-directed DNA polymerase [4,5] – have been implicated in various types of human and animal leukemia's and other tumors. Although there are many compounds that exhibit potent anti-viral, and possibly anti-tumor, activity in cell culture and in experimental animals, only very few synthetic compounds and one natural product – alpha interferon – have so far been approved for treatment of viral infections in man. Alpha interferon has been approved for treatment of hairy cell leukemia, of Karposi's sarcoma and of genital warts caused by papiloma virus [6].

A class of natural products with low mammalian toxicity that are currently regarded as having antitumor activity are polysaccharides of biological origin, e.g., polysaccharides from yeasts, algae, bacteria, higher plants and fungi [7–9]. Of interest in this context are polysaccharides produced by some species of red algae; these compounds have shown promising activity against a variety of animal viruses [10–13]. In general, polysaccharides exhibiting antiviral potential are highly sulfated [10,14–17]. Dextran sulfate and polysaccharides from marine algae, for example, have been found to be potent *in vitro* inhibitors of HIV types 1 and 2 [15,18–20]. They inhibit HIV-1-induced cytopathogenicity and HIV-1 antigen expression

[13,18,21,22]. These sulfated polysaccharides also inhibit the activity of purified reverse transcriptase and RNase H, which are essential for retrovirus replication [18,20]. Some previous studies showed that algal polysaccharides exert their inhibitory action at a very early stage (adsorption, fusion or penetration), in the viral infection cycle [9,18,20,23–25], whereas others showed that these polysaccharides did not interfere with virus attachment or penetration, but they did prevent viral protein synthesis [11,26,27].

Our previous research has shown potent antiviral activity of a highly sulfated polysaccharide extracted from red microalga. The polysaccharide, which consists mainly of xylose, glucose and galactose [28], exhibits antiviral activity against various members of the herpes family of viruses [29]. In the current study, the activity of this red microalgal polysaccharide against the replication and the transforming ability of the retroviruses, Moloney murine sarcoma virus (MuSV) and Moloney murine leukemia virus (MuLV), was studied.

Results and Discussion

Characterization of cell transformation by MuSV-124 and MuSV/MuLV

NIH/3T3 cells grown in plastic dishes in RPMI medium with 2% NBCS appear as flat cells (Fig 1a); these cells are completely unable to grow in agar. When these cells were infected with an appropriate dilution of MuSV-124, tiny foci of transformed cells, with a highly refractile spindle shape, growing randomly in a criss-cross fashion, could be detected by microscopic observation within five days of infection (Fig. 1b). Later, these foci gradually increased in size and compactness until they became visible to the naked eye on day 12 to 14 after infection with MuSV-124. The number of foci remained unchanged in these cultures during the entire culture period. When the cells were infected with high titer of MuSV-124 (1 ffu/cell), most of the cells were transformed two-three days after infection. In MuSV/MuLV-infected cultures, the number of foci increased continuously, and at any time foci of various sizes (tiny to large) could be detected. Moreover, if these cultures were maintained for a sufficiently long time, all the cells eventually became transformed. Examination of the culture medium for the presence of viral reverse transcriptase revealed that MuSV/MuLV infection yielded virus-producing cells, whereas MuSV-124 infection resulted in the formation of transformed cells not producing virus. It is therefore likely that the increasing number of foci in the productively infected cells resulted from multiple secondary infections by the virus progenies, the smaller foci being formed by these infections. It was found that the later the infection, the smaller the foci. Both MuSV-124- and MuSV/MuLV-transformed cells could grow efficiently in agar.

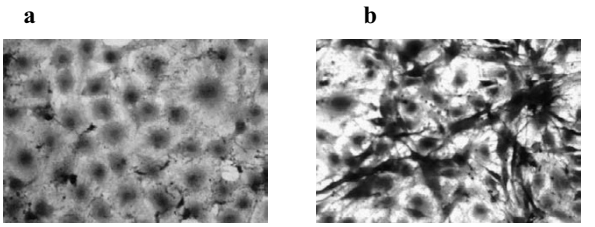

Figure 1
(a) Control uninfected NIH/3T3 cells. (b) NIH/3T3 cells infected and transformed by MuSV (5 days p.i.). The magnification of these photos is ×40.

Antiretroviral effect of red microalgal polysaccharides

The polysaccharide extracted from *Porphyridium* sp. was more effective in inhibiting retrovirus replication and cell transformation by MuSV than the polysaccharides obtained from *P. aerugineum* or *Rhodella reticulata* (Table 1). The concentration of *Porphyridium* sp. polysaccharide required for 50% protection against the formation of foci of transformed cells by MuSV or for a 50% reduction in MuLV production (as evaluated in terms of reverse transcriptase activity) was one or two orders of magnitude lower than that needed when *P. aerugineum* or *R. reticulata* polysaccharide was applied. We therefore focused our research on the anti-retroviral effects of *Porphyridium* sp. polysaccharide.

Table 1: Inhibiting effect of red microalgal polysaccharides on formation of foci by MuSV-124 and on virus production, as measured by reverse transcriptase (RT) activity

Algal polysaccharide	Virus	ffu_{50} protection (μg/ml)	RT_{50} reduction (μg/ml)
Porphyridium sp.	MuSV-124	10	-
	MuSV/MuLV	-	5
P. aerugineum	MuSV-124	500	-
	MuSV/MuLV	-	200
Rhodella reticulata	MuSV-124	150	-
	MuSV/MuLV	-	50

NIH/3T3 cells were infected with MuSV-124 or MuSV/MuLV in the presence of various concentrations of algal polysaccharides. ffu_{50} protection and RT_{50} reduction represent the concentration of polysaccharide that offers 50% protection against the formation of foci of malignant cells and 50% reduction of RT activity, respectively. Data represent mean values of five separate experiments.

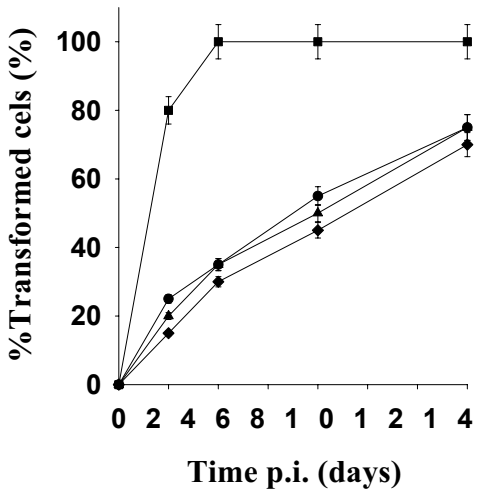

Figure 2
Kinetics of the development of MuSV-malignant cell transformation in the presence of *Porphyridium* sp. polysaccharide, dextran sulfate or carragenaan NIH/3T3 cells were infected with 10^5 ffu of MuSV-124 in the absence or presence of 10 μg/ml of *Porhyridium* sp. polysaccharide (■), dextran sulfate (♦) and carrageenan k (●). The treatment with the polysaccharide was continued up to the end of the experiment. Data are mean ± SD, (n = 4).

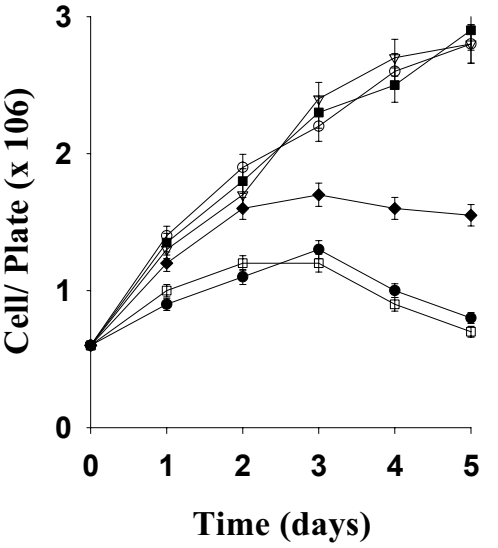

Figure 3
Effect of polysaccharides on cell proliferation of NIH/3T3 cells NIH/3T3 at 37°C in the absence (○) or presence of 10 (△), 100 (■) or 1000 μg/ml (♦) of *Porphyridium* sp. polysaccharide or 100μg/ml of either dextran cells were seeded at a concentration of 0.5×10^6 cells per 9.6-cm^2 plate and incubated sulfate (●) or carrageenan (□). Data are mean ± SD, (n = 4).

Porphyridium sp. polysaccharide was also superior to other polysaccharides, such as carrageenan and dextran sulfate 500,000, in preventing the transformation of NIH/3T3 cells. Although the anti-transforming activity of *Porphyridium* sp. polysaccharide did not seem to be much higher than that of carrageenan or dextran sulfate at a concentration of 10 μg/ml (Fig. 2), at the higher concentrations of *Porphyridium* sp. polysaccharide was not toxic to the cells whereas the other two biopolymers were extremely toxic to the cells (Fig. 3). *Porphyridium* sp. polysaccharide had no effect on the proliferation of NIH/3T3 cells, even up to a concentration of 500 μg/ml (results not shown). However, at a concentration of 1,000 μg/ml, *Porphyridium* sp. polysaccharide caused the cells to stop growing three days after the beginning of the treatment (Fig. 3).

Microscopy showed that there were there were no changes in cell morphology in the presence of *Porphyridium* sp. polysaccharide, even at a concentration of 1000 μg/ml.

Effect of dosage of Porphyridium sp. polysaccharide on cell transformation

The algal polysaccharide significantly inhibited malignant transformation of NIH/3T3 cells by MuSV or MuSV/MuLV. As can be seen from Table 2, continuous treatment with = 100 μg/ml of Porphyridium sp. polysaccharide, from the time of infection until 14 days after infection (scoring time), fully inhibited formation of foci by both virus stocks.

Effect of time of addition of Porphyridium sp. polysaccharide on cell transformation

The algal polysaccharide (100 μg/ml) was added to NIH/3T3 cells at various times before and after infection with either MuSV-124 or MuSV/MuLV. As can be seen from Table 3, the polysaccharide fully inhibited formation of foci in MuSV-124- and MuSV/MuLV-infected cultures if it was added before or at the time of infection. If the polysaccharide was added post-infection, it was less effective. In MuSV/MuLV-infected cultures the polysaccharide was still significantly inhibitory to the formation of foci when added 48 h after infection. The protective effect of the polysaccharide in these cultures was lost only if it was added 72 h after infection, whereas in MuSV-124-infected cultures the effectiveness of the polysaccharide was lost when addition was as early as 48 h after infection.

Table 2: Effect of dosage of *Porphyridium* sp. polysaccharide on NIH/3T3 cell transformation by MuSV-124 and MuSV/MuLV

Porphyridium sp. polysaccharide (μg/ml)	Number of foci[1]	
	MuSV-124	MuSV/MuLV
0	200 ± 10	120 ± 8
5	150 ± 10	90 ± 7
10	110 ± 8	65 ± 5
50	15 ± 6	10 ± 3
100	0	0
250	0	0

[1]Mean of 5 cultures ± SD. Foci scored 14 days after infection.

Table 3: Effect of time of addition of *Porphyridium* sp. polysacharide on NIH/3T3 cell transformation

Timing of *Porphyridiumsp.* addition of polysaccharide	Number of foci[1]	
	MuSV-124	MuSV/MuLV
None	80	70
24 h before infection	0	0
2 h before infection	0	0
0 h at infection	0	0
2 h after infection	15 ± 4	6 ± 2
24 h after infection	50 ± 7	14 ± 4
48 h after infection	73 ± 6	30 ± 5
72 h after infection	75 ± 7	64 ± 6

[1]Means of five cultures ± SD Foci were scored 12 days after infection. h means hours.

The obtained differences between cell cultures infected with either of these virus stocks (MuSV/MuLV or MuSV-124) are due to the different characteristics of these infections. In the case of MuSV-124-infected cell cultures, the inhibitory effect of *Porphyridium* sp. polysaccharide could not be explained in terms of the inhibition of secondary viral infections, since this virus yielded a virus nonproducing infection [30,31]. However, in MuSV/MuLV-infected cultures, part of the inhibitory effect of the polysaccharide against cell transformation could be a result of inhibiting secondary viral infections, since the majority of the foci that appeared in the cultures seemed to result from multiple secondary infections by the progeny of the primary infection.

Effect of time of addition of Porphyridium sp. polysaccharide on release of progeny virus from MuSV/MuLV-infected cells

The algal polysaccharide probably exerted its effect by preventing secondary infections, which were the major source of foci scored in these cultures under our experimental conditions. To determine whether the effect of the polysaccharide was due to the arrest of virus release from the primary infected cells or merely from blocking the establishment of secondary infections, the polysaccharide was added at various times before and after infection, and the release of progeny virus was followed by assaying viral reverse transcriptase activity in aliquots taken from the culture medium at different times post infection. The results presented in Fig. 4 showed that the infection cycle is completed within 20–24 h after inoculation, this being the time at which the appearance of the first progeny could be detected. The polysaccharide had a significant inhibitory effect on the release of virus progeny even when it was added 48 h after infection. Therefore, the significant prevention of formation of malignant foci by the polysaccharide at this late time was most likely due to its action against the subsequent secondary infection cycle. These results are in agreement with our suggestion that in MuSV/MuLV-infected cultures, part of the inhibitory effect of the polysaccharide against cell transformation could be a result of inhibiting secondary viral infections.

Effect of time of removal of Porphyridium sp. polysaccharide on cell transformation

To determine whether it is necessary for *Porphyridium* sp. polysaccharide to remain in the culture during the whole time until scoring of foci, cells were treated with the polysaccharide 2 h before infection and polysaccharide was removed at various times after infection. Foci were scored 12 days after infection. As can be seen from Table 4, about 75 and 65% of the transforming capacity of MuSV-124 and MuSV/MuLV, respectively, were recovered when the polysaccharide was removed at the time of infection. When *Porphyridium* sp. polysaccharide was removed 72 h post-infection, only 55% and 20% of transforming capacity of MuSV-124 and MuSV/MuLV, respectively, were recovered.

As a consequence of the different character of the infection by these two virus stocks, their focus-forming capacity responded quite differently to the timing of polysaccharide addition and removal. In the case of MuSV/MuLV infected cultures, focus formation was significantly inhibited even when the polysaccharide was added or removed at longer times after infection compared to MuSV-124 infected cultures. These findings indicate that the continuous presence of thepolysaccharide in the culture medium after infection with the virus was essential for full prevention of malignant transformation

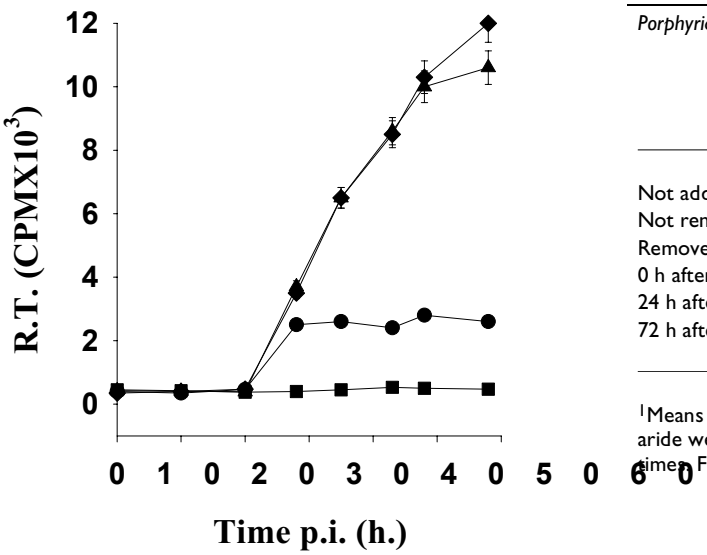

Figure 4
Effect of time of *Porphyridium* sp. polysaccharide addition on MuLV progeny release Polysaccharide (100 μg/ml) untreated cells (♦) or treated at the time of infection (■), 24 h (●) or 48 h (♦) postinfection (p.i.), were inoculated with MuSV/MuLV. Two hours after inoculation, unadsorbed virus was removed by three washes, and the cells were covered with fresh medium containing the polysaccharide in all treated cultures. Virus release was followed by assaying viral reverse transcriptase activity in aliquots taken from the culture medium at various times after inoculation. h means hours. Data are mean ± SD, (n = 4).

Table 4: Focus formation after removal of *Porphyridium* sp. polysaccharide

Porphyridium sp. polysaccharide	Number of foci[1]	
	MuSV-124	MuSV/MuLV
Not added	100 ± 6	70 ± 5
Not removed	0	0
Removed at:		
0 h after infection	75 ± 7	65 ± 4
24 h after infection	64 ± 6	50 ± 5
72 h after infection	55 ±	20 ± 4

[1]Means of five cultures ± SD 100 μg/ml of *Porphyridium*sp. polysachariide were added 2 h before infection and removed at the indicated times. Foci were scored 12 days postinfection. h means hours.

over the tested period (about two weeks). When the treatment with the polysaccharide was terminated immediately post-infection (Table 4), there was a significant recovery in the appearance of malignant transformed cells for all tested concentrations of the polysaccharide. This reversibility strongly suggests that the polysaccharide, partially at least, exerted its inhibitory effect on a certain event occurring after proviral integration. In addition, this reversibility could not be explained only by the possibility of preventing viral reinfections by the polysaccharide because in the case of MuSV-124 infections there are no reifections [30,31]. The inhibitory effect does not seem to be mediated by interferon or by an interferon-like antiviral state, since interferon has been found to inhibit certain events occurring before proviral integration [31].

Our results do not rule out the possibility that at least part of the inhibitory effect of the polysaccharide was due to blocking some of the viral receptors, thus interfering with the penetration of the virus into the cells. This possibility was supported by our results showing that treatment of the cells with the polysaccharide post-infection caused a significant inhibition of cell transformation, but that this inhibition was less impressive than that obtained when treatment with the polysaccharide was started before or at the time of infection (Table 3). This possibility is also in agreement with various previous studies [9,17,18,22,24,32] that suggested that sulfated polysaccharides prevent early steps in the viral life cycle. In addition, some of our data not presented here showed that the inhibitory effect of *Porphyridium* sp. polysaccharide on cell malignant transformation by MuSV was not a result of a direct interaction between the polysaccharide and the virus particles. In contrast, our previous data (29) showed a strong interaction between *Herpes simplex* virus (HSV 1 and HSV-2) particles and *Porphyridium* sp. polysaccharide. This contradiction could be due to differences in viral envelope composition. Herpes viruses envelope is positively charged, whereas retroviruses are negatively charged. Therefore, the sulfate groups of the polysaccharide could easily interact with positively charged viruses.

Conclusions

The present data show that the red microalgal polysaccharides profoundly inhibited retroviral malignant cell transformation and retrovirus replication. Most effective inhibitory activity of these polysaccarides on cell transformation was obtained when the cells were treated with polysaccharide before or at the time of infection. These results support the possibility that at least part of the inhibitory effect of the polysaccharide was due to blocking some of the viral receptors, thus interfering with the penetration of the virus into the cells. On the other hand, the reversibility of this inhibitory activity strongly suggests that the polysaccharide exerted its inhibitory effect also on

a certain event occurring after proviral integration. Thus, it appears that Porphyridium sp. polysaccharide has a pleiotropic mode of action during the infection cycle of MuSV. The exact steps (or step) during the viral replication cycle that are affected by Porphyridium sp. polysaccharide remain to be elucidated.

Materials and Methods

Cells and viruses

NIH/3T3 cells (mouse fibroblast cells) were grown at 37°C in RPMI medium supplemented with 10% new born calf serum (NBCS) and the antibiotics penicillin, streptomycin and neomycin. Clone 124 of TB cells chronically releasing Moloney murine sarcoma virus (MuSV-124) (31) was used to prepare a virus stock that contained an approximately 30-fold excess of MuSV particles over Moloney murine leukemia virus (MuLV) particles. MuLV and MuSV used in this research were grown on NIH/3T3 cells. The virus concentration was determined by counting the number of foci (ffu-focus-forming units) in the case of MuSV and by the reverse transcriptase assay in the case of MuLV.

Preparation and purification of microalgal polysaccharide

Polysaccharides produced from three species of red microalga; *Porphyridium* sp., *P. aerugineum* and *Rhodella reticulata*, were used in this study. The polysaccharides were collected and purified as previously described [33]. Briefly, these polysaccharides are produced and secreted into the growth medium by the appropriate red microalgae. The medium was collected, cells were removed by centrifugation and the supernatant containing the polysaccharides was dialyzed and lyophilized.

Cell infection and determination of viral infection

A monolayer of NIH/3T3 cells was grown in 9-cm^2 tissue culture plates and treated with 0.8 μg/ml of polybrene (a cationic polymer required for neutralizing the negative charge of the cell membrane) for 24 h before infection with the virus. Free polybrene was then removed, and the cells were incubated at 37°C for 2 h with the infecting virus (MuSV-124) at various concentrations in RPMI medium containing 2% of NBCS. The unabsorbed virus particles were removed, fresh medium containing 2% NBCS was added, and the monolayers were incubated at 37°C. After 2–3 days, the cell cultures were examined for the appearance of malignant transformed cells. The amount of malignant transformed cells was expressed as the percentage of transformed cells in the inspection field or as the number of foci in the infected culture 10 days after infection.

Reverse transcriptase assay

Viral reverse transcriptase activity was assayed as previously described [34].

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