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Sulfated fucan from marine alga inhibits HeLa cells infection by HTLV-1 free particles: semi-quantitative analysis

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Article

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Abstract: A sulfated fucan from *Laminaria abyssalis* marine alga prevented the interaction of HTLV-1 particles, purified from the MT-2 cell line, with HeLa cells. The infection obtained using a concentrated virus suspension was detected only by amplification of the newly synthesized HTLV-1 proviral cDNA by the nested-polymerase chain reaction (PCR). The sulfated polysaccharide was not toxic to the cells at a concentration of 100 µg/mL and prevented infection by the viral particles when added to the cell monolayers. The proviral cDNA was only detected when the sulfated polysaccharide was added to the cells three hours post-infection, indicating that the inhibitory activity occurred in the initial stages of virus-cell interaction. Our results demonstrate, for the first time, the ability of a sulfated fucan from marine algae to inhibit virus transmission through free virus particles.

Keywords:
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Introduction

The human T-cell lymphotropic virus type 1 (HTLV-1) was initially identified by Poiesz et al. (1980) and has been associated with a hematopoietic malignancy, adult T-cell leukemia/lymphoma (ATLL), and with a degenerative neurologic disorder called tropical spastic paraparesis or human T-cell lymphotropic type 1-associated myelopathy (TSP/HAM). Prognosis of adult T-cell leukemia/lymphoma remains poor because of immunosuppression and intrinsic resistance to chemotherapy.

Treatment of ATLL with a combination of zidovudine (AZT) and interferon-alpha (IFN) prolongs the survival of patients (Bazarbachi & Hermine, 2001). In spite of this success, the search for new anti-retroviral agents with different mechanisms of action is encouraged.

Sulfated polysaccharides obtained from marine algae have been shown to inhibit the propagation of different viruses, including HTLV-1. The mechanism by which these polysaccharides exert an antiviral effect can be attributed to interference in the virus binding to cell receptors (Witvrouw & De Clercq, 1997). Furthermore, Ida et al. (1994) and Romanos et al. (2002a) have also shown that sulfated polysaccharides are able to inhibit cell-to-cell contact, essential for HTLV-1 transmission.

Cell-to-cell contact is needed for effective infection with HTLV-1 (Miyoshi et al., 1981; Yamamoto et al., 1982; Popovic et al., 1983; Delamarre et al., 1997; Lairmore & Franchini, 2007). However infection with free virus particles has also been described *in vitro* (Clapham et al., 1983; Hoxie et al., 1984; Trejo & Ratner, 2000).

In this study we report that a sulfated fucan from the *Laminaria abyssalis* marine alga inhibits HeLa cells infection by free HTLV-1 particles *in vitro*.

Materials and Methods

Cells and virus

HTLV-I infected T-cell line MT-2 (established cell line from umbilical cord lymphocytes transformed by HTLV-1 following co-cultivation with leukemia-lymphoma cells) and non-infected cell line HeLa (human cervical carcinoma) were maintained in RPMI 1640 (Sigma) supplemented with 2 mM L-glutamine (Sigma), 50 µg/mL garamicin (Shering-Plough), and 2.5 µg/mL fungizon (Gibco) plus 10% of heat-inactivated fetal bovine serum (FBS) (Cultilab) and maintained at

37 °C in atmosphere of 5% of CO_2.

Sulfated polysaccharide from marine algae

Laminaria abyssalis A. B. Joly & E. C. Oliveira (Phaeophyta) marine alga, a brown seaweed, was collected from 60 m depth of Macaé City at (22° 30'S; 40° 59'W), Rio de Janeiro State, Brazil. After collection the algae were kept alive until arrival at the laboratory. A voucher of this specimen has been deposited in the Botany Department, Biology Institute, Federal University of Rio de Janeiro, Brazil. The polysaccharide was extracted from the dried alga by papain digestion and the sulfated fucan was purified by *N*-cetyl-*N,N,N*-trimethilammonium bromide and ethanol precipitations, followed by anion-exchange chromatography on DEAE-cellulose, as previously described (Pereira et al., 1999). The purity of the sulfated fucan was evaluated by chemical analysis, agarose gel electrophoresis and ^{1}H-NMR spectra, as described (Pereira et al., 1999).

Cytotoxicity assays

The cytotoxicity of the sulfated fucan to HeLa cells was assessed via the *in situ* reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to blue formazan by mitochondrial dehydrogenases of metabolically active cells. HeLa cell confluent monolayers (2 x 10^5 cells/mL), cultivated in 96-well flat-bottomed plates, were incubated in the presence of the sulfated fucan at different concentrations. After 24 h, 20 µL of MTT (7.5 mg/mL in phosphate-buffered saline, pH 7.2) was added to each well and the cell cultures were incubated for 5 h at 37 °C. Solubilization of the formazan crystals was achieved by adding 100 µL of 0.4% HCl and 10% Triton X-100 in isopropanol. The absorbance was read in a spectrophotometer (Bio-Rad Model 3550-UV) at a wavelength of 595 nm (Romanos et al., 2002b).

Concentration and purification of virus particles

The viral suspension was obtained from the growth media of the virus-producing cell line MT-2. The cell suspension containing HTLV-1 particles was clarified by centrifugation (Sorval RC5-B - rotor HS-4) at 6,500 *g* for 30 min and the supernatant was concentrated by ultracentrifugation (Beckman LE 80K, rotor Beckman 45 ti) at 30,000 *g* for 2 h in 17% sucrose cushion. The pellet was resuspended to 1:100 (in relation to the original volume of virus suspension) in TNE buffer (20 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH7.5). HTLV-1 was purified by ultracentrifugation (Beckman LE 80K, rotor Beckman SW40) through a 20 to 60% sucrose gradient at 100,000 *g* for 18 h.

Detection of the purified virus

Before carrying out the cell-free infection assay, a RT-PCR assay was performed for detection of the HTLV-1 particles in the virus preparation. RNA was extracted from the purified virus suspension in Catrimox-14 solution (Iowa Biotechnology Corp., Coralville, IA) following the manufacturer's specification with minor modification. Two hundred microliters Purified virus suspension (200 µL) were added to 1 mL of Catrimox-14. After centrifugation (Heraeus Instruments Microcentrifuge) at 1,000 *g*/5 min, the supernatant was discarded and the pellet was resuspended in 500 µL of 2M lithium chloride. The mixture was centrifuged (1,000 *g*/5 min), the supernatant was discarded, and the pellet was washed in 70% ethanol and solubilized in deionized water. The RNA from MT-2 cell was also extracted using the same procedure.

To discard the possibility of DNA contamination, the extracted RNA was treated with 500 U/mL DNase (Pharmacia) for 2 h.

The purified RNA was assayed by RT-PCR. RNA (10 µL) were added to PCR tubes containing 7 µL dimethylsulfoxide (Sigma) and denatured at 97 °C for 5 min. The suspension was then cooled in ice for 5 min and the following reaction mixture was added: 52 µL water; 16 µL deoxynucleoside triphosphate mixture (containing 1.25 mM each of dATP, dGTP, dCTP, and dTTP [Life Technologies]); 10 µL 10x buffer (containing 200 mM Tris-HCl, pH8.4, 500 mM KCl [Life Technologies]); 3 µL 50 mM $MgCl_2$ (Life Technologies); 1 µL of primers (containing 50 pmol/µL each of SK43 (+) (5'-CGGATACCCAGTCTACGTGT-3') and SK44 (-) (5'-GAGCCGATAACGCGTCCATCG-3'); 2.5 units of *Taq* DNA polymerase (Life Technologies); and 3.3 units of reverse transcriptase (AMV-RT [Molecular Genetic Resources]). The mixture was incubated at 42 °C for 45 min for reverse transcription, followed by an incubation of 94 °C/5 min, and submitted to 35 cycles of PCR (1 min. at 94 °C, 30 s at 60 °C, 30 s at 70 °C), and a final 7 min incubation at 68 °C. The primers amplified a 159-bp segment from the pX gene.

Amplified DNA fragments were submitted to Nested-PCR. PCR products (5 µL) were added to PCR tubes containing 7 µL DMSO and 88 µL of the reaction mixture were added: 57 µL water; 16 µL deoxynucleoside triphosphate mixture (containing 1.25 mM each of dATP, dGTP, dCTP, and dTTP [Life Technologies]); 10 µL 10x buffer (containing 200 mM Tris-HCl, pH8.4, 500 mM KCl [Life Technologies]); 3 µL 50mM $MgCl_2$ (Life Technologies); 1 µL of

primers (containing 40 pmol/µL each of TN1 (+) (5'-ACGTGTTTGGCGATTGTGT-3') and TN2 (-) (5'-CATCGATGGGGTCCCAGGTGA-3'); 2.5 units of *Taq* DNA polymerase (Life Technologies)). PCR was performed as described above. The primers amplified a 129-bp segment.

PCR products (10 µL) were loaded on 1.2% agarose gel in TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). The electrophoresis was performed at 120V for 1 h in 0.5X TBE buffer containing 0.5 µL of ethidium bromide/mL.

Antiviral assay

HeLa cells monolayers (10^6 cells/mL) were infected with 400 µL of purified HTLV-1 suspension and sulfated fucan (100 µg/mL) added at different times (0, 1, 2 and 3 h) after infection. Four hours after infection, cell monolayers were washed three times and the DNA was extracted in DNAzol reagent (Life Technologies) following the manufacturer's specification. Five hundred microliters of DNAzol reagent were added to the cell monolayer, and the DNA was precipited by the addition of 250 µL of 100% ethanol. The sample was mixed by inversion, stored at room temperature for 3 min. and centrifuged (Heraeus Instruments Microcentrifuge) for 10 min. at 2,500 g. The pellet was washed in 70% ethanol and solubilized in deionized water. The sample obtained was analysed for HTLV-1 proviral DNA by PCR and Nested-PCR as above described.

Results

Initially we tested whether algal sulfated fucan is toxic to HeLa cells. The cells were exposed to a high concentration of the sulfated fucan (100 µg/mL) and no cytotoxicity was observed, as indicated by *in situ* assay of reduction of MTT to blue formazan by mitochondrial dehydrogenases of metabolically active cells (not shown).

Subsequently, we purified the virus particles from the growth media of the virus-producing cell line MT-2. RT-PCR was carried out to detect viral RNA in a purified HTLV-1 suspension. After RT-PCR, the cDNA was detected only on virus harvested from MT-2 cells. To detect the cDNA from purified virus suspensions, the RT-PCR product was submitted to nested-PCR (Figure 1).

Finally, we evaluated the antiviral activity of the sulfated fucan. For this experiment, 400 µL of purified virus suspension were added to HeLa cells monolayers. Sulfated fucan was added to the cell cultures at different times after the infection.

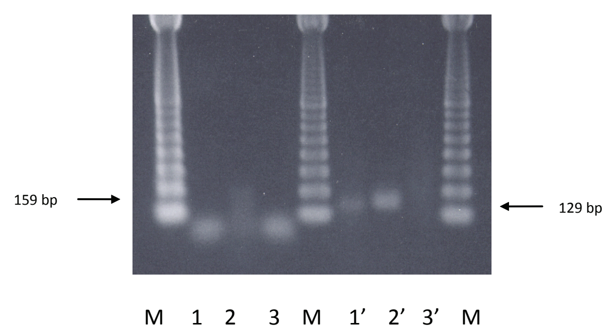

Figure 1. RT-PCR and Nested-PCR for detection of HTLV-1 in the purified virus suspension. RNA extracted from the purified HTLV-1 suspension and HTLV-1-producing MT-2 cell were submitted to RT-PCR (lanes 1- 3) and Nested-PCR (lanes 1'-3'). Amplified products were observed in agarose gel. Molecular markers (M); RNA from purified HTLV-1 (lanes 1 and 1'); RNA from HTLV-1-producing MT-2 cell (lanes 2 and 2') and; Blank (lanes 3 and 3').

The inhibitory effect of virus infection was evaluated by PCR and Nested-PCR to detect the cDNA, as an indicator of cellular infection by the virus. Figure 2 shows that no cDNA was detected when the sulfated polysaccharide was added up to 2 hours post-infection, indicating that the inhibitory activity occurred in the initial stages of virus-cell interaction, possibly during adsorption and/or penetration of the virus into the cells.

Discussion

The antiviral effect of sulfated polysaccharides has been associated with their capability to prevent the interaction between molecules present on the surface of the virus and cellular receptors (Nakashima et al., 1989; Baba et al., 1990; Ida et al., 1994; Witvrouw et al., 1994; Sekine et al., 1995; Hayashi et al., 1996; Romanos et al., 2002b; Matsuhiro et al., 2005; Talarico et al., 2005; Adhikari et al., 2006). In a previous study, we demonstrated that a sulfated fucan from the marine alga *Laminaria abyssalis* was able to inhibit the interaction between HTLV-1 infected cells and indicator cells, avoiding the syncytium formation, essential for an efficient virus infection (Romanos et al., 2002a).

Infection with cell-free HTLV-1 particles is less efficient compared to cell to cell transmission. Nevertheless this mode of transmission may be relevant under particular situations. In fact, in this study we observed that free-particles of the virus induced infection *in vitro*, although a high viral load was necessary. The infection obtained using a concentrated virus suspension was detected only by amplification of the proviral cDNA by nested-PCR.

We also evaluated the capacity of a sulfated fucan to prevent the interaction of HTLV-1 particles

with surface cellular receptors. The sulfated fucan was added at different times after infection. Four hours after infection, the cellular DNA was extracted and the HTLV-1 proviral cDNA was analyzed. Our results confirmed that the sulfated fucan from *L. abyssalis* was able to inhibit the initial stages of the viral infection.

The results presented here demonstrate, for the first time, the ability of a sulfated fucan from marine alga to inhibit virus transmission through free virus particles.

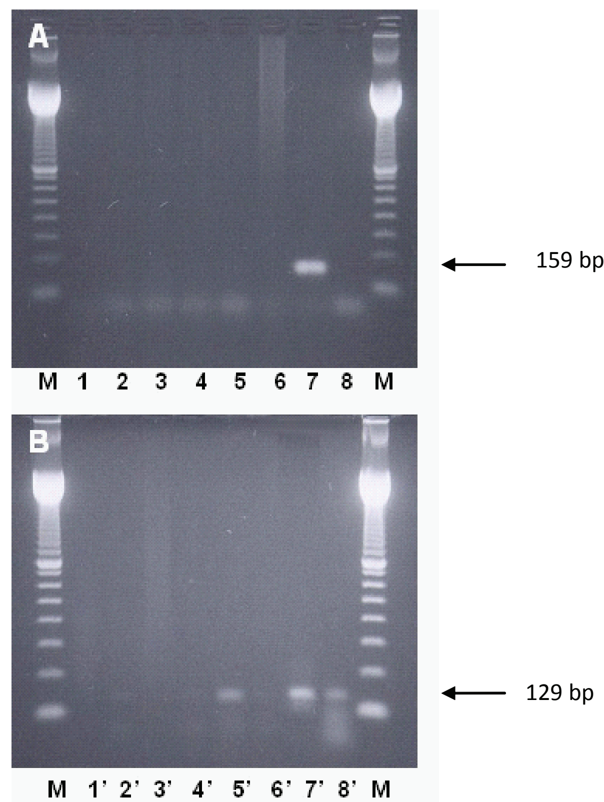

Figure 2. cDNA detection in HTLV-1 infected HeLa cells in the presence of the sulfated fucan. HTLV-1 infected HeLa cells were treated with the sulfated fucan at different times (0, 1, 2 and 3 h) after infection. After 4 h, the cDNA was extracted and detected by PCR (A) and Nested-PCR (B). Molecular markers (M); HeLa cell (Negative Control) (lanes 1 and 1'); HTLV-1 infected HeLa cell in the presence of sulfated fucan at 0 h (lanes 2 and 2'); after 1 h (lanes 3 and 3'); after 2 h (lanes 4 and 4'); after 3 h (lanes 5 and 5'); Blank (lanes 6 and 6'); cDNA from MT-2 cell (positive control) (lanes 7 and 7'); HTLV-1 infected HeLa cell without treatment (lanes 8 and 8').

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