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Short Communication

A sulphated fucan from the *Laminaria abyssalis* inhibits the human T cell lymphotropic virus type 1-induced syncytium formation in HeLa cells

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This work evaluated the effect of a sulphated fucan extracted from the *Laminaria abyssalis* marine algae on the human T cell lymphotropic virus type 1 (HTLV-1)-induced syncytium formation. The experiments were carried out in HeLa cells cocultured with a HTLV-1-infected T cell line (C91/PL cells) in the presence of the sulphated polysaccharide at concentration below that corresponding to the ED_{50}. The sulphated fucan inhibited almost 100% of the syncytium formation at concentration of 100 µg/ml and was still active (>95%) at a concentration of 25 µg/ml. It was also observed that the best inhibition occurred when the compound was added in the first 2 h of the cell-to-cell contact. This is the first report showing that a purified sulphated polysaccharide, extracted from marine algae, is able to inhibit the cell-to-cell contact essential for the spreading of the HTLV-1.

Keywords: human T cell lymphotropic virus type 1 (HTLV-1), *Laminaria abyssalis*, marine algae, sulphated fucan, syncytium inhibition

Introduction

The marine world has an extensive diversity of plant species that have been arising as a potentially valuable alternative source of natural bioactive products (Hatch *et al.* 1979).

The first paper reporting the antiviral activity of marine algal extracts was published in 1958. In this work, Gerber *et al.* (1958) showed that some seaweed extracts had antiviral activity against mumps and influenza B viruses. After that, this inhibitory activity was also extended to other enveloped viruses, including herpes simplex virus (HSV), cytomegalovirus (CMV) and human immunodeficiency virus (HIV) (reviewed by Witvrouw & De Clercq, 1997).

Initially identified by Poiesz *et al.* in 1980, the human T cell lymphotropic virus type 1 (HTLV-1) has been associated with a haematopoietic malignancy, adult T cell leukaemia/lymphoma and to a degenerative neurologic disorder called tropical spastic paraparesis or human T cell lymphotropic type 1-associated myelopathy. These diseases are difficult to treat and consequently have a poor prognosis (Blattner, 1997).

In a previous study (Romanos *et al.*, 2002) we reported a screening performed with extracts of four Brazilian marine algae regarding to their inhibitory effect of HTLV-1-induced syncytium formation. This is a critical event for the interaction of HTLV-1-infected cells and non-infected cells, and the consequent viral transmission. We now demonstrate that the sulphated fucan isolated from one of the alga, *Laminaria abyssalis*, is one of the active substance in the aqueous extract responsible for the inhibitory activity.

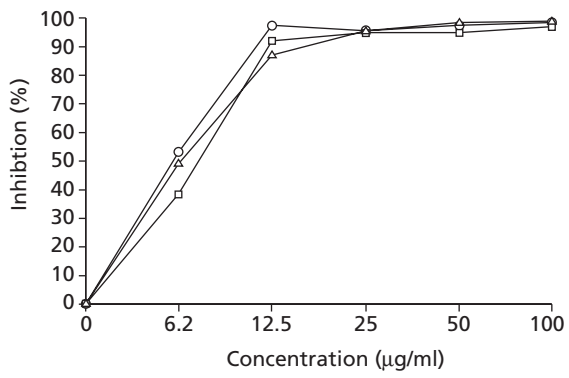
Laminaria abyssalis (Phaeophyta) is a Brazilian endemic brown marine algae that was collected from 60 m depth of Macaé City (22°30′S; 40°59′W) at Rio de Janeiro State, Brazil. A voucher of this specimen is kept at the Botanical Department, Biology Institute, Federal University of Rio de Janeiro, Brazil. The algae was washed with tapwater and three times with distilled water to withdraw the salt, dried and the aqueous extract prepared by grinding the material with distilled water at concentration of 20% (w/v) in a mixer for 10 min. The homogenate was centrifuged at 1500×*g* for 10 min and the resulting supernatant was diluted (1:2) in RPMI 1640 2X, sterilized by filtration with Millipore membrane 0.22 μm, lyophilized and stored at -20°C. The lyophilized extract was dissolved in RPMI 1640 at concentration of 100 μg/ml before use. The dried algae was submitted to papain digestion to obtain the sulphated fucan that was purified by N-cetyl-N,N,N-trimethilammonium bromide and ethanol precipitation, followed by anion-exchange chromatography on DEAE-cellulose, as previously described (Pereira *et al.*, 1999). The purity of the sulphated fucan was evaluated by chemical analysis, agarose gel electrophoresis and ^{1}H-NMR spectra, as described (Mulloy *et al.*, 1994; Pereira *et al.*, 1999). The sulphated fucan from *L. abyssalis* has a complex and heterogeneous structure. It is a branched polymer with fucosyl units at nonreducing ends containing 3- and 4-linked residues, sulphated at the O-2 and O-4 positions. Dextran sulphate (MW aproximately 8000 – Sigma-Aldrich Química Brazil Ltd, São Paulo, Brazil) was used as standard.

The cytotoxicity of the polysaccharide was assessed by a spectrophotometric method based on the *in situ* reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan by mitochondrial dehydrogenases of metabolically active cells. In these experiments C91/PL cells (HTLV-1-infected T cell line) and HeLa cells (human cervical carcinoma) were cultivated in 96-well flat-bottomed plates. C91/PL cells (10^6 cells/ml) and HeLa cells (2×10^5 cells/ml) were maintained in RPMI 1640 medium (Sigma) supplemented with 2 mM L-glutamine, 50 mg/ml gentamicin, 2.5 mg/ml fungizon plus 10% of heat-inactivated foetal bovine serum (FBS) and incubated at 37°C in an atmosphere of 5% CO_2. After 24 h of incubation with different concentrations of the algal extract, purified sulphated fucan or dextran sulphate, 20 μl of MTT (7.5 mg/ml in phosphate-buffered saline, pH 7.2) were added to each well and the cells were reincubated for 5 h at 37°C. The formazan crystals were dissolved by the addition of 100 μl of 0.4% HCl and 10% Triton X-100 in isopropanol and their absorbance was then measured in a spectrophotometer (Bio-Rad Model 3550 UV) at a wavelength of 595 nm. No significant toxicity was observed when these cells were exposed to algal extract or purified sulphated fucan, even at high concentration (100 μg/ml), while the standard dextran sulphate had a slight cytotoxic effect.

In the syncytium inhibition assay, C91/PL cells were cocultured with HeLa cells in the presence of several concentrations of the algal extract, sulphated fucan and dextran sulphate. After 24 h, C91/PL and medium were removed, the HeLa cells were washed three times using phosphate-buffered saline (PBS), fixed with 95% methanol in PBS for 20 min. and stained with 0.3% Wright in methanol. The cells were then examined on an inverted microscope (Olympus CK 2) and those with five or more nuclei were scored as syncytium. Syncytia were counted in six fields at random and the sum of six fields was scored as syncytium numbers (Ida *et al.*, 1994). The percentage of inhibition was calculated comparing treated and untreated cells. No syncytium was observed when HeLa cells were cultured in the absence of C91/PL cells. The cocultivation of HeLa indicator monolayer culture with C91/PL led to the formation of multinucleated giant cells (data not shown). A strong inhibition (~100%) of the syncytium formation was observed at a concentration of 100 μg/ml and was still significant (>95%) at a concentration of 25 μg/ml (Figure 1).

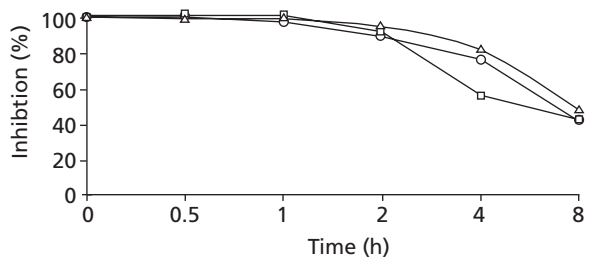
The formation of syncytium could result from to cell-to-cell contact or as a consequence of viral protein synthesis. The time course of this event increases after 1 h of coculture and reaches a maximum effect at 8 h (data not shown). A similar result was observed by Ida *et al.* (1994) using

Figure 1. Inhibitory effect of the *Laminaria abyssalis* extract, sulphated fucan and dextran sulphate on syncytium formation



C91/PL cells were cocultured with HeLa cells in the presence of several concentrations of *L. abyssalis* algal extract, sulphated fucan and dextran sulphate. After 24 h, C91/PL and medium were removed, the HeLa cells were washed with PBS, stained with 0.3% Wright in methanol. Syncytia were counted in six fields at random by inverted microscope and the sum of six fields was scored as syncytium numbers. The percentage of inhibition was calculated comparing treated and untreated cells. *Laminaria abyssalis* extract (circles), sulphated fucan (squares), dextran sulphate (triangles).

Figure 2. Syncytium formation inhibition by the sulphated fucan at different times of contact between infected cells and non-infected cells



C91/PL cells were added to HeLa cell monolayer. At different times (0, 0.5, 1, 2, 4, 8 h) the algal extract, sulphated fucan or dextran sulphate (100 µg/ml) were added to the cocultive and re-incubated up to 24 h. C91/PL cells and medium containing the extract or compounds were removed, the HeLa cells were washed, fixed, stained and the number of syncytia counted by inverted microscope. The percentage of inhibition was calculated comparing treated and untreated cells. *Laminaria abyssalis* extract (circles), sulphated fucan (squares), dextran sulphate (triangles).

HCT-1 and XC cell lines as indicator cells. These data indicate that syncytium formation comes from cell-to-cell contact and is not due to the viral protein synthesis by indicator cells. Furthermore, no changes were observed on the syncytium formation when HeLa cells were treated with actinomycin D before coculture with C91/PL (data not shown).

At different times of coculture (0, 0.5, 1, 2, 4, 8 h), the algal extract, sulphated fucan or dextran sulphate at concentration of 100 µg/ml were added and incubated for up to 24 h. Following this, the C91/PL cells and medium containing the extract or compounds were removed, the HeLa cells were washed, fixed and stained as mentioned above. The best inhibition occurred when the extract or compounds were added in the first 2 h of the cell-to-cell contact (Figure 2). This result is in agreement with the study of Ida *et al.* (1994) who demonstrated the inhibitory effect of dextran sulphate and heparin on HTLV-1-induced syncytium formation, particularly when the compounds were added in the early phase of the assay. Probably, the sulphated fucan from *L. abyssalis* seaweed inhibits the attachment of the viral protein expressed by the infected cells to the receptors on the target cells.

Our results suggest that the sulphated fucan extracted from *L. abyssalis* brown marine algae is an attractive candidate for inhibition of the HTLV-1 transmission. Further studies are necessary to determine the efficacy of this compound in the therapy and/or prevention of the HTLV-1 infection. The anticoagulant activity of sulphated polysaccharides is a common side effect that can limit the use of these compounds as antiviral agents. The observation that the sulphated fucan from *L. abyssalis* has a lower anticoagulant activity than mammalian heparin (Pereira *et al.*, 1999) makes this polysaccharide even more attractive for antiviral therapy.

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