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Antiviral Activity of Carrageenans from Marine Red Algae

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***SUMMARY*. Three carrageenan representatives of each structural type: λ- and ι –family *(Gigartina acicularis)*, ι-family *(Euchema denticulatum)* and κ –family *(Kappaphycus cottonii)* have been tested for their in vitro antiviral activity. The carrageenans proved to be potent inhibitors of herpes human virus type 1 (HHV-1) and Poliovirus. The best results were obtained with carrageenans from *Gigartina acicularis* and *Euchema denticulatum,* which are more sulfated than those from *Kappaphycus cottonii.* The selective index values (CC_{50}/ID_{50}) ranged from more than 22 to more than 545 for HHV-1 and more than 6.6 to more than 32 for Poliovirus. No citotoxic effects were observed. At 0.75 mg/ml, none of the carrageenans tested showed a virucidal activity against HHV-1 or Poliovirus. Carrageenans from *Euchema denticulatum* (ι-family) and *Gigartina acicularis* (λ- and ι –family) exerted their antiviral effect via, in part, by a lower inhibition of the virus attachment and by the interference in a subsequent stage of the virus replicative cycle. The κ-carrageenan from *Kappaphycus cottonii* exerted its antiviral effect mainly by a lower inhibition of the virus attachment. In cultures treated with carrageenans from *Euchema denticulatum* (ι-family) and *Gigartina acicularis* (λ- and ι –family), the HHV-1 viral DNA synthesis had a reduction of threefold and twofold with 0.75 mg/ml, respectively.**

INTRODUCTION

Carrageenans are sulfated polydigalactosides (20-50% $^{-}OSO_3Na$) with molecular weight between 10^5 and 10^6 that can be extracted from several red seaweeds. They comprise a broad range of structures and are divided into families: the κ-family is defined by the presence of a C-4-sulfate group on the β-D-galactose unit; the ι -family is characterized by a C-4-sulfate group on the β-D-galactose unit and a C-2-sulfate group on the 3-6-anhydro-α-D-galactose; the λ-family is characterized by a sulfate group on the C-2 of β-D-galactose unit and 2,6-dissulfate on α-D-galactose unit [1]. Polysaccharides is a complex group of peculiar molecules exhibiting a wide range of biological activities such as antiinflammatory, anticoagulant, antithrombotic, antitumoral, antimetastatic, antifertilizing and antiviral [2]. Unlike antimicrobial drugs against bacteria and fungi, only a few effective antiviral drugs are available. One of the most important reasons for the lack of success in developing antiviral drugs is due to the nature of the infectious viral agents, which totally depend upon the cell they infect for multiplication and survival, so, compounds that may cause the death of viruses also are very likely to injure the host cell that harbour them [3].

Efforts have been made to evaluate the antiviral activity of natural products, including those from algaes, in order to characterize new compounds which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules. Numerous carrageenans and other polysaccarides have been tested for their antiviral activity [4-7].

KEY WORDS: Antiviral, *Carrageenan,* Herpes Simplex viruses, Poliovirus, Polysaccharide, Red algae.

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We report herein our results concerning the *in vitro* activity against HHV-1 and Poliovirus of three carrageenan representatives of each structural type: λ- and ι –family *(Gigartina acicularis),* ι-family *(Euchema denticulatum)* and κ –family *(Kappaphycus cottonii).* Secondly, the mechanism of viral inhibition was determined.

MATERIALS AND METHODS

Carrageenan

The carrageenans tested were extracted from three marine red algae (Rhodophyceae) collected in different countries: *Euchema denticulatum* (Philippines) *Gigartina acicularis* (Bretagne, France) and *Kappaphycus cottonii* (Philippines). They were extracted with hot distilled water, filtered on diatomaceous earth and the filtrate was poured into absolute ethanol, with stirring. The precipitate was recovered and washed with 95° ethanol, dehydrated with diethyl ether and dried overnight at 50 °C [8]. The chemical composition of each extract differs in the content of sulfated galactans and type of carrageenan. Each algae seems to have different types of carrageenan: *Euchema denticulatum* (ι-family) [9,10], *Gigartina acicularis* (λ- and ι –family) [11] and *Kappaphycus cottonii* (κ –family) [10].

The carrageenan samples were provided by Prof. J.M. Kornprobst (SMAB/ISOMer – Nantes/ France). Stock solutions (10 mg/ml) were prepared in PBS buffer and stored at -20 °C; and, for the experiments, aliquots were diluted with MEM to obtain the indicated concentrations.

Cells and viruses

African green monkey kidney cells (VERO cell line ATCC CCL81) were grown in Eagle's minimum essential medium (MEM) supplemented with 10 % newborn calf serum, 160 Units ml-1 penicillin and 80 mg.ml^{-1} gentamicin. Cells were routinely passed every three days. The viruses used were the following: *Human Herpesvirus* 1 (HHV-1 strain H29S) and poliovirus type 2, a vaccinal strain Sabin II. Virus stocks were propagated by serial passages on VERO cells at a low multiplicity, incubated for 2 days, then frozen and thawed three times. Afterwards the preparation was cleaned by centrifugation at low speed in order to remove the cell debris. Virus titration was performed by the limiting dilution method [12]. The virus titre was estimated from cytopathogenicity and expressed as 50 % tissue culture infectious doses ml^{-1} ($TCID_{50}$/ml). It was 2.0 x $10^{5.5}$ $TCID_{50}$/ml for HHV-1 and 2.0 x $10^{6.57}$ $TCID_{50}$ /ml for poliovirus.

Evaluation of cytotoxicity

Aiming assess the effect of carrageenan on uninfected Vero cells, dilutions ranging from 6 mg/ml to 11.7 µg/ml (i.e. final concentrations in wells from 1.5 mg/ml to 2.9 µg/ml) in the maintenance medium were added to VERO monolayers (a 96-well microplate with 4.0 x 104 cells per well). After incubation for 72 h, cytotoxicity was determined by a microscopic examination of the cell morphology. The concentration at which the cell number was reduced to 50% in relation to that of the controls was taken as the 50% cytotoxic concentration (CC_{50}). The maximum tolerated concentration (MTC) was not possible to determine because the carrageenan solutions were very viscous. All assays were carried out in triplicate.

Antiviral activity

Antiviral assays were carried out as described previously [13]. Dilutions of the carrageenan extracts ranging from 0.75 mg/ml to 0.003 µg/ml were prepared in the maintenance medium and added to confluent 1-day-old monolayers of Vero cells. These cells were grown in microtitre tissue culture plates just before inoculation, at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. Toxicity, cell, and virus controls were run simultaneously. Plates were incubated at 37 °C for 72 h for HHV-1 or 32 h for poliovirus (multiple replicative cycles). Then, the monolayers were observed for cytopathic effect. The concentration which inhibited 50% the viral cytopathic effect (compared to the virus control) was expressed as the 50% inhibitory dose (ID_{50}). In order to quantify the antiviral activity, the same plates were frozen and thawed three times. The contents of the identical wells were harvested, mixed and clarified by low-speed centrifugation; and, virus titrations were performed on the supernatant fluids by the limiting dilution method [12]. The antiviral activity of each carrageenan extract was determined as the reduction factor ($\log_{10}$) of the viral titre by the comparison with untreated controls. All experiments were carried out three times.

Study of mode of action of the carrageenan extracts against HHV-1 and Poliovirus

All experiments with carrageenans from *Gigartina acicularis* (λ- and ι –family), *Euchema denticulatum* (ι-family) and *Kappaphycus cottonii* (κ–family) were performed at non toxic concentration of 0.75 mg/ml.

Virus inactivation

Equal volumes of carrageenan extracts and HHV-1 or Poliovirus stock suspension were mixed and incubated for 1 h at 37 °C in order to test possible virucidal activity. Thereafter, each mixture was diluted 10-fold serially and infectious titres were compared to those of controls [14].

Yield reduction after a single cycle of replication

Vero cell monolayers cultured in 4-well culture plates were infected with HHV-1 or Poliovirus at a multiplicity of infections of about 1. After 60 min at 37 °C, the unadsorbed viruses were removed and the monolayers were washed twice with MEM; subsequently, carrageenan extract dissolved in MEM was added. After incubation of 18 h for HHV-1 or 8 h for Poliovirus, the cultures were frozen and thawed three times the cell debris was removed by low-speed centrifugation. The supernatant virus titres were determined by the limiting dilution method and compared to that of the controls without carrageenan extracts [14].

Culture pretreatment

Vero cell monolayers were pretreated with each carrageenan extract for 24 h at 37 °C. After being washed with MEM, the cells were exposed to HHV-1 or Poliovirus at an Multiplicity of Infections (MOI) of about 1. Following incubation, the experiment was performed as described above but no carrageenan extract was added to the medium [14].

Study of viral DNA synthesis inhibition by nucleic acid hybridization

Purification of HHV-1 DNA

Vero cells grown in 25 cm^2 tissue culture flasks were infected at a high multiplicity (MOI = 1), and HHV-1 adsorbed for 1 h at room temperature, followed by washing with MEM to remove unadsorbed virus. From then on, only maintenance medium (control) or carrageenan extract dissolved in MEM (assays) was added. The cultures were reincubated at 37 °C for 18 h. The medium was discarded and the cells were disrupted with 100 µl of lysis buffer. Subsequently, DNA was purified, as previously described by Boom *et al.* [15], using a diatom suspension. Samples were dissolved in 100 µl of sterile water.

Hybridization probe

The digoxigenin-labelled DNA probe was a non radioactive probe prepared by polymerase chain reaction according to the method described by Griffais *et al.* [16]. The DNA template was the U57 gene of HHV-1 obtained by a first PCR [16]. This template was amplified using two primers: 5'-CTCACAGCCCCGAT-3' and 5'-GTCCCGCGTTGC-3'. They were mixed with *Thermus aquaticus* polymerase (Perkin Elmer Cetus, Norwalk, USA) and deoxynucleotide triphosphates: d ATP, d CTP, d GTP and d UTP linked to digoxigenin, dig-d UTP (Boehringer Mannheim, Fr.). The solution was subjected to 38 cycles of amplification in 3 steps: 92 °C, 55 °C and 72 °C. Amplification was verified using agarose gel electrophoresis.

Hybridization procedure

A twofold dilution of DNA extracts was performed serially from 1/10 to 1/320 and dilutions were heated up to 95 °C to denature DNA. The samples were deposited on a Nylon membrane HYBOND N (Amersham International Plc, UK) and fixed to a HYBRI-SLOT manifold (BRL, Maryland, USA). Afterwards, the filters were prehybridized in thermally sealed plastic bags for 3 h at 42 °C in a prehybridization solution containing 1% of a blocking agent (Boehringer Mannheim, Fr.). After denaturation, a non-radioactive probe was added into the bag and hybridization was carried out at 42 °C for 18 h, with gentle shaking.

Detection of HHV-1 DNA probe binding

The hybrids were detected by enzyme-linked immuno-assay using an antibody conjugate: antidigoxigenin sheep antibody conjugated to alkaline phosphatase. The colour reaction was initiated at alkaline pH by the addition of 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT). (The detection kit was purchased from Boehringer Mannheim, Fr.). A blue precipitate indicated the presence of HHV-1 DNA.

RESULTS AND DISCUSSION

We have investigated the anti-HHV-1 and the antipoliovirus activity *"in vitro"* of three carrageenans extracts from three different algae species collected in different countries.

The antiviral activity was first evaluated by the inhibition of the cytopathic effect (CPE) in cultures inoculated at a multiplicity of infection of 0.01. The ID_{50} (mg/ml) values required for a 50% CPE inhibition and the selectivity indexes (CC_{50}/ID_{50}) are indicated in Table 1.

Carrageenan (type)	CPE inhibition (MOI 0.01)				Yield reduction [a] (MOI 0.01)	
	HHV-1		Poliovirus		HHV-1	Poliovirus
	ID_{50}	SI	ID_{50}	SI	log_{10}	
Gigartina acicularis (λ and τ)	0.00275	>545	0.047	>32	3.00 ± 0.86	2.50 ± 0.50
Euchema denticulatum (τ)	0.026	>71	0.115	>13	3.00 ± 0.25	1.50 ± 0.25
Kappaphycus cottonii (κ)	0.068	>22	0.227	>6.6	2.00 ± 0.25	1.50 ± 0.25

Table 1. *In vitro* antiviral activity of carrageenans against HHV-1 and Poliovirus. For three carrageenans the CC50 is > 1.5 mg/ml; ID_{50} (mg/ml); SI (CC_{50}/ID_{50}). [a] when compared with controls HHV-1 virus titre. Results are presented as the mean ± SD from three independent tests.

As shown in Table 1, the CC50 for the three carrageenans was higher than 1.5 mg/ml (maximal concentration tested). Thus, the selectivity index (ratio CC_{50}/ID_{50}) in the confluent cultures used in antiviral assays range from 22 to more than 545 for HHV-1; and from more than 6.6 to more than 32 for Poliovirus at 0.01 MOI. Human Herpes virus type I was more susceptible than poliovirus to all carrageenan extracts. For HHV-1 the highest selectivity index values were in the same range as those previously found for polysaccharides dextran sulfate and heparin which were tested against HIV replication in MT-4 and Molt-4 cell cultures [17] and for λ-carrageenan and pentosan polysulfate tested against ASFV *"in vitro"* [18].

The effect on HHV-1 and Poliovirus replication was precisely quantified by infectious titre reduction after several rounds of multiplication, being the culture inoculated at 0.01 MOI. To be considered active, a sample should induce at least a 2 log_{10} decrease in virus titre in comparison with untreated virus control [19]. According to this criterion, all carrageenans tested were active since the compounds reduced the virus titre between 2.0 and 3.0 log_{10} against HHV-1; whereas, the activity against Poliovirus was lower with titre reduction between 1.5 and 2.25 log_{10}. These results are shown in Table 1. The best results were obtained with carragenans from *Gigartina acicularis* and *Euchema denticulatum,* the more sulfated according to Amat [1].

Virus inactivation

No loss of infectivity was observed when HHV-1 or Poliovirus was incubated for 1 h at 37 °C with *Gigartina acicularis* (λ- and ι–family), *Euchema denticulatum* (ι-family) and *Kappaphycus cottonii* (κ–family) extracts of 0.75 mg/ml; it can be concluded that none of them has a virucidal for the two viruses tested.

Effect of cell culture pretreatment

After a single round of replication, the HHV-1 yield reduced 0.9, 0.9 and 0 log_{10} with *Euchema denticulatum* (ι-family), *Gigartina acicularis* (λ- and ι–family) and *Kappaphycus cottonii* (κ–family), respectively. These results suggest that the sulfated polysaccharides from *Euchema denticulatum* and *Gigartina acicularis* have a little effect on virus attachment or virus entry.

Effect of carrageenans on the yield of virus from a single round of replication

In this experiment, Vero cells were infected at a MOI of about 1; then, the carrageenans were added to the culture medium 1 h posterior to inoculation to exclude any effects on adsorption and penetration. After a single round of replication, the HHV-1 yield reduced 2.0, 1.9 and 1.1 log_{10} with *Euchema denticulatum* (ι-family), *Gigartina acicularis* λ- and ι–family) and *Kappaphycus cottonii* (κ–family), respectively. These results suggest that carrageenans from *Euchema denticulatum* (ι-family) and *Gigartina acicularis* exert their antiviral effect, in part, by the inhibition of the virus attachment and the interference in a subsequent stage of the virus attachment. To determine this step, HHV-1 DNA synthesis was evaluated by a hybridization technique in cultures where the most active carrageenans (*Euchema denticulatum* - ι-family and *Gigartina acicularis* - λ- and ι–family) were added. The effect of carrageenans on HHV-1 DNA synthesis was measured by nucleic acid hybridization with a digoxigenin-labelled HHV-1 DNA probe. The DNA of untreated virus-infected controls, serially diluted twofold, was detectable up to a 1:320 dilution whereas the DNA of uninfected untreated control cells could not be detected even at a 1:10 dilution, which proved the probe specificity. In cultures treated with carrageenans from *Euchema denticulatum*

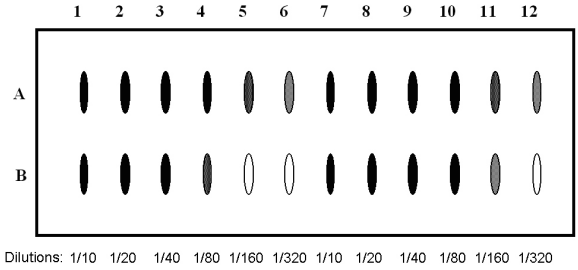

Figure 1. Slot hybridization of DNA extracts from HHV-1 infected cells untreated (A1 to A6) and treated with carrageenans (A7 to A12, B1 to B6 and B7 to B12).A1 to A6: DNA of untreated virus control (HHV-1). A7 to A12: DNA of cultures treated with 0.37 mg/ml of *Euchema denticulatum* (τ-family). B1 to B6: DNA of cultures treated with 0.75 mg/ml of *Euchema denticulatum* (τ-family). B7 to B12: DNA of cultures treated with 0.75 mg/ml of *Gigartina acicularis* (λ- and τ–family).

and *Gigartina acicularis,* DNA synthesis was reduced threefold and twofold, respectively, with 0.75 mg/ml (Fig. 1). These results indicate that carrageenans interfere with viral DNA synthesis. This test did not, however, allow us to conclude that replication was actually disrupted. Inhibition could result from another phenomenon occurring upstream, such as DNA polymerase interference.

These results are consistent with the yield reduction after a single cycle of replication, although the decrease of DNA synthesis alone could not explain the reduction of virus titre. Other events of the replication cycle, such as synthesis of late proteins or assembly step can be inhibited by these polysaccharides. These findings suggest that these sulfated polysaccharides inhibit a subsequent step in virus replication to viral internalization but prior to the onset of late viral protein synthesis as described by Gonzáles *et al.* [20] for other polysaccharides (Table 2).

If we consider the structure-activity relationships, the more actives carrageenans *(Gigartina acicularis* -λ- and ι –family and *Euchema denticulatum* -ι-family) are more sulfated than the less active ones (*Kappaphycus cottonii,* κ–family). These data were also observed by Gonzáles *et al.* [20]. Regarding the spectrum the of action of the carrageenans tested, they were more active against HHV-1 (enveloped virus) and less active against poliovirus (a naked virus). These results are also in agreement with the results obtained by Gonzáles *et al.* [20].

We conclude that these extracts have an important antiviral activity and are potential candidates for further studies of mechanism of action.

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Carrageenan (type)	Yield reduction log_{10} MOI = 1			
	Culture pretreatment		After a single cycle of replication	
	HHV-1	Poliovirus	HHV-1	Poliovirus
Gigartina acicularis (λ and τ)	0.9 ± 0.25	0.3 ± 0.10	1.9 ± 0.25	0.5± 0.25
Euchema denticulatum (τ)	0.9 ± 0.25	0.5 ± 0.20	2.0 ± 0.50	0.5 ± 0.10
Kappaphycus cottonii (κ)	0	0.2 ± 0.25	1.1 ± 0.50	0.3 ± 0.10

Table 2. Mechanism of action of the carrageenans. Results are presented as the mean ± SD from three independent tests.

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