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Interference in dengue virus adsorption and uncoating by carrageenans

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Abstract

This study demonstrated that the λ- and ι-carrageenans, sulfated polysaccharides containing linear chains of galactopyranosyl residues, are potent inhibitors of dengue virus type 2 (DENV-2) and 3 (DENV-3) multiplication in Vero and HepG2 cells, with values of effective concentration 50% from 0.14 to 4.1 µg/ml. This activity was assayed by plaque reduction, virus yield inhibition and antigen expression tests, and was independent of the input multiplicity of infection in the range 0.001–1. The inhibitory action of the λ-carrageenan, an heparan sulfate (HS)-imitative compound, was exerted by a dual interference with virus adsorption and internalization of nucleocapsid into the cytoplasm. Although virus particles may enter the cell when compound was added after DENV-2 adsorption, as shown by intracellular uptake of radiolabeled DENV-2 particles and quantitative RT-PCR, infectious center and virion uncoating assays have shown that carrageenan-treated virions cannot be released from the endosomes. Viral protein synthesis, the first step of macromolecular synthesis after DENV entry to the host cell, was not affected by the carrageenan. Furthermore, no inhibition of virus multiplication was detected when the entry process was bypassed through DENV-2 RNA transfection into the cell. The dual sites of action of an HS-like molecule suggest that, at least in monkey kidney and human hepatic cells, the HS residues in the cell membrane appear to act as mediators for DENV-2 entry, an interesting alternative target for flavivirus therapy.
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Introduction

Dengue virus (DENV), a member of family *Flaviviridae* genus *Flavivirus*, is the agent of the most prevalent arthropod-borne viral disease in humans. Currently, it is estimated that the virus is endemic in more than 100 countries, producing about 50 million infections each year (Gubler, 2002). DENV exists as four antigenically distinct serotypes, DENV type 1 (DENV-1) to DENV-4, which cocirculate in tropical and subtropical regions transmitted to human by two species of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Although most primary infections are asymptomatic, the four serotypes can cause dengue fever (DF), a mild febrile illness that in a minority of cases progresses to the severe and life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Primary infection provides immunity against the infecting serotype, but the secondary infection with another serotype appears to enhance the risk of developing DHF/DSS through an immunopathological process named antibody-dependent enhancement (ADE) (Halstead, 1979; Kliks et al., 1989). Host genetic and immunological factors as well as genetic variation in virulence among DENV strains also appear to contribute to DHF and DSS pathogenesis (Holmes and Burch, 2000; Leitmeyer et al., 1999; Rico-Hesse et al., 1997; Rothman and Ennis, 1999). Despite the increasing incidence and emergence of dengue infections around the world, there are no antiviral agents or vaccines available against DENV, and little is known about the entire replication cycle of DENV in mosquito or vertebrate cells.

The virion contains a single-stranded, positive-sense RNA molecule of approximately 11 kb in length, inserted in an icosahedral nucleocapsid and surrounded by a lipid envelope covered with peplomers. The genome is translated into three structural proteins (the capsid protein C, a small nonglycosylated membrane protein M and the envelope glycoprotein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

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Previous studies in our laboratory were directed to evaluate the antiviral efficacy of diverse natural and synthetic products against DENV, including sulfated polysaccharides obtained from South American seaweeds (de S.F-Tischer et al., 2006; Pujol et al., 2002; Rodríguez et al., 2005; Talarico et al., 2005), essential oils extracted from aromatic plants (Duschatzky et al., 2005; García et al., 2003) and carbonucleoside analogues (Martins Alho et al., 2005). Among this variety of tested compounds, sulfated polysaccharides were as far the more successful inhibitors of DENV in vitro infection, apparently based on the structural similarities with the heparan sulfate (HS) residues of cell membrane proteoglycans. HS is constituted by polyanionic carbohydrate chains and is present abundantly on the surface of most animal cells serving as a receptor for various ligands (Bernfield et al., 1999; Rostand and Esko, 1997; Spillmann, 2001). An involvement of HS during initial stages of DENV replicative cycle has been suggested by several reports, but the specific functional role of HS during infection is still unresolved. Controversial results have suggested that HS could act directly as a receptor for attachment and entry (Chen et al., 1997; Germi et al., 2002; Hilgard and Stockert, 2000; Hung et al., 1999), HS may be required only to recruit virions on the cell surface to facilitate the interaction with a high-affinity co-receptor of protein nature (Martínez-Barragán and del Angel, 2001; Thepparit and Smith, 2004), or even HS may not be necessary for DENV infection (Bielefeldt-Ohmann et al., 2001). Given the wide host range of DENV, these discrepancies may be ascribed to differences in the interactions leading to virus entry,

as receptor usage and internalization, depending on DENV serotype, passage history of the virus and host cell type.
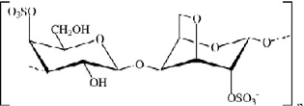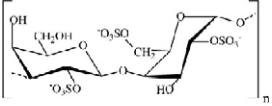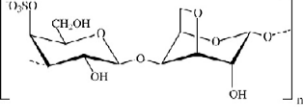
Here, we report on the analysis of the effect of HS-imitative compounds on DENV infection of monkey kidney and human hepatic cells. Based on our previous studies with algal-derived polysaccharides against herpesvirus (Damonte et al., 2004a) and DENV infections, three chemical classes of carrageenans were chosen as potential inhibitors. Carrageenans are sulfated galactans consisting of linear chains of alternating 1–3 β-D-galactopyranosyl residues (A units) and 1–4 α-D-galactopyranosyl or D-3,6-anhydrogalactosyl residues (B units), which comprise a broad range of structural classes (Stortz and Cerezo, 2000). The steps in the DENV-2 life cycle inhibited by carrageenans were localized providing evidence that HS functions for virus attachment and entry in both types of vertebrate cells and showing an effective alternative for antiviral strategies.

Results

Inhibition of DENV infection by carrageenans

To evaluate the antiviral activity of carrageenans on DENV infection, ι-, λ- and κ-carrageenans were assayed for their ability to inhibit DENV plaque formation in Vero cells. As previous studies have reported differential antiviral susceptibility among DENV serotypes (Lin et al., 2002; Talarico et al., 2005), the four serotypes were initially evaluated in a plaque reduction assay. As can be seen in Table 1, the three types of

Table 1
Chemical structure of carrageenans and spectrum of antiviral activity against DENV serotypes

Compound	CC_{50} (μg/ml) [a]	EC_{50} (μg/ml) [b]			
		DENV-1	DENV-2	DENV-3	DENV-4
ι-carrageenan	>1000	40.7±4.9	0.4±0.1	4.1±0.1	8.2±0.1
λ-carrageenan	>1000	>50	0.15±0.04	2.0±0.3	4.2±1.1
κ-carrageenan	>1000	>50	1.8±0.3	6.3±0.1	>50

[a] Cytotoxic concentration 50%, concentration required to reduce Vero cell viability by 50%.
[b] Effective concentration 50%, concentration required to reduce DENV plaque number in Vero cells by 50%. Each value is the mean of duplicate assays±standard deviation.

carrageenans exerted inhibitory effect against DENV infection but with a variable level of effectiveness according to the compound and the virus serotype. DENV-2 and DENV-3 were the most susceptible viruses to these polysaccharides with values of effective concentration 50% (EC_{50}) lower than 10 μg/ml for the three compounds. The λ- and ι-carrageenan were, in that order, the most active agents, and particularly DENV-2 exhibited the highest inhibition with EC_{50} values lower than 1 μg/ml for these two types of carrageenans. By contrast, the susceptibility of the other two serotypes, DENV-4 and DENV-1, was very irregular. Again, the λ- and ι-carrageenan exhibited a higher degree of inhibitory action against DENV-4 in comparison to the κ-carrageenan, whereas DENV-1 was as far the less sensitive serotype. Vero cell viability was not affected by the three compounds as determined by the MTT assay at concentrations up to 1000 μg/ml (Table 1). Thus, the inhibition of ι- and λ-carrageenan against DENV-2 and DENV-3 can be considered highly specific since the selectivity indices, i.e. the relationship between cytotoxic concentration 50% (CC_{50}) and EC_{50}, are in the range 2500–6666, for DENV-2, and 244–500, for DENV-3, respectively.

To confirm the potent antiviral action of the most effective compounds, λ- and ι-carrageenans, against DENV-2 and DENV-3, the inhibitory activity was assessed in an assay performed at a multiplicity of infection higher than that allowable in a plaque reduction test. To this end, a virus yield inhibition assay was performed by inoculation of cells at an m.o.i. of 0.1 and titration of virus production after 48 h of infection in the presence of serial two-fold drug concentrations. Clinical studies have shown the presence of DENV antigen and nucleic acid in the liver of DF patients (Couvelard et al., 1999; Rosen et al., 1999), indicating that hepatocytes may be a target capable of supporting in vivo DENV replication. Consequently, we decided to perform in parallel a virus yield reduction assay in the human hepatoma-derived cell line HepG2, representative of the host tissue in natural infection. Results are shown in Table 2. For Vero cells, the values of EC_{50} against DENV-2 and DENV-3 are of similar order as those obtained for the plaque reduction assay (Table 1). With respect to the human HepG2 cells, the reduction in DENV-2 and DENV-3 production in the presence of the carrageenans was also dose-dependent as in Vero cells and with high effectiveness since the EC_{50} values were consistently lower than 1 μg/ml. As reported for Vero cells, there was no difference in cell viability between untreated HepG2 cells and cells treated with as much as 1000 μg/ml of carrageenans (data not shown).

Results presented in Tables 1 and 2 suggest that the antiviral activity of carrageenans against DENV-2 and DENV-3 is independent of the method employed for evaluation (plaque or yield reduction) and, even more important, of the input multiplicity of infection (0.001 in plaque assay vs. 0.1 in yield reduction test). To assess the independence of carrageenan effectiveness with virus inoculum, dose response curves for DENV-2 and DENV-3 with both carrageenans were performed by virus yield inhibition assay at a range of m.o.i. from 0.01 to 1. As seen in Fig. 1, the inhibitory effects of λ- and ι-carrageenan were not dependent of the inoculating dose of DENV-2 and DENV-3, with only slight variations in the percentage of inhibition at very low compound concentrations. In fact, the values of EC_{50} for λ-carrageenan calculated from data in Figs. 1A and C were in the range 0.15–0.39 μg/ml against DENV-2 and 1.40–2.28 μg/ml against DENV-3, respectively. Thus, with a 100-fold higher virus inoculum, the EC_{50} value was about or less than 2-fold higher than the value obtained when evaluated at the lower inoculum. Similar variation in EC_{50} values was observed for ι-carrageenan.

The effects of carrageenans on DENV infection were also monitored using an indirect immunofluorescence assay to determine viral antigen expression. Vero and HepG2 cells were exposed to carrageenans simultaneously with virus infection, and at 48 h p.i. viral antigen expression was detected by immunostaining with anti-DENV antibodies. The results of a representative experiment are shown in Fig. 2. In DENV-2 infected Vero and HepG2 cells, a regular dotted distribution of viral proteins in cell cytoplasm was revealed (Figs. 2A and E). The number of DENV-2 antigen-expressing cells was drastically reduced by λ-carrageenan, with the detection of very few sporadic positive cells (Figs. 2B and F). In fact, the percentage of infected cells per microscope field showing DENV-2 immunofluorescence in the presence of λ-carrageenan with respect to infected untreated controls was lower than 1%. A similar pattern of reduction in DENV-2 antigen expression was observed after treatment with ι-carrageenan (Figs. 2C and G).

Influence of time of treatment with carrageenans on DENV infectivity

The DENV-2 serotype and the λ-carrageenan were chosen as model system to further study the mode of action of carrageenans against DENV infection. Mechanistic studies were performed at a carrageenan concentration of 20 μg/ml in order to assess a high inhibitory effect in all the assays designed to elucidate the mode of action of the compounds. As an initial approach, a time course study was done to analyze the influence of the time of addition of the compound during the virus multiplication cycle. The λ-carrageenan was added to Vero and HepG2 cells simultaneously with virus or at hourly intervals after adsorption of virus to the cells. A high inhibitory effect was observed when the compound was added to cells together with the virus (time 0) or immediately

Table 2
DENV-2 and DENV-3 yield inhibition in Vero and HepG2 cells

Compound	EC_{50} (μg/ml) [a]	
	Vero	HepG2
DENV-2		
ι-carrageenan	0.4±0.1	0.14±0.01
λ-carrageenan	0.22±0.02	0.17±0.01
DENV-3		
ι-carrageenan	1.1±0.1	0.63±0.01
λ-carrageenan	0.6±0.1	0.63±0.01

[a] Effective concentration 50%: concentration required to inhibit virus yield at 48 h post-infection by 50%. Each value represents the mean of duplicate assays±standard deviation.
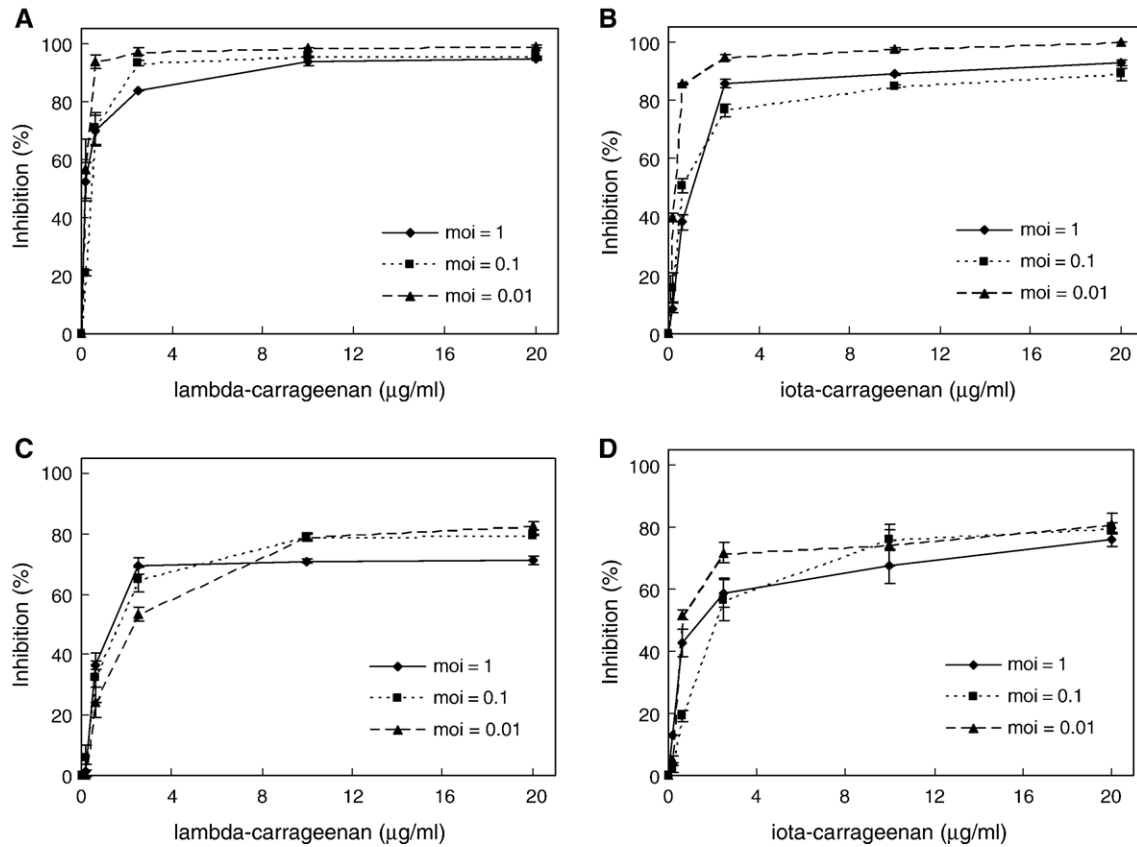
Fig. 1. Inhibition of DENV-2 and DENV-3 production by carrageenans at different multiplicities of infection. Vero cells were infected at a range of m.o.i. from 0.01 to 1 with DENV-2 (A, B) or DENV-3 (C, D) in the absence or presence of increasing concentrations of λ-carrageenan (A, C) or ι-carrageenan (B, D). Extracellular virus yields were determined at 48 h p.i. by a plaque assay in Vero cells. Results are expressed as percent of inhibition in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays±standard deviation.

after adsorption at 4 °C, at 1 h post-infection. Under these two experimental conditions, virus yields were reduced more than 95% in both Vero and HepG2 cells (Fig. 3A). By contrast, no significant reduction in virus multiplication was observed when the carrageenan was incorporated after 2 h of infection. These

results locate the antiviral target of these polysaccharides in a very early stage of the virus cycle, during the first hour of infection and previous to the second hour of infection.

Based on the results obtained, we next investigated the stage in the viral life cycle at which the carrageenan blocked infection
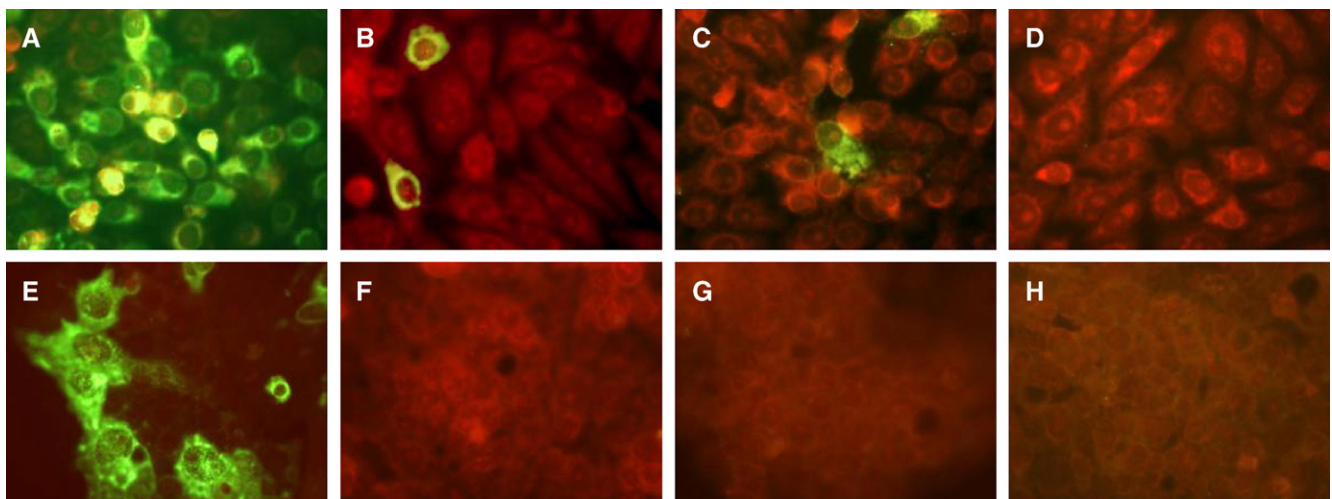

Fig. 2. Effect of carrageenans on DENV-2 antigen expression. Vero (A–D) and HepG2 (E–H) cells were mock infected (D and H) or infected with DENV-2 in the absence (A and E) or presence of 20 μg/ml of λ-carrageenan (B and F) or ι-carrageenan (C and G). At 48 h p.i., immunofluorescence staining was carried out using anti-DENV mouse antibodies. Magnification: 400×.
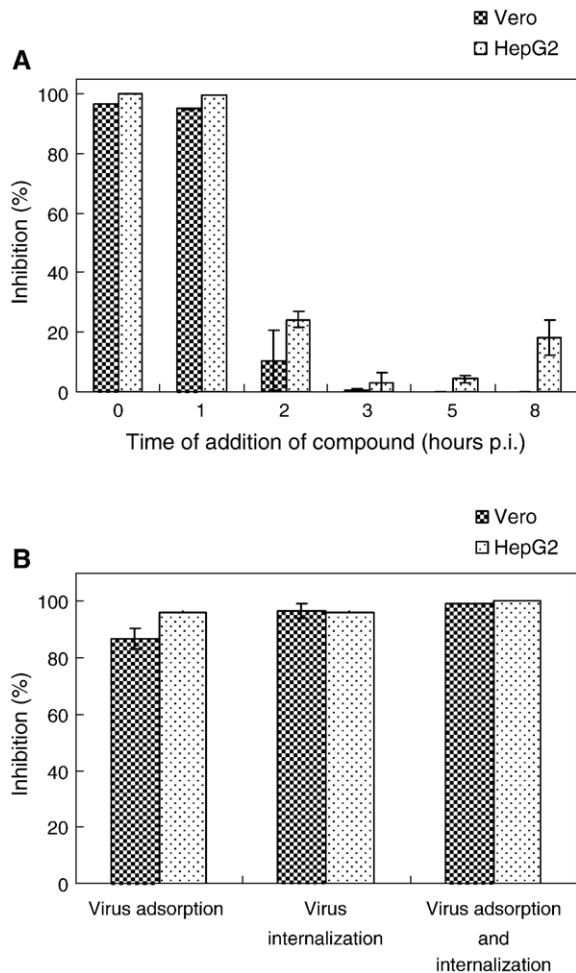
Fig. 3. Influence of time of treatment with λ-carrageenan on anti-DENV-2 activity. (A) Vero and HepG2 cells were infected with DENV-2 and compound (20 μg/ml) was added simultaneously with virus (time 0) or at the indicated times after infection. At 10 h p.i. medium was discarded and an infectious center (IC) assay was performed in Vero cells. (B) Vero and HepG2 cells were infected with DENV-2 under three different treatment conditions. Virus adsorption: cells were infected in MM containing 20 μg/ml of compound and, after 1 h adsorption at 4 °C, were overlaid with compound-free MM. Virus internalization: DENV-2 was adsorbed to Vero cells at 4 °C, then MM containing 20 μg/ml of compound was added and incubation continued at 37 °C for 2 h. Then, cells were treated with citrate buffer and covered with MM. Virus adsorption and internalization: cells were infected and maintained in the presence of 20 μg/ml of compound during 1 h adsorption at 4 °C and 2 h internalization at 37 °C and then processed as above. For the three treatments, virus yields were determined at 48 h p.i. by a plaque assay in Vero cells. Results are expressed as percent of inhibition in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays±standard deviation.

focusing the efforts on the early stages of multiplication occurring during the first hour of infection. First, the effect of carrageenan on the events leading to viral entry in the host cell was studied by evaluation of the inhibitory action of the compound on virus yield after 48 h of infection when present exclusively during virus adsorption or/and virus internalization. As shown in Fig. 3B, a significant antiviral effect (inhibition in the range 86.6–96.2%) occurred when the carrageenan was present only during virus adsorption at 4 °C. If adsorption took place in a drug-free

medium, and the compound was added at culture supernatants when the temperature was raised at 37 °C and maintained only during 2 h of incubation, the virus yield determined at 48 h p.i. was also significantly affected in comparison with untreated cultures (96.1–96.6% inhibition). As expected, the presence of the carrageenan during both adsorption at 4 °C and internalization at 37 °C exerted the maximum level of reduction in virus yield with 99.1–99.9% inhibition.

Inhibition of DENV-2 adsorption and internalization

To confirm the effect of λ-carrageenan on DENV-2 adsorption and penetration, different experimental approaches were intended. For both stages of the multiplication cycle, the amount of bound or internalized virus in the presence of compound was measured by infectivity assays, quantification of cell-associated viral RNA molecules and radiolabeled virus particles.

The inhibition of virus adsorption by the carrageenan was demonstrated by the three assays abovementioned. After DENV-2 adsorption at 4 °C in the presence of 20 μg/ml of compound, the amount of Vero cell-bound infectivity was inhibited about 90% (Fig. 4A). When the amount of cell-bound viral RNA was quantified by real-time RT-PCR in other set of cultures under the same treatment conditions, a similar reduction in the number of RNA molecules attached to the cells was observed (Fig. 4B). Finally, the carrageenan also inhibited the binding of radiolabeled DENV-2 particles to Vero cells (Fig. 4C).

Next, the effect of carrageenan on DENV-2 penetration was analyzed. Vero cells were allowed to adsorb virus in the absence of compound, and then infected cultures were incubated at 37 °C for 1 h in the presence of carrageenan. Thereafter, in a set of cultures the amount of internalized virus was quantified by an infectious center assay, whereas another set of cultures was processed to determine the amount of viral RNA inside the cells. As seen in Fig. 4D the number of infectious centers was reduced about 2 log (99% inhibition) in cells incubated with the carrageenan during 1 h after DENV-2 adsorption. By contrast, under the same treatment conditions, the number of internalized DENV-2 RNA molecules per culture was similar in compound-treated and untreated cultures (Fig. 4E), suggesting that when the carrageenan was added immediately after adsorption virus particles entered the cell but they could not complete the infectious process. To assess this possibility, a suspension of purified radiolabeled DENV-2 particles was adsorbed to Vero cells at 4 °C, then cultures were shifted to 37 °C in the presence of carrageenan, and after 1 h of incubation, the amount of radioactivity inside the cells was determined. The amount of radiolabeled internalized virus was not affected by the carrageenan (Fig. 4F).

Although not clearly elucidated for all the family members, flaviviruses are believed to enter cells by receptor-mediated endocytosis (Lindenbach and Rice, 2001). Thus, the acid environment of the endosome should induce conformational changes in the E protein to allow the transport of nucleocapsid to the cytoplasm. The controversial results shown in Figs. 4D–F could be explained if carrageenan-treated DENV virions can enter the cells but are not able to be uncoated and released from
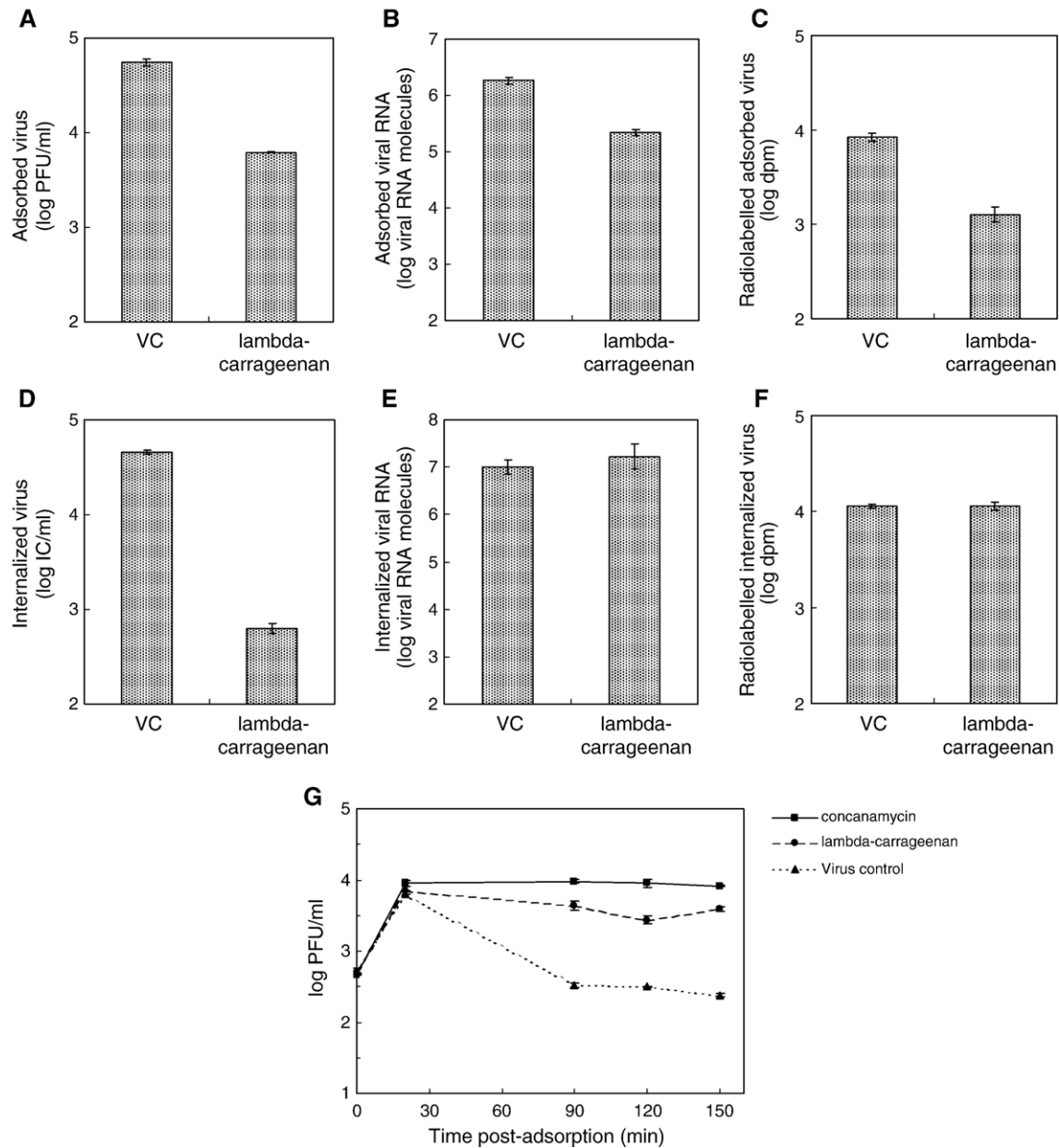
Fig. 4. Effect of λ-carrageenan on virus adsorption and internalization. (A) DENV-2 was adsorbed to Vero cells at 4 °C for 1 h in MM containing or not 20 μg/ml of λ-carrageenan. Then, cell-bound infectious virus was determined by plaque assay. (B) Vero cells were infected and treated as in A, then total RNA was extracted and the amount of adsorbed DENV-2 RNA molecules was determined by quantitative real-time RT-PCR. (C) Cells were incubated with ^{35}S-labeled DENV-2 at 4 °C for 1 h in the absence or presence of 20 μg/ml of λ-carrageenan, and then cell-bound radioactivity was determined. (D) DENV-2 was adsorbed to Vero cells at 4 °C for 1 h. After incubation at 37 °C for 1 h in MM containing 20 μg/ml of λ-carrageenan, cell monolayers were treated with proteinase K and the cell pellet was plated onto Vero cells to determine internalized virus by an infectious center (IC) assay. (E) Vero cells were infected and processed as in D, total RNA was extracted and real-time RT-PCR was performed to determine the amount of internalized viral RNA molecules. (F) Cells were incubated with ^{35}S-labeled DENV-2 at 4 °C for 1 h, then at 37 °C for 1 h in MM with carrageenan, processed as in D, and cell-internalized radioactivity was then measured. (G) DENV-2 was adsorbed to Vero cells at 4 °C for 1 h, and then cells were incubated at 37 °C in MM containing or not carrageenan or concanamycin. At different times post-adsorption, non-internalized virus was removed, cells were disrupted by freeze–thawing and intracellular infectivity was determined. VC: virus control, compound-untreated infected cells; λ-carrageenan: compound-treated infected cells. Each value represents the mean of duplicate assays±standard deviation.

the endosomes. To test this possibility, DENV-2 was adsorbed to Vero cells at 4 °C, and then infected cultures were further incubated at 37 °C in the presence of carrageenan. At different times post-adsorption, non-internalized virus was removed with proteinase K and cell-associated infectivity was determined. Virus uncoating and the consequent nucleocapsid delivery to the cytoplasm seem to be the post-adsorption step affected by

carrageenan since no significant decrease in cell-associated DENV infectivity was detected in treated cells whereas a consistent reduction occurred in untreated infected cells (Fig. 4G). The accumulation of intracellular infectivity in carrageenan-treated cells resembles that observed in concanamycin-treated cultures (Fig. 4G). This compound was simultaneously assayed as a positive control because it has been reported for other

enveloped viruses that concanamycin allows virus endocytosis but prevents membrane fusion between virus envelope and the endosomal membrane leading to nucleocapsid release into the cytoplasm (Guinea and Carrasco, 1994; Irurzun et al., 1997).

Lack of inhibitory effect of carrageenan after DENV entry

After viral entry to the host cell, the first step of macromolecular biosynthesis in DENV replication cycle is the synthesis of viral proteins from the RNA positive strand genome. To investigate whether carrageenan affects viral RNA translation, Vero cells were pulsed with [^{35}S]-cysteine-methionine in the presence of the compound, during an early period in the infection process after internalization is completed (12–

16 h) but compatible with an adequate detection of the main viral polypeptides after immunoprecipitation. As seen in Fig. 5A, under these conditions, viral bands corresponding to the E and prM polypeptides were clearly revealed in control untreated infected cells (lane 2) as well as in carrageenan-treated cells (lane 3). Altogether, these results indicate that the inhibitory action of the carrageenan located in the first hour after infection is specifically targeted to the stages of adsorption and nucleocapsid internalization, previous to the macromolecular biosynthetic events.

To assess that the carrageenan target is exclusively at the DENV entry to the host cell and there is no effect in any further stage, the inhibitory action of λ-carrageenan in cells transfected with viral RNA was also evaluated. To this end, DENV-2 RNA
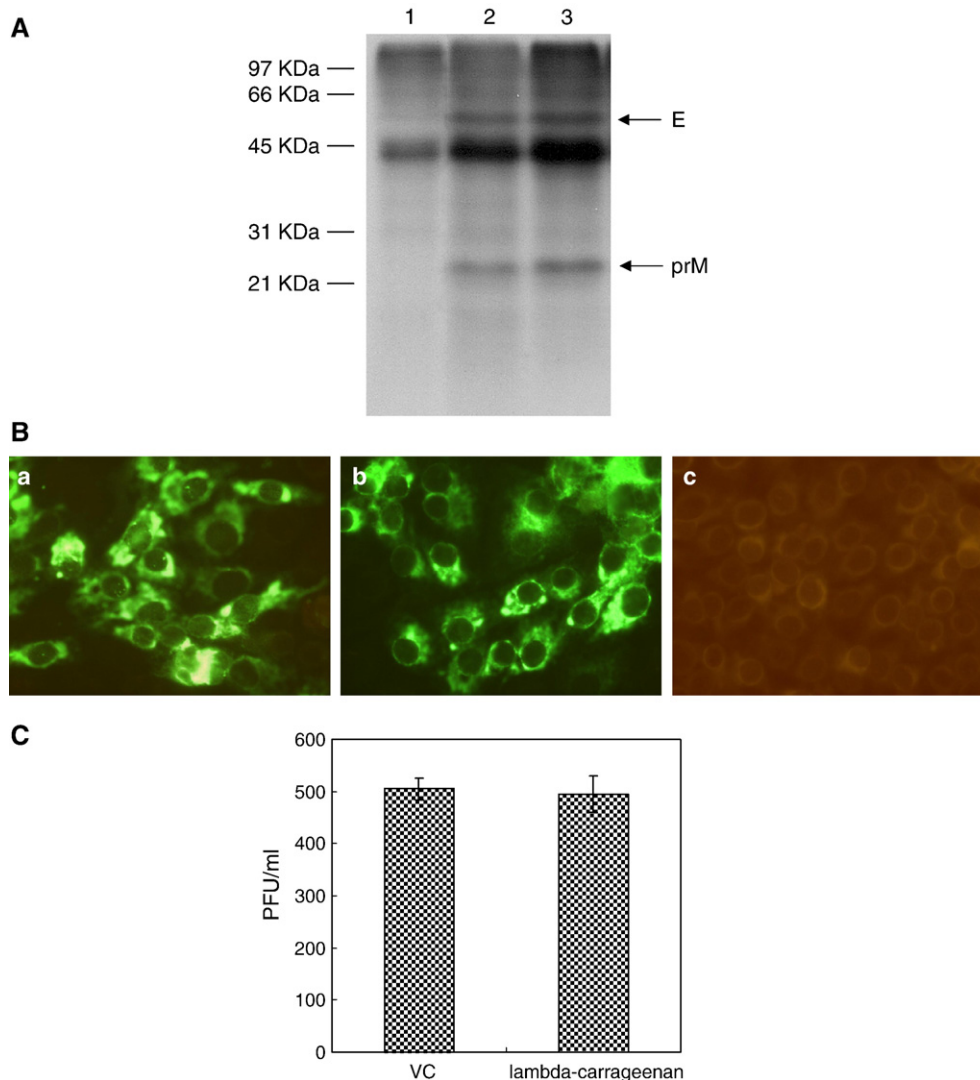

Fig. 5. Lack of inhibitory effect of carrageenan after DENV entry. (A) Synthesis of viral proteins. Vero cells were infected with DENV-2, and after 1 h at 37 °C, cells were incubated with MM in the presence or absence of 20 µg/ml of λ-carrageenan. At 12 h p.i., cells were labeled with 100 µCi/ml of EXPRE^{35}S-^{35}S for 4 h and then viral polypeptides were immunoprecipitated from cell lysates with rabbit anti-DENV-2 serum and analyzed by electrophoresis. Lanes: 1, mock infected cells; 2, untreated infected cells; 3, λ-carrageenan-treated infected cells. Molecular mass markers are indicated on the left; arrows on the right indicate the positions of the main viral polypeptides. (B and C) Transfection of Vero cells with DENV-2 RNA. Vero cells grown in coverslips were transfected with DENV-2 RNA, and 2 h later monolayers were incubated in MM containing or not 20 µg/ml of λ-carrageenan during 18 h. Thereafter, cells were washed and further incubated in MM without compound. At 96 h post-transfection, cells were stained with anti-DENV-2 mouse antibody plus FITC-goat anti-mouse IgG (B) and supernatants were harvested for PFU titration (C). a: untreated transfected cells; b: λ-carrageenan-treated transfected cells; c: mock transfected cells. VC: virus control, compound-untreated transfected cells.

was obtained from purified virions and transfected into Vero cells grown in coverslips with Lipofectamine as vehicle. After 96 h of transfection, the effect of λ-carrageenan on viral protein expression and infectious particle production was determined. The results show no inhibition of DENV multiplication in RNA-transfected cells. A similar level of antigen-positive cells, visualized as single cells or focuses containing 2–20 cells, was detected by indirect immunofluorescence in λ-carrageenan-treated or untreated cells. Fig. 5B shows large focuses representative of those observed in microscope fields of untreated and treated transfected cultures. Concomitantly, no reduction in the amount of infectious particles released to cell supernatants was detected by quantitative titration of PFU in λ-carrageenan-treated cultures (Fig. 5C), indicating that when DENV-2 RNA entered Vero cells through transfection bypassing the entry process of virus particles the compound was not able to block the formation of infectious virions.

Comparison of virucidal and antiviral activities

Finally, the possibility of an inactivating effect of carrageenans against cell-free DENV virions was also studied. In particular, λ-carrageenans of natural origin were reported as inactivating agents against herpes simplex virus (HSV) whereas other structural types of carrageenans and sulfated polysaccharides in general usually lack these inactivating properties and only act blocking the binding of virions to the cell membrane (Carlucci et al., 1999; Damonte et al., 2004a; McClure et al., 1991). Thus, we decided to evaluate the virucidal activity of λ-carrageenan by incubation of a DENV-2 suspension with the compound at 37 °C during 45 min. Then, remaining infectivity in the mixture was determined by plaque formation in Vero cells and previous dilution of the sample to assure that, when the compound virus suspensions are incubated on the cell monolayers, the compound concentration is below the antiviral EC_{50} value. For comparative purposes, the antiviral plaque reduction assay was simultaneously performed with the same compound concentrations. As seen in Fig. 6, the λ-carrageenan was able to
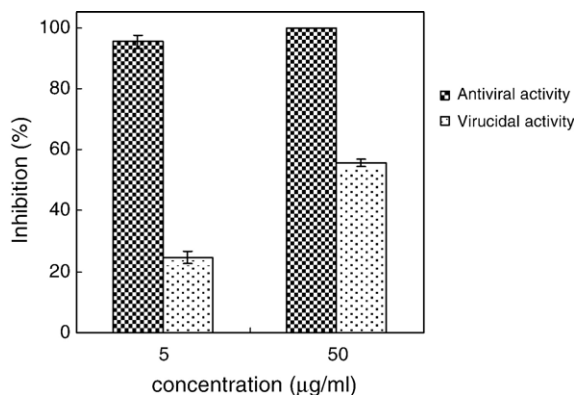

Fig. 6. Comparison of virucidal and antiviral activities. Virucidal: DENV-2 suspensions were incubated with 5 or 50 μg/ml of λ-carrageenan at 37 °C during 45 min and then the remaining infectivity was determined. Antiviral: plaque reduction assay in Vero cells. Results are expressed as percent of inhibition in carrageenan-treated samples compared to untreated ones. Each value is the mean of duplicate assays ± standard deviation.

produce a very weak virucidal effect at very high concentrations in comparison with its antiviral effectiveness. Only at a concentration of 50 μg/ml a significant but low level of virus inactivation, with 55.7% inhibition in remaining infectivity, was detected. Thus, the ratio between VC_{50} (virucidal concentration 50%, concentration required to inactivate 50% of virions) and antiviral EC_{50} was 211, indicating that the blockade observed in the multiplication of DENV-2 in Vero and HepG2 cells can be totally ascribed to an interference with the viral entry process.

Discussion

Studies reported here have proved that λ- and ι-carrageenans, sulfated polysaccharides containing linear chains of galactopyranosyl residues, are potent inhibitors of DENV-2 and DENV-3 multiplication in cells of monkey and human origin, with values of selectivity indices in the range >244–>7143. This activity was initially tested by plaque reduction assays, necessarily performed at a low m.o.i., but then confirmed by inhibition of virus yield and antigen expression assays, accomplished at high m.o.i., indicating that the ability of carrageenans to interfere with DENV multiplication was independent of the testing method and the input virus dose. In addition, when viral antigen expression was measured by immunofluorescence staining, the spreading ability of DENV-2 was also shown to be greatly suppressed in the presence of the carrageenans, with a drastic reduction in the number of infected cells as well as an inhibition in the formation of giant fluorescent focus.

The lack of dependence of the antiviral potency of carrageenans with the infecting virus inoculum was even more evident when virus yield reduction assays were performed simultaneously at a wide range of multiplicities (Fig. 1). This important property is not unusual for antiviral agents, including other types of DENV inhibitors (Whitby et al., 2005), but represents a clear advantage for those compounds able to block infection even in the presence of high initial virus doses. These results provide support for the potential therapeutic use of these carrageenans, suggesting that they might suppress the multiplication of sufficiently high concentrations of DENV-2 and DENV-3 and, thus, prevent de novo infection of cells and virus spreading.

Carrageenans of natural and commercial origin have been evaluated as inhibitors of other enveloped viruses, including HSV, cytomegalovirus and human immunodeficiency virus (Baba et al., 1988; Bourne et al., 1999; Cáceres et al., 2000; Carlucci et al., 1999; González et al., 1987; Hamasuna et al., 1993; Talarico et al., 2004; Yamada et al., 2000). It was reported that carrageenans act against these viruses by prevention of the binding of virus to the HS residues in cellular proteoglycans and consequently are responsible for blocking virus adsorption. In the present study, different experimental approaches have shown that the target for carrageenan in DENV-2 seems to be extended not only to adsorption, as mentioned for other viruses, but also to a post-adsorption event blocking the viral nucleocapsid internalization into the cytoplasm. The first inhibitory action in the prevention of virus adsorption when DENV was adsorbed to the host cell in the presence of

carrageenan was proved by determination of infectious adsorbed virus, quantification of attached viral RNA molecules and measure of radiolabeled virus particles (90% inhibition). When the compound was added to infected cells after virus adsorption, more than 95% inhibition of virus yield was observed at 48 h p.i. Since no inhibition of virus multiplication by carrageenan was detected when DENV-2 RNA was introduced into the cell by transfection, the process affected by carrageenan when added after adsorption must be an event related to virus entry such as virus penetration or uncoating.

At present, the mechanism of DENV entry to the cell is a controversial subject. Electron microscopic studies have shown that DENV-2 penetrated directly into the cytoplasm of C6/36 and BHK cells by fusion of the virion envelope with the plasma membrane at physiologic pH (Hase et al., 1989; Lim and Ng, 1999), but it is now generally accepted that for productive flavivirus infection viral uptake occurs through receptor-mediated endocytosis (Lindenbach and Rice, 2001). After binding to the cell surface, the virion is supposed to be directed to the endocytic pathway, and as response to the acidic pH of the endosomes a conformational rearrangement occurs in E protein, inducing fusion of the viral and cell membranes to allow viral genome to enter the cytoplasm (Modis et al., 2003, 2004). The inhibition here described for carrageenan when added after adsorption of DENV-2 to the host cell suggests that the interaction of the polysaccharide with the virion bound to the cell surface will avoid the normal transit of the viral nucleocapsid into the cytoplasm. The uptake of virion particles by the cell occurs in the presence of carrageenan, as shown by the presence of radiolabeled DENV virions and RNA molecules, after compound treatment during the 1 h internalization period at 37 °C (Figs. 4E–F). But, under the same experimental conditions, the amount of internalized virus able to complete a productive cycle in the original infected cell was reduced 2 log, as determined by infectious center formation (Fig. 4D). In addition, in the presence of the carrageenan the proteinase K-resistant virus infectivity accumulated and remained inside the cell until 150 min after adsorption similarly as occurred with concanamycin treatment (Fig. 4G), indicating the intracellular permanence of enveloped virions. Altogether, these results suggest that virions enter the cell but the fusion event leading to uncoating of the nucleocapsid and escape from the endosome is blocked, probably due to the association of the carrageenan with the E virion glycoprotein.

The dual sites of action of an HS-like molecule such as the λ-carrageenan shown here on virus adsorption and uncoating suggest that, at least in vertebrate cells, the HS residues present in the cell membrane appear to act as attachment receptors for DENV-2 and also, through its binding to the E protein, may serve as mediators for virus uncoating into the host cell. At present, many questions concerning attachment and entry of flaviviruses are still unresolved, and particularly, the involvement of HS in these processes is controversial. Several previous studies have demonstrated attachment of DENV to HS, and, accordingly, positively charged patches of basic residues proposed as putative glycosaminoglycan-binding motifs were identified within domains I, II and III of the E glycoprotein

(Chen et al., 1997; Hung et al., 2004; Modis et al., 2005; Lee et al., 2006). However, only a few studies have also provided evidence for the requirement of HS for viral entry to mammalian cells (Chen et al., 1997; Germi et al., 2002; Hilgard and Stockert, 2000; Hung et al., 1999). In this study we were able to demonstrate that an HS-imitative compound had the ability to interfere with DENV-2 replication when added after virus adsorption and, even, under these conditions the antiviral potential of λ-carrageenan was higher than its ability to affect virus adsorption (Figs. 3B, 4A and D).

Furthermore, the mode of action of carrageenans on DENV multiplication allows to explain the differential susceptibility to this type of compound shown by the four virus serotypes. DENV-2 and DENV-3 were the more susceptible serotypes whereas DENV-4 and DENV-1 exhibited a very weak inhibition (Table 1). These results are in agreement with previous studies about the antiviral activity of heparin and natural galactans against DENV serotypes, reporting the high susceptibility of DENV-2 and the lack of response by DENV-1 (Hung et al., 1999; Talarico et al., 2005). This behavior is not surprising given the discrepant data reported in several publications about the cell receptors and co-receptors for DENV according to the virus serotype and/or the host cell.

In addition to the information provided about the putative dual function of HS for DENV-2 entry into Vero and HepG2 cells, our studies are indicative of the promising perspectives of HS-imitative compounds such as the carrageenans as potential antivirals to prevent virus multiplication. Given the worldwide importance of disease caused by DENV (as described in Introduction), several classes of inhibitors for this virus have been reported (reviewed in Damonte et al., 2004b; Leyssen et al., 2000). They include polyanionic substances affecting viral binding; inhibitors of intracellular virus multiplication, blocking nucleoside triphosphate synthesis, helicase and protease activities; maturation inhibitors able to interfere with the post-translational processing of E glycoprotein by cellular glucosidases; nucleic acid-based antisense viral therapy; other miscellaneous inhibitors with a presently unknown target. In line with these objectives, the carrageenan studied here was very effective in a treatment simultaneous with infection and also showed strong post-exposure activity on DENV-infected cells, proving the concept that virus entry is a valid and interesting alternative target for anti-flavivirus therapy.

Materials and methods

Compounds and antibodies

Carrageenans type ι, λ and κ were purchased from Sigma-Aldrich. Stock solutions of compounds were prepared in distilled water at 2 mg/ml.

Hyperimmune sera to DENV-2 strain NGC were prepared in rabbit by five intravenous injections at 10-day intervals of a concentrated virus suspension containing 10^6 PFU in 1 ml TES buffer (10 mM Tris–HCl pH 7.2, 100 mM NaCl, 1 mM EDTA) and UV-irradiated during 5 min. Serum was obtained from the central ear vein 1 week after the last injection of virus. Murine

hyperimmune ascitic fluid against DENV-2 for immunofluorescence assays was gently provided by Dr. Andrea Gamarnik (Fundación Instituto Leloir, Buenos Aires, Argentina).

Cell culture and virus infection

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO) supplemented with 5% fetal bovine serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 HT mosquito cell line from *A. albopictus*, adapted to grow at 33 °C, was cultured in L-15 Medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% fetal bovine serum. The human hepatoma cell line HepG2 was propagated in MEM containing 0.03% glutamine, 0.01% sodium pyruvate and 10% fetal bovine serum.

The stocks of DENV-1 strain Hawaii, DENV-2 strain NGC, DENV-3 strain H87 and DENV-4 strain 8124 were prepared in C6/36 HT cells and titrated by plaque formation in Vero cells.

To obtain a concentrated stock of DENV for immune serum preparation and RNA extraction, C6/36 HT cells were infected with DENV-2 and supernatants were harvested at 4 and 5 days after infection. After clarification by low-speed centrifugation, supernatants were concentrated by pelleting for 2 h at $100,000 \times g$. Then, the pellet was resuspended in TES buffer by brief sonication.

To obtain radiolabeled DENV-2, C6/36 HT cells were infected with DENV-2 and at 72 h p.i. 23 µCi/ml of EXPRE^{35}S-^{35}S (NEN Dupont) was added in methionine-cysteine-free MM. Cell supernatants were harvested at 96 h p.i., and radiolabeling was repeated between 96 and 120 h p.i. Supernatants were clarified and concentrated as described above, and the sonicated virus pellet was layered onto a 15–60% sucrose gradient. After centrifugation at $100,000 \times g$ for 2 h, the virus band was pelleted for 2 h at $100,000 \times g$ and resuspended by sonication.

Cytotoxicity and infectivity antiviral assays

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) method. Confluent cultures in 96-well plates were exposed to different concentrations of the carrageenans, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 µl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 µl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The CC_{50} was calculated as the compound concentration necessary to reduce cell viability by 50%.

Antiviral activity was evaluated by two methods: plaque reduction and virus yield inhibition assays. In the plaque reduction test, Vero cell monolayers grown in 24-well plates were infected with about 50 PFU/well of DENV serotypes in the absence or presence of serial two-fold concentrations of the compounds. After adsorption, residual inoculum was replaced by MM containing 1% methylcellulose and the corresponding dose of each compound. Plaques were counted after 6–12 days of incubation at 37 °C, according to virus serotype. The EC_{50} was calculated as the compound concentration able to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

In virus yield inhibition assays, Vero and HepG2 cells were infected with DENV-2 or DENV-3 at a multiplicity of infection (m.o.i.) of 0.01–1 in the presence of different concentrations of the compounds, 2 wells per concentration. After 48 h of incubation at 37 °C, cell supernatants were collected and the virus yields were determined by plaque formation in Vero cells. The EC_{50} values were calculated as above.

Indirect immunofluorescence assay

Vero and HepG2 cells grown in coverslips were infected with DENV-2 (m.o.i. 0.1) in the presence or absence of λ- or ι-carrageenan (20 µg/ml). At 48 h post-infection, cell monolayers were washed with cold PBS and fixed in methanol for 15 min at −20 °C for cytoplasmic immunofluorescence. Indirect staining was carried out by using anti-DENV mouse antibodies and fluorescein (FITC)-labeled goat anti-mouse IgG. After a final washing with PBS, cells were stained with Evans Blue and mounted in a glycerol solution containing 1,4-diazabicyclo[2, 2, 2]octane (DABCO).

Influence of time of treatment on antiviral activity

First, a time-of-addition experiment was done by infecting Vero and HepG2 cells with DENV-2 (m.o.i. 1) in either MM containing 20 µg/ml of λ-carrageenan (time 0) or MM without compound. After 1 h adsorption at 4 °C, medium containing unadsorbed virus was removed and cell cultures were washed with PBS. Then, MM with compound was added immediately (time 1 h p.i.) or at 2, 3, 5 and 8 h p.i. At 10 h after infection, cell supernatants were withdrawn, the monolayers were washed with PBS and trypsinized with 0.25% trypsin–EDTA to disperse cells. Protease treatment was stopped by adding 2 mM phenyl-methyl-sulphonyl-fluoride (PMSF) in PBS containing 3% bovine serum albumin (PBS-BSA), then cells were washed in PBS containing 0.2% BSA by low-speed centrifugation, resuspended in MM and the final cell suspensions were serially 10-fold diluted and plated onto confluent monolayers of Vero cells. After 1.5 h incubation at 37 °C, monolayers were overlaid with MM containing 1% methylcellulose. Infectious centers were counted after 6 days of incubation.

The influence of three different compound treatment conditions during adsorption and/or internalization was also determined in Vero and HepG2 cells infected with DENV-2 (m.o.i. 1). Virus adsorption: cells were exposed to DENV-2 in the presence of 20 µg/ml of compound, and after 1 h adsorption at 4 °C, both compound and unadsorbed virus were removed, the cells were washed with cold PBS and overlaid with MM. Virus internalization: cells were infected in compound-free MM

and after 1 h adsorption at 4 °C, unadsorbed virus was removed, the cells were washed and further incubated at 37 °C during 2 h in MM containing 20 μg/ml of compound. Thereafter, cells were washed with PBS and treated with citrate buffer (citric acid 20 mM, potassium chloride 10 mM, sodium chloride 135 mM, pH 3) for 1 min to inactivate adsorbed but not internalized virus. Then, cells were washed with PBS and covered with MM. Virus adsorption and internalization: cells were infected with DENV-2 and maintained in MM containing 20 μg/ml of compound during both the 1 h adsorption period at 4 °C and the 2 h internalization period at 37 °C. Then, cells were washed, processed with citrate buffer as above and refed with MM. For the three treatments, cell supernatants were harvested at 48 h after infection and virus yields were determined by a plaque assay in Vero cells.

Virus adsorption assays

The inhibitory effect of carrageenans on virus adsorption was measured by different experimental approaches.

(a) Infectivity assay of DENV-2 adsorption

Vero cells were infected with DENV-2 at an m.o.i. of 1 in the presence or absence of 20 μg/ml of λ-carrageenan. After 1 h adsorption at 4 °C, cells were washed with cold PBS to remove unadsorbed virus and disrupted by freezing and thawing. The amount of infectious bound virus was then measured by plaque formation.

(b) Adsorption of DENV-2 RNA by real-time RT-PCR

Cells were infected and processed as described in (a), except that after adsorption and extensive washing with cold PBS, total RNA was extracted from cells by using TRIzol (Invitrogen) according to the manufacturer's instructions. For quantification of the amount of cell-bound viral RNA, an iCycler IQ (Bio-Rad) employing TaqMan technology was used. The primers and probe targeted to amplify nucleotides 10,419 to 10,493 within the viral 3′UTR were previously described (Alvarez et al., 2005). Each 50 μl reaction mix contained 5 μl of RNA sample and final concentrations of 1× RT-PCR buffer (10 mM Tris–HCl pH 8.4, 50 mM KCl, 0.01% w/v gelatin and 10 mM DTT), 2.5 mM MgCl$_2$, 250 μM deoxynucleoside triphosphates, 100 nM of each primer and probe and 100 units M-MLV RT (Promega). Reverse transcription was allowed to proceed for 1 h at 37 °C, and then 2 units of Taq DNA polymerase (Invitrogen) were added to each reaction tube. PCR amplification and detection were performed using the following conditions: 95 °C for 3 min (1 cycle), and then 40 cycles of 95 °C for 15 s and 61 °C for 1 min. A standard curve was generated using in vitro transcribed DENV replicon RNA (Alvarez et al., 2005).

(c) Binding assay of radiolabeled DENV-2 particles

Vero cells were pretreated with PBS containing 1% calf serum, 0.1% glucose and 0.5% BSA at 37 °C for 1 h, and infected with ^{35}S-radiolabeled virions in the presence or absence of 20 μg/ml of λ-carrageenan. After 1 h adsorption at

4 °C, cells were extensively washed with cold PBS, lysed in NaOH 0.1 N–SDS 1% solution, and cell-associated radioactivity was quantified using a liquid scintillation counter.

Virus internalization and uncoating

(a) Internalization assay of DENV-2 by infectivity determination

Vero cells were infected with DENV-2 at an m.o.i. of 1. After 1 h adsorption at 4 °C, cells were washed with PBS and incubated at 37 °C for 1 h in MM containing or not 20 μg/ml of λ-carrageenan. Then, cells were washed with PBS and treated with proteinase K (Invitrogen) for 45 min at 4 °C to remove adsorbed but not internalized virus. After proteinase K inactivation with 2 mM PMSF in PBS-BSA, cells were washed with PBS containing 0.2% BSA by low-speed centrifugation, and the pellet was resuspended in MM. Different serial dilutions of the cell suspension were plated onto Vero cell monolayers to quantify productive internalized virus by infectious center formation.

(b) Internalization of DENV-2 RNA by real-time RT-PCR

Cells were infected and processed as described in (a), except that the final cell pellet was extensively washed with PBS containing 0.2% BSA and total RNA was extracted from cells by using TRIzol (Invitrogen) and the amount of internalized viral RNA was quantified by real-time RT-PCR as described above. The number of internalized DENV RNA molecules per culture was expressed after subtracting the amount of DENV RNA molecules present in cultures in which immediately after virus adsorption at 4 °C for 1 h, proteinase K treatment was performed.

(c) Internalization assay of radiolabeled DENV-2 particles

Vero cells were pretreated with PBS containing 1% calf serum, 0.1% glucose and 0.5% BSA at 37 °C for 1 h. Then, radiolabeled virions were adsorbed to cells at 4 °C for 1 h, cells were washed, incubated at 37 °C in MM with 20 μg/ml of λ-carrageenan and processed as described in (a), except that the final cell pellet was lysed in NaOH 0.1 N–SDS 1% solution, and cell-associated radioactivity was quantified.

(d) Uncoating of DENV-2

DENV-2 was adsorbed to Vero cells at 4 °C for 1 h, and then cells were washed and incubated at 37 °C with MM containing or not either carrageenan (20 μg/ml) or concanamycin (50 nM). At different times post-adsorption, non-internalized virus was removed as described in (a) and the final cell pellet was resuspended in MM, disrupted by freezing and thawing and intracellular infectivity was determined by plaque assay.

Viral protein synthesis

Vero cells were infected with DENV-2 at an m.o.i. of 1. After 1 h at 37 °C to allow virus adsorption and internalization, cells were washed with PBS and refed with MM in the presence or absence of 20 μg/ml of carrageenan. At 11 h p.i., cells were

incubated in methionine-cysteine-free medium in the presence or absence of compound for 1 h, and then labeled by addition of 100 μCi/ml of EXPRE³⁵S-³⁵S (NEN Dupont) for 4 h. After labeling, cells were washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 0.4 mM PMSF, 30 μl/ml aprotinine and 1% sodium deoxycholate in 0.01 M Tris–HCl, pH 7.4. After three cycles of freeze–thawing, samples of the clarified cell lysates were mixed with polyclonal rabbit anti-DENV-2 serum and incubated for 30 min at 37 °C, and 90 min at 4 °C. Antibody–antigen complexes were collected with protein A–sepharose, incubated for 30 min at 37 °C, and 90 min at 4 °C, washed three times in RIPA buffer and solubilized by boiling in sample electrophoresis buffer (5% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris–HCl, pH 6.8). Polypeptides were then electrophoresed on 15% SDS-polyacrylamide slab gels and visualized by fluorography.

RNA transfection

A DENV-2 suspension containing 10^6 PFU/ml was purified and RNA was extracted from virions using the commercial kit TOTALLY RNA (Ambion) and eluted in RNAse-free water. RNA was quantitated by UV absorption at 260 nm. To prepare liposomes, 5 μl of Lipofectamine 2000 (Invitrogen) was diluted in 45 μl of Opti-MEM I medium (GIBCO). After gently mixing, DENV-2 RNA (16 μg of RNA diluted in 50 μl of Opti-MEM I) was added and the mixtures were incubated during 20 min. Vero cells grown in coverslips at about 90% confluence were washed with Opti-MEM I and then incubated with the RNA–liposome complexes for 2 h at 37 °C. Subsequently, the supernatant containing the liposome suspension was removed, monolayers were washed with PBS and refed with growth medium containing or not 20 μg/ml of λ-carrageenan. After 18 h of incubation at 37 °C, supernatants were removed, cells were washed with PBS and overlaid with MM in the absence of compound. At 96 h post-transfection, supernatants were harvested for plaque assay and cells were fixed with methanol for immunofluorescence staining.

Virucidal assay

A DENV-2 suspension containing 6×10^5 PFU/ml was incubated with an equal volume of MM with or without different concentrations of carrageenan for 45 min at 37 °C. The samples were then chilled, diluted in MM and residual infectivity was determined by plaque formation.

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