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Characterization of In Vitro Dengue Virus Resistance to Carrageenan

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The λ-carrageenan (λ-car) is a potent and selective inhibitor of dengue virus (DENV) infection targeted to virus adsorption and internalization, due to the structural similarities with the mammalian cell receptor heparan sulfate. To further characterize the antiviral activity of λ-car, the selection and the phenotypic and genomic features of λ-car resistant DENV-2 variants are studied here in comparison to control virus. Resistant variants were rapidly selected in Vero cells after three passages in presence of the drug. No difference was detected in the growth profiles in Vero and C6/36 cells between resistant and control viruses. By contrast, the kinetics of adsorption and internalization of resistant variants in Vero cells was significantly diminished whereas entry to C6/36 cells was unaffected. By plaque purification and sequence analysis of the population, two types of resistant clones were found: some clones presented two mutations in E protein, K126E, and F422L; but other equally λ-car resistant clones had no mutations in E. Furthermore, no mutations were found in other viral proteins like prM, C, or NS1. The genomic disparity in E protein was also associated to differences in phenotype stability. The stable genomic resistance here described provides information about determinants in E protein involved in receptor binding and membrane fusion for uncoating. ***J. Med. Virol. 88:1120–1129, 2016***.

KEY WORDS: dengue virus; sulfated polysaccharides; carrageenans; virus entry; antiviral resistance

INTRODUCTION

Dengue is the most prevalent human arthropod-borne viral disease in tropical and sub-tropical regions of the world [Guzmán et al., 2010]. Approximately 2.5 billion people are living in endemic areas and it is estimated that 350 million apparent and inapparent infections occur each year [Bhatt et al., 2013]. Dengue virus (DENV), a member of *Flaviviridae*, comprises four serotypes named DENV-1 to DENV-4, which co-circulate worldwide by human transmission through infected mosquitoes of the genus *Aedes* and can cause a mild illness known as dengue fever or the severe forms of dengue hemorrhagic fever/dengue shock syndrome responsible of 25,000 annual deaths [Halstead, 2007].

DENV is an enveloped virion containing a single stranded, positive sense RNA included in an inner nucleocapsid. The genome codes for a single polyprotein that is cleaved into three structural proteins (the capsid protein C, a small membrane protein M, which matures from the precursor prM, and the envelope glycoprotein E) and seven nonstructural NS polypeptides with varied functions.

Despite the rapid expansion of dengue, at present neither a specific chemotherapy nor a preventive vaccine are available. The development of vaccines is a hard challenge because simultaneous full protection against all serotypes is required to avoid the chance of antibody-dependent enhancement, a phenomenon induced by heterotypic non-neutralizing antibodies and usually associated to severe clinical manifestations [Halstead, 2007; Flipse et al., 2013].

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On this basis, the search of antiviral agents for specific chemotherapy is an urgent need. Among the different strategies targeted to inhibit virus or cell components involved in DENV multiplication, virus entry has become an attractive alternative for therapeutic intervention since it represents a barrier to block the beginning of infection and drugs must not necessary enter the cells. Several classes of sulfated polysaccharides were found to be potent inhibitors of DENV-2 entry [Talarico et al., 2005; Talarico and Damonte, 2007; Pujol et al., 2012; Ichiyama et al., 2013; Vervaeke et al., 2013], due to the structural similarities with heparan sulfate (HS), a glycosaminoglycan (GAG) present in membrane proteoglycans and proposed as a main primary receptor for DENV-2 in mammalian cells [Chen et al., 1997; Hilgard and Stockert, 2000; Germi et al., 2002; Dalrymple and Mackow, 2011]. Since E protein is involved in both receptor recognition and membrane fusion events leading to virus entry, the polysaccharides act by blockage of the HS-E protein interaction. The N- terminal ectodomain of E protein has three domains (DI, DII, and DIII) whereas the C-terminal comprises a membrane proximal stem region containing two α-helices, connected by a stretch of conserved sequences [Kuhn et al., 2002]. The presence of GAG-binding determinants participating in DENV attachment and fusion in the three domains was postulated after solving the crystal structure of E glycoprotein [Modis et al., 2003, 2005] and also experimentally demonstrated in DII and DIII [Crill and Roehrig, 2001; Hung et al., 2004; Lee et al., 2006; Watterson et al., 2012].

Among HS-mimicking agents, the λ-carrageenan (λ-car) is one of most effective anti-DENV-2 inhibitors [Talarico and Damonte, 2007; Talarico et al., 2011; Acosta et al., 2014]. Our previous studies have shown the potent and selective antiviral activity of λ-car against DENV-2 in Vero and HepG2 cells, by dual interference with virus adsorption and penetration of nucleocapsid into the cytoplasm through virus envelope-endosome membrane fusion [Talarico and Damonte, 2007]. To further characterize the target of carrageenan on DENV-2, the selection and the phenotypic and genomic features of λ-car resistant variants are reported here.

MATERIALS AND METHODS

Compounds

Commercial λ-car, ι-car, heparin and dextran sulfate with an average molecular weight of 8,000 (DS8000) were purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions of compounds were prepared in distilled water at 2 mg/ml.

Cells and Virus

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS). For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 mosquito cell line from *Aedes 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% FBS.

The original stock of DENV-2 strain NGC was prepared in C6/36 cells and titrated by plaque formation in Vero cells.

Antiviral Assay

Antiviral activity was evaluated by a plaque reduction assay. Vero cells grown in 24-well microplates (3.0×10^5 cells per well) were infected with about 50 PFU per well of each viral variant in the absence or presence of different concentrations of the compounds, 2 wells per concentration. After 1 hr incubation at 37°C, residual inoculum was replaced by MM containing 1% methylcellulose (Sigma–Aldrich) (plaquing medium) containing the corresponding dose of compound. Plaques were counted after 6 days of incubation at 37°C. The effective concentration 50% (EC_{50}) values were calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed thrice and each in duplicate.

Isolation of Drug Resistant Variant Viruses

Vero cells grown in 6-well microplates (1.2×10^6 cells per well) were infected with DENV-2 at a multiplicity of infection (m.o.i.) of 0.1 in the presence of λ-car during virus adsorption and throughout the period of incubation. Supernatants were collected at 4–7 days p.i. and titrated by plaque formation in Vero cells. The supernatant with the highest virus titer was selected to perform the next passage and serial passages were continued in this manner. The compound concentration was 1 μg/ml for the initial two passages and 2 μg/ml for passage 3. The EC_{50} of each passage against λ-car was determined in Vero cells to monitor the appearance of resistant variant viruses. Simultaneously, control passages of DENV-2 in Vero cells without carrageenan were also performed.

For obtaining viral clones from the viral passages, Vero cells grown in 6-well microplates were infected with the selected passage. After 1 hr incubation at 37°C, viral inoculum was removed, cell monolayers were overlaid with MM containing 0.5% agarose (Invitrogen, Waltham, MA) and further incubated during 7 days at 37°C. Thereafter, MM containing 33 μg/ml neutral red (GIBCO) and 0.5% agarose was added to cells and microplates were incubated for 5 hr at 37°C. Viral clones were isolated, suspended in 500 μl of MM and subjected to vigorous stirring and one cycle of freezing–thawing. For amplification of viral clones, Vero cells grown in 24-well microplates were infected

with each viral clone in duplicate. Cell supernatants were collected at 4–8 days p.i. and titrated by plaque formation in Vero cells. The supernatant with the highest virus titer for each clone was selected to determine the EC_{50} of λ-car by a plaque reduction assay.

Assays for Virus Adsorption and Internalization

For virus adsorption, Vero and C6/36 cells grown in 6-well microplates were infected with DENV-2 variants (m.o.i. of 0.1) and incubated for 0, 15, 30, or 60 min at 4°C. Medium containing unadsorbed virus was then removed, cells were washed with PBS, covered with MM, scraped, and lysed by cycles of freezing–thawing. Afterward, cellular debris was removed by centrifugation at low speed, and adsorbed virus was determined in supernatants by a plaque assay in Vero cells.

For internalization, DENV-2 was adsorbed to cells for 1 hr at 4°C. Unadsorbed virus was then removed and cells were washed with PBS and incubated at 37°C with MM. At different times post-adsorption, cells were washed with PBS and treated with 1 mg/ml of 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 containing 3% BSA, cells were washed with PBS containing 0.2% BSA and resuspended in MM. Ten-fold serial dilutions of cell suspensions were plated onto Vero cells to quantify productive internalized virus by infectious center formation.

The effects of λ-car on variant virus adsorption and internalization were determined by a plaque reduction assay under different treatment conditions during each step as previously described [Talarico et al., 2005].

Ammonium Chloride Inhibition of DENV-2 Infection

Vero cells grown on cover slips in 24-well microplates were pretreated with ammonium chloride (10–50 mM) for 2 hr at 37°C, and then infected with DENV-2 at an m.o.i. of 0.1 in the presence of drug. After 1 hr of infection at 37°C, virus inocula were removed and cultures were further incubated at 37°C in MM without drug. At 48 hr p.i. cells were fixed with methanol for 10 min at −20°C for indirect immunofluorescence staining. Cells were incubated first with monoclonal antibody against the E glycoprotein (Abcam, Cambridge, UK) followed by FITC-labeled goat anti-mouse IgG (Sigma–Aldrich). The percentage of fluorescent cells in each sample was calculated from 20 randomly selected fields, counting approximately 50–60 total cells/microscope field.

Sequencing of DENV-2 Variants

Viral RNA was isolated from supernatants of infected Vero cells by using TRIzol (Invitrogen) according to the manufacturer's instructions. The extracted RNA was used in a reverse transcription reaction to obtain the cDNA of the region of interest followed by PCR amplification. Oligonucleotides used to amplify and sequence C, prM, E, and NS1 proteins of DENV-2 variants are described in Supplemental Table SI. Sequences were aligned using the software *Blast 2 Sequences* [Tatusova and Madden, 1999] and alignments were edited with the software *GeneDoc 2.5* [Nicholas et al., 1997], and *Vector NTI 9.0* (InforMax Inc., Bethesda, MD).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software. Comparison of means was tested by Student's unpaired *t*-test. Statistical significance is depicted in figures: $^{*}P < 0.01$, $^{**}P < 0.001$.

RESULTS

Isolation of λ-car-Resistant Variants and Antiviral Susceptibility to Sulfated Polysaccharides

The λ-car-resistant variants were selected by culturing the original DENV-2 virus stock (designated as DENV-2-vc 0) in Vero cells in the presence of increasing concentrations of λ-car. Simultaneously, control passages of DENV-2 in Vero cells without λ-car were also performed. The selection of drug resistance was very rapid: after three passages of DENV-2-vc 0 in the presence of λ-car, the virus designated DENV-2-λ 3 exhibited a high resistance to λ-car in virus plaque reduction assay whereas control virus similarly passaged in the absence of λ-car (designated DENV-2-vc 3) maintained a dose-dependent antiviral susceptibility with very little differences in comparison to the original DENV-2-vc 0 (Fig. 1A). The profiles of resistance or susceptibility of DENV-2-λ 3 and DENV-2-vc 3 to other sulfated polysaccharides such as ι-car, heparin, and DS8000 were similar to those observed for λ-car (Fig. 1B–D). From dose-response curves, the EC_{50}s against the polysulfates were extrapolated and the values corroborated the resistance of DENV-2-λ 3 and the susceptibility of DENV-2-vc 3 to all polysaccharides, with EC_{50} values in the range 1.4 ± 0.2–6.0 ± 1.4 μg/ml versus 0.08 ± 0.02–1.9 ± 0.2 μg/ml, respectively (Table I).

Multiplication and Entry of DENV-2 Variants in Vero and C6/36 Cells

The resistant population was further characterized for replication ability comparatively with control viruses. To this end, the growth curves of DENV-2-vc 3 and DENV-2-λ 3 were determined in Vero cells as well as in the mosquito cell line C6/36, usually employed to propagate DENV stocks. Despite slight initial difference in virus production at 24 hr p.i., the multiplication of DENV-2-λ 3 and DENV-2-vc 3 exhibited similar levels of growth rate and virus yields in both cell types after 48 hr of infection (Fig. 2A).

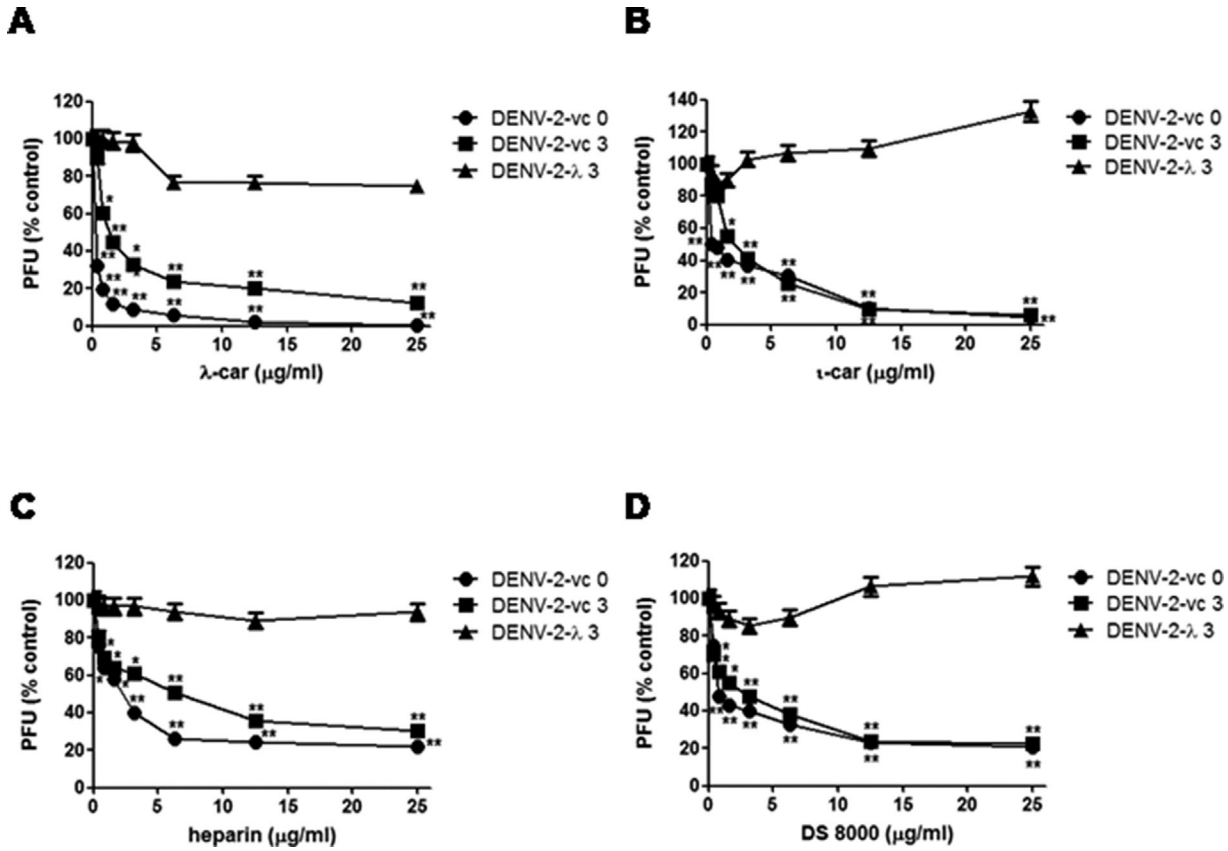

Fig. 1. Dose-response curves of DENV-2 variants against polysaccharides in Vero cells. The antiviral activity of λ-car (**A**), ι-car (**B**), heparin (**C**), and DS 8000 (**D**) was determined by a plaque reduction assay. Results are expressed as % of virus plaque formation in compound-treated cultures compared to untreated ones. Each value represents the mean of triplicate assays ± SD. Asterisks indicate statistical significance between treated and control infected cells for each variant ($^{*}P < 0.01$; $^{**}P < 0.001$).

Given the inhibitory effect of carrageenans on virus entry, any possible alteration in the initial steps of the DENV-2 replicative cycle was next analyzed. The adsorption kinetics was determined by measuring virus binding after different times of infection of Vero and C6/36 cells at 4°C. As seen in Fig. 2B, a very significant difference in amount of infective virions bound to Vero cells was observed between DENV-2-λ 3 and DENV-2-vc 3, with a 2–3 fold reduction of resistant virus binding. By contrast, in C6/36 cells the binding kinetics of both viruses was comparable.

TABLE I. Susceptibility of DENV-2 Variants to Sulfated Polysaccharides

	EC_{50} (μg/ml)[a]			
Virus variant	λ-car	ι-car	Heparin	DS8000
DENV-2-vc 0	0.08 ± 0.02	0.39 ± 0.06	1.9 ± 0.2	0.9 ± 0.1
DENV-2-vc 3	1.4 ± 0.2	2.0 ± 0.3	6.0 ± 1.4	2.6 ± 0.6
DENV-2-λ 3	>50	>50	>50	>50

[a]EC50 (effective concentration 50%): compound concentration required to reduce DENV-2 plaque number in Vero cells by 50%. Each value is the mean of triplicate assays ± standard deviation.

Next, the subsequent step of virus internalization was evaluated. Both variants were adsorbed to cells at 4°C and then infected cultures were incubated at 37°C for different time periods. Internalized virus at each time point was determined by an infectious centre assay. The cellular uptake of DENV-2-λ 3 in Vero cells was reduced in comparison to control virus but the differences were much less pronounced than those observed for virus adsorption whereas there were no significant variations between both virus populations in the pattern of internalization in mosquito cells (Fig. 2C). Although the rate of virus entry into Vero cells has been altered in λ-car resistant DENV-2, the low pH-dependence for cell penetration remained unaffected. The treatment of Vero cells before infection and during the first hour of DENV-2 infection with ammonium chloride, a lysosomotropic weak base that immediately raises the pH of acidic vesicles [Ohkuma and Poole, 1978], produced a very important inhibition in the number of infected cells expressing viral antigen (Fig. 2D and E). Consequently, the described conformational changes in E protein that depend on acid pH to trigger membrane fusion [Modis et al., 2004] appeared

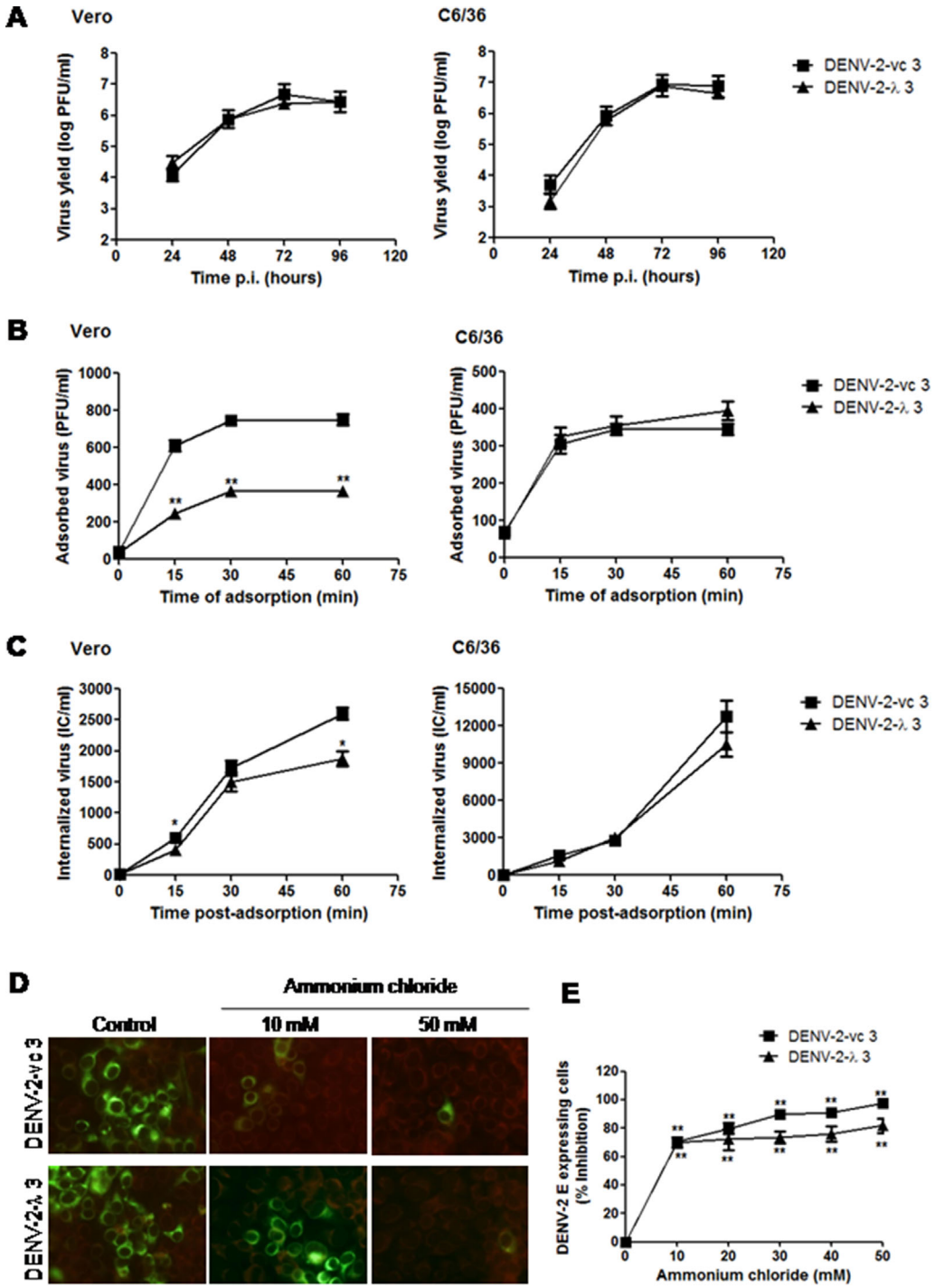

Fig. 2. (**A**) Growth curves of DENV-2 variants in Vero and C6/36 cells. Cells were infected with DENV-2 variants (m.o.i. of 0.1) and incubated at 37°C or 33°C depending on the cell type. At different times after infection extracellular virus yields were determined by a plaque assay. (**B**) Adsorption kinetics of DENV-2 variants in Vero and C6/36 cells. Cells were infected with DENV-2 variants (m.o.i of 0.1) and incubated at 4°C. Cell bound infectious virus was determined by plaque assay. (**C**) Internalization kinetics of DENV-2 variants in Vero and C6/36 cells. DENV-2 variants were adsorbed to cells during 1 hr at 4°C and then incubated at 37°C or 33°C. Thereafter, cell monolayers were treated with proteinase K and the cell pellet was inoculated on Vero cells to determine internalized virus by an infectious center (IC) assay. (**D, E**) Vero cells were treated with increasing concentrations of ammonium chloride or untreated (control), and then infected with DENV-2 variants. At 48 hr p.i., immunofluorescence staining was carried out using mouse anti-E glycoprotein antibody. The percentage of DENV-2 E expressing cells was calculated from 20 randomly selected fields and results are expressed as % inhibition with respect to infected cells without drug treatment. Each value represents the mean of triplicate assays ± SD. Asterisks indicate statistical significance between DENV-2 variants for each time point (**A–C**) and between treated and control infected cells for each variant (**E**) ($^{*}P < 0.01$; $^{**}P < 0.001$).

also to be required for entry of λ-car resistant DENV-2 into Vero cells.

Phenotypic and Genetic Characterization of Resistant Variant Clones

Due to the quasispecies nature of RNA viruses, there likely coexist drug-resistant and drug-susceptible virions in both DENV-2-λ 3 and DENV-2-vc 3 populations. To analyze the heterogeneity of susceptible and resistant variants, 10 to 15 independent virus plaques were picked from each virus stock and were amplified by a single passage in Vero cells. Those clones able to produce adequate virus titers after only one passage in Vero cells were further analyzed by determining the EC_{50} against λ-car. The 10 clones obtained from DENV-2-λ 3, designated DENV-2-λ 3-1 to DENV-2-λ 3-10, exhibited a highly resistant profile to λ -car (100% of resistant clones) as the original DENV-2-λ 3 (Supplemental Table SII). The dose-response curves of two clones, DENV-2-λ 3-5 and DENV-2-λ 3-9, are shown in Figure 3A. On the other hand, the DENV-2-vc 3 population was more heterogeneous alternating very susceptible clones, with EC_{50} in the range 0.07–1.5 μg/ml like the original DENV-2-vc suspension, and other more resistant clones with EC_{50} values between 17.3 and 50 μg/ml (Supplemental Table SII). The dose-response curves of two representative clones, DENV-2-vc 3-11 (susceptible, EC_{50} 0.07 ± 0.03 μg/ml) and DENV-2-vc 3-6 (partially resistant, EC_{50} 17.3 ± 0.1 μg/ml), are presented in Figure 3B. The presence of a fraction of drug-resistant clones in DENV-2-vc 3 (about 50% of the analyzed population) is not surprising since drug-resistant variants always exist at low levels in drug-susceptible virus populations, and additionally, the serial passaging of DENV-2 in Vero cells selects for viruses with lower susceptibility to sulfated polysaccharide inhibition [Acosta et al., 2014].

In an attempt to identify the specific DENV-2 molecular targets responsible of λ-car resistance, nucleotide sequences were obtained for the E gene of susceptible and resistant viruses. No changes in nucleotide sequences were detected between the parental DENV-2-vc 0 and DENV-2-vc 3, whereas four nucleotide changes were observed in DENV-2-λ 3 pool population (Table II): two silent mutations at positions 732 and 1335 and two changes at positions 376 and 1264, resulting in the amino acid substitutions K126E and F422L, respectively. Based on the published DENV-2 and DENV-3 sE protein structures [Modis et al., 2003, 2005; Roehrig et al., 2013], reported HS-binding clusters composed of positively charged amino acid residues on protein surface are indicated in domains I, II, and III (Fig. 4). The amino acid 126 is located at the HS-binding patch in domain II of E (indicated in the model of sE presented in Fig. 4) whereas the substitution in 422 is located at the stalk linking domain III with the transmembrane region of E protein (not shown on the model in Fig. 4).

The sequencing of individual clones isolated from DENV-2-λ 3 revealed two contrasting possibilities for clones equally resistant to λ-car: the presence of the same two amino acid substitutions detected in the pool population in clones like DENV-2-λ 3-5, but surprisingly clones like DENV-2-λ 3-9, with the same resistance profile to λ-car in the plaque reduction assay, did not exhibit any mutation (Table II). Furthermore, both clones DENV-2-λ 3-5 and DENV-2-λ 3-9 were also equally cross-resistant to other sulfated polysaccharides, like ι-car, with EC_{50} values > 50 μg/ml and relative resistance ratio as observed with the uncloned population (Table II). As control, no mutations were observed in the clones isolated from DENV-2-vc 3 (data not shown).

Besides E glycoprotein, the protein known to bind HS for DENV-2 attachment and internalization, we also sequenced the other two structural proteins, C, and prM, as well as NS1, a DENV nonstructural

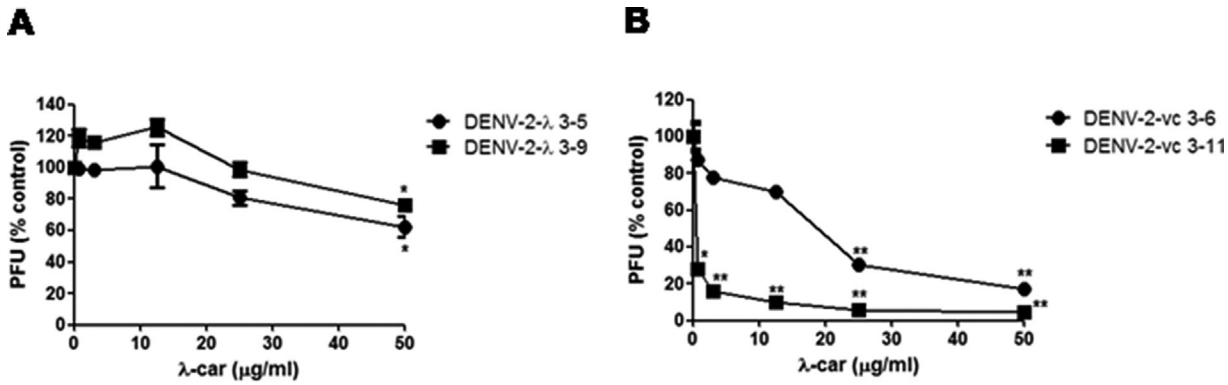

Fig. 3. Dose-response curves of clones obtained from DENV-2-λ 3 (**A**) and DENV-2-vc 3 (**B**) against λ-car in Vero cells. The antiviral activity was determined by a plaque reduction assay. Results are expressed as % of virus plaque formation in compound-treated cultures compared to untreated ones. Each value represents the mean of triplicate assays ± SD. Asterisks indicate statistical significance between treated and control infected cells for each variant ($^{*}P < 0.01$; $^{**}P < 0.001$).

TABLE II. Genetic Changes in E Protein of DENV-2 Variants

Viral variant	Nucleotide change[b]	Amino acid change[c]	Relative resistance against[a] λ-car	Relative resistance against[a] ι-car
DENV-2-vc 0	—	—	1	1
DENV-2-vc 3	—	—	17.5 ± 6.9	5.1 ± 1.6
DENV-2-λ 3	$A^{376} \rightarrow G$	$K^{126} \rightarrow E$	>625	>128
	$T^{732} \rightarrow C$	—		
	$T^{1264} \rightarrow C$	$F^{422} \rightarrow L$		
	$G^{1335} \rightarrow A$	—		
DENV-2-λ 3-5	$A^{376} \rightarrow G$	$K^{126} \rightarrow E$	>625	>128
	$T^{732} \rightarrow C$	—		
	$T^{1264} \rightarrow C$	$F^{422} \rightarrow L$		
	$G^{1335} \rightarrow A$	—		
DENV-2-λ 3-9	—	—	>625	>128

[a]EC_{50} of each DENV-2 variant/EC_{50} of DENV-2-vc 0 against λ-car and ι-car.
[b]Nucleotide changes are numbered from the 5′ end nucleotide of E protein sequence.
[c]Amino acid changes are numbered from the N-terminal residue of E protein.

protein reported to interact with HS [Avirutnan et al., 2007]. No nucleotide changes were detected in the three proteins for any DENV-2 variant in comparison with DENV-2-vc 0 (data not shown).

With the aim to understand the apparently different λ-car-resistance mechanism of DENV-2-λ clones 3-5 and 3-9 and fully assess the involvement of adsorption and internalization in this phenomenon, we evaluated the inhibitory effect of λ-car on these two stages of virus entry into Vero cells. The dose-response curves of adsorption and internalization in the presence of the carrageenan for the pool populations DENV-2-vc 3 and DENV-2-λ 3 as well as the two clones DENV-2-λ 3-5 and DENV-2-λ 3-9 were determined (Fig. 5). As expected from previous studies [Talarico and Damonte, 2007], DENV-2-vc 3 attachment and internalization into Vero cells was highly inhibited with more than 90% reduction in comparison to compound-untreated infected cells (Fig. 5A). By contrast, an almost total resistance for both early events was observed for DENV-2-λ 3 (Fig. 5B). The two clones obtained from DENV-2-λ 3 presented a distinctive pattern of resistance to λ-car in adsorption and internalization. DENV-2-λ 3-5 exhibited a performance similar to the uncloned DENV-2-λ 3 since both populations were equally resistant to λ-car when the compound was present during virus adsorption or internalization (Fig. 5C), a behavior that might be related to the mutations detected in E protein. By contrast, DENV-2-λ 3-9, the clone lacking E mutations, was resistant to λ-car during adsorption but partially susceptible when the compound was added during internalization (Fig. 5D). In fact, an increase in the amount of adsorbed virus was consistently observed in the presence of low concentrations of carrageenan.

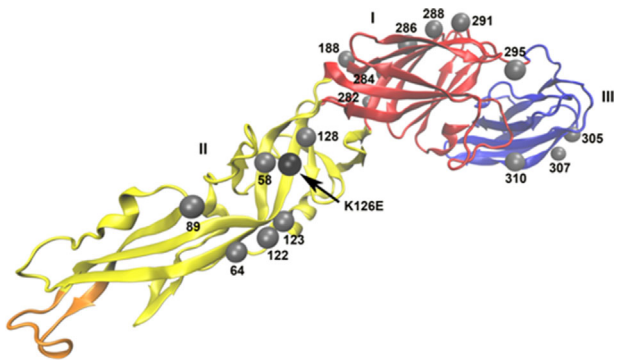

Fig. 4. Localization of K126E mutation and HS-binding amino acid sites in three-dimensional structure of DENV-2 NGC E protein. Position of K126E and reported HS-binding amino acid sites [Modis et al., 2003, 2005] are viewed in the sE monomer structure with domain I (red), domain II (yellow), domain III (blue), and the fusion peptide (orange) based on published structure [Modis et al., 2003].

The genomic disparity between DENV-2-λ 3-5 and DENV-2-λ 3-9 was also associated to relevant differences in the stability of the resistance phenotype of the variants. When DENV-2-λ 3-9 was propagated on Vero cells without λ-car in the culture medium, the virus became susceptible to carrageenans turning the EC_{50} from >50 μg/ml to 2.1 ± 0.5 μg/ml after the first passage in compound-free conditions. On the other hand, the clone DENV-2-λ 3-5 maintained a stable resistance phenotype even after several passages in Vero cells in the absence of carrageenan as well as the two mutations detected in E protein (data not shown).

DISCUSSION

The aim of this study was the characterization of the carrageenan-resistance profile of DENV-2 in cell cultures. Previous studies demonstrated the effective antiviral activity of λ-car in mammalian Vero and HepG2 cells whereas no inhibition was displayed in mosquito C6/36 cells [Talarico and Damonte, 2007; Talarico et al., 2011]. Carrageenan-resistant DENV-2 variants were rapidly selected in Vero cells after three passages in presence of the drug. Furthermore, the DENV-2-λ 3 variant population

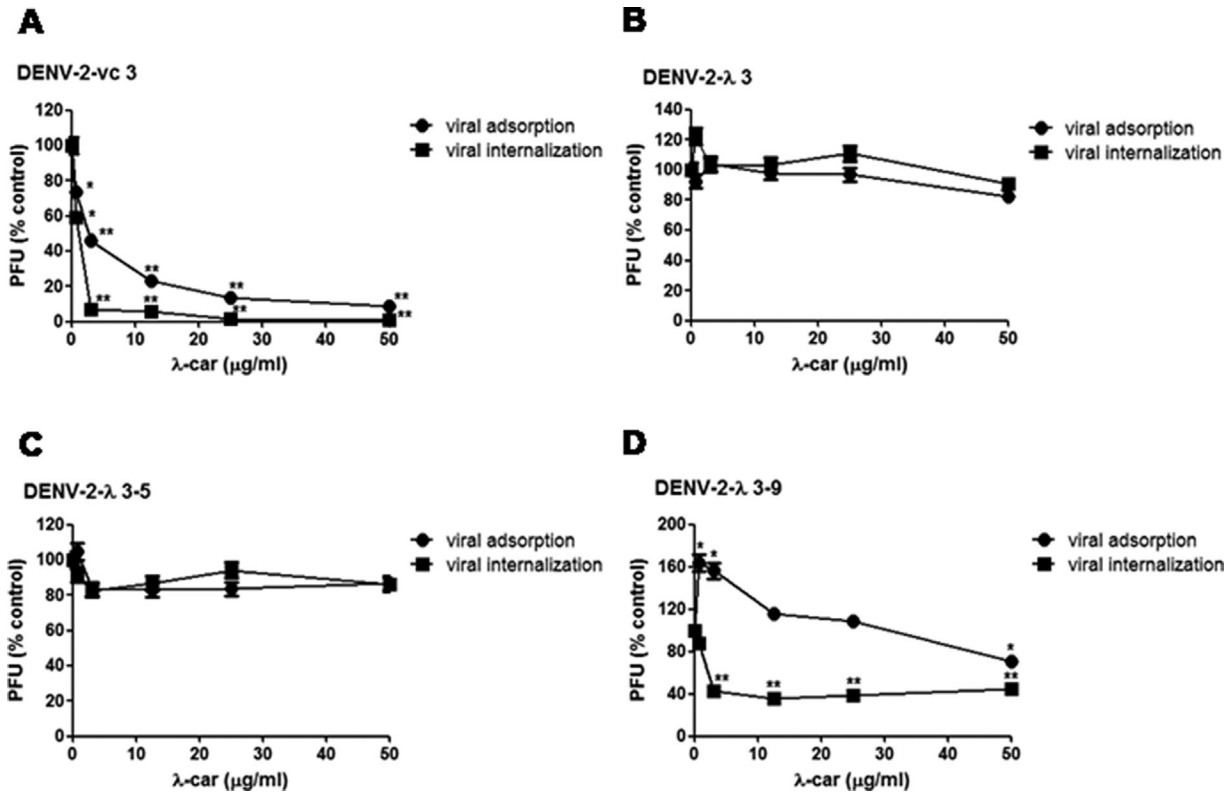

Fig. 5. Effect of λ-car on DENV-2 variants adsorption and internalization to Vero cells. Viral adsorption: Vero cells were infected with each DENV-2 variant in the presence of λ-car and after 1 hr adsorption at 4°C, cells were washed and refed with plaquing medium. Viral internalization: DENV-2 variants were adsorbed to Vero cells in the absence of compound; then cells were further incubated for 1 hr at 37°C in the presence of λ-car, treated with citrate buffer and overlaid with plaquing medium in the absence of compound. For both treatments, results are expressed as % of virus plaque formation in compound-treated cultures compared to untreated ones. Each value represents the mean of triplicate assays ± SD. Asterisks indicate statistical significance between treated and control infected cells for each variant ($^{*}P < 0.01$; $^{**}P < 0.001$).

was cross-resistant to ι-car, heparin, and DS8000 confirming that a similar DENV target is recognized by these sulfated polysaccharides. When DENV-2 was simultaneously passaged in Vero cells in the absence of λ-car, a small increase was observed in the antiviral resistance of DENV-2-vc 3 respect to the parental DENV-2-vc 0 but the variation is not comparable to that observed for DENV-2-λ 3, since the relative resistance (ratio EC_{50} DENV-2-λ 3/EC_{50} DENV-2-vc 0) for λ-car is >625. The slight variation in antiviral susceptibility between DENV-2-vc 0 and DENV-2-vc 3 (relative resistance for λ-car is 17.5 ± 6.9) is in agreement with a previous report showing that DENV-2 became increasingly resistant to sulfated polysaccharide inhibition after serial passaging in Vero cells [Acosta et al., 2014]. Therefore, the selection of λ-car resistance appears to be substantially due to the selective pressure exerted by the compound presence and also complemented by the continuous propagation in Vero cells.

The phenotype and genotype of DENV-2-λ 3 was then characterized in comparison to DENV-2-vc 3. No substantial difference was detected in the growth profiles in Vero and C6/36 cells for both viruses, suggesting that the viral fitness was not affected. This finding is in accordance with observations reported for DENV-2 variants resistant to other class of virus entry inhibitors like carbohydrate-binding agents [Alen et al., 2012]. By contrast, the kinetics of adsorption and internalization of DENV-2-λ 3 in Vero cells was significantly diminished in comparison to control virus. These modifications in the entry steps suggest that the mechanism leading to resistance to λ-car in Vero cells is an alteration in virion ability to interact with cell surface HS, the GAG with reported relevance as DENV receptor for entry in several mammalian cell lines [Chen et al., 1997; Hung et al., 1999; Hilgard and Stockert, 2000; Martinez-Barragan and del Angel, 2001; Germi et al., 2002; Dalrymple and Mackow, 2011]. By contrast DENV-2-λ 3 adsorption and internalization to C6/36 cells were not impaired, confirming previous studies about the differential participation of HS as receptor in mammalian and mosquito cells and the lack of antiviral activity of λ-car, and other sulfated polysaccharides, in C6/36 cells [Hung et al., 2004; Talarico et al., 2005; Thaisomboonsuk et al., 2005; Watterson et al., 2012].

The isolation of clones from DENV-2 selected suspensions allowed to detect 100% resistance

homogeneity in DENV-2-λ 3 whereas the control DENV-2-vc 3 population was heterogeneous including λ-car-susceptible and resistant clones. However, by sequence analysis two types of DENV-2-λ 3 resistant clones were found: some clones, such as DENV-2-λ 3-5, presented the two mutations K126E and F422L in E protein, also detected in the uncloned DENV-2-λ 3. Unexpectedly, other clones like DENV-2-λ 3-9 were equally resistant to λ-car ($EC_{50} > 50\,\mu g/ml$) as DENV-2-λ 3-5 but had no amino acid mutations in E. Also no mutations were found in other viral proteins like prM, C, or NS1. The E genomic diversity between both clones was also linked to a different stability of the highly resistant phenotype after virus propagation in the absence of drug. Consequently, the stable acquisition of carrageenan resistance by DENV-2 appeared to be dependent of the two mutant residues in E protein and is expressed by a complete lack of inhibitory effect of the polysaccharide on virus entry. By contrast, a transient state of resistance can be occasionally achieved through a selection basis still unknown that may be related to the complex mechanism of DENV-2 entry, at present not fully elucidated [Smit et al., 2011; Castilla et al., 2015].

As shown in the structural model of sE presented in Figure 4, the change K126E resulted in the loss of a basic amino acid (Lys) and the incorporation of an acid one (Glu) at a position located in the HS-binding patch present in E domain II. This amino acid change produced a loss of positive charge and the acquisition of negative charge at the virion surface and could explain the lower susceptibility to polysaccharides of DENV-2-λ 3 and DENV-2-λ 3-5 in comparison to DENV-2-vc 3. In fact, the role of amino acid 126 for polysulfate binding was also demonstrated by Lee et al. (2006) who reported that the low GAG affinity displayed by the PUO-218 strain of DENV-2 compared to NGC strain was due to the change of Lys by Glu at E residue 126. Other authors also reported mutations in basic amino acids located in HS-binding cluster of domain II around site 126. By site-directed mutagenesis, K122E and K123E mutants were found less susceptible to heparin inhibition than the original wt DENV-2 16681 strain [Roehrig et al., 2013] whereas the amino acid changes K124E and N128E introduced in DENV-2 PL046 strain resulted in reduced heparin binding affinity [Prestwood et al., 2008]. Interestingly, the weaker HS-affinity detected in mutants with negatively charged amino acids in the GAG-binding cluster of domain II appeared to be linked to increased DENV virulence in mice, indicating a role of the interaction of E protein with HS in modulating the outcome of infection and severity of disease [Lee et al., 2006; Prestwood et al., 2008]. A relatedness among HS affinity, cell tropism, and virulence has been also described for variants of serotype DENV-4 selected by passage in fetal rhesus cells [Añez et al., 2009].

The other substitution found in DENV-2-λ 3 and DENV-2-λ 3-5 E protein is a change between neutral amino acids (Phe by Leu) in the residue 422 located at the stalk linking domain III with the transmembrane region of E protein. This is a conserved mutation involving an amino acid change between groups of strongly similar properties. The stalk is a conserved sequence in several flavivirus and would be involved in the pore formation for fusion between the envelope virion and the endosomal membrane [Modis et al., 2004; Stiasny and Heinz, 2006]. Since both E mutations found in DENV-2-λ 3 and DENV-2-λ 3-5 correspond to residues involved in HS binding and membrane fusion, respectively, it is easy to understand the reduced level of adsorption and internalization displayed by these mutants in Vero cells (Fig. 2B and C) as well as the resistance to λ-car in both processes (Fig. 5B and C).

In conclusion, the resistance to carrageenan, representative of HS-mimicking DENV-2 inhibitors, appears to be a complex biological event mainly triggered by selective pressure of the drug but also regulated by extended in vitro cell passage. It may be related to genomic and non genomic changes in E protein. In the first case, the stable pattern of resistance here described also provides information about determinants in E protein involved in receptor binding and membrane fusion for uncoating. The resistance non related to E mutations requires further research to be fully elucidated.

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