



Viral entry inhibitors block dengue antibody-dependent enhancement *in vitro*

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ABSTRACT

Severe dengue virus (DENV) disease symptoms, including dengue hemorrhagic fever and dengue shock syndrome, have been correlated with the presence of pre-existing antibodies that enhance rather than neutralize infections in Fc receptor bearing cells. These antibodies can originate from previous infection with a different serotype of dengue, or from waning antibody titers that occur in infants and young children as they are weaned from breast milk that contains protective dengue-specific antibodies. Despite the apparent importance of this antibody dependent enhancement (ADE) effect, there has been no description of any specific inhibitors of this process. We explored DENV entry inhibitors as a potential strategy to block ADE. Two different peptide entry inhibitors were tested for the ability to block antibody-mediated DENV-2 infection of human, FcRII bearing K562 cells *in vitro*. Both peptides were able to inhibit ADE, showing that entry inhibitors are possible candidates for the development of specific treatment for severe DENV infection.

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1. Introduction

Dengue is the most important insect-transmitted viral disease and is prevalent in developing tropical and subtropical countries where the main mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, can breed year round. Worldwide, there are an estimated 50–100 million cases of dengue infection per year and 2.5 billion people who live in areas at risk (World Health Organization, 2009). An estimated 500,000 people are hospitalized annually with severe dengue symptoms; dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), a very large proportion of whom are children (World Health Organization, 2009). Currently there are no specific treatments or vaccines against dengue virus (DENV) and cases are treated using only supportive care.

Abbreviations: DENV, dengue virus; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FFU, focus forming unit; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

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The four distinct serotypes of dengue virus co-circulate in many areas and give rise to sequential epidemic outbreaks when the number of susceptible individuals in the local human population reaches a critical threshold and weather conditions favor reproduction of the mosquito vectors. Initial infection with one DENV serotype usually generates a protective and long-lasting immune response against re-infection with the same serotype. While antibody cross-reactivity between serotypes is common, cross-serotype protection is only short lived. Low levels of neutralizing antibodies, cross-reactive but non-neutralizing antibodies, or both, from previous infections have been shown to bind virions of other serotypes and target them to Fc receptors on macrophages and certain other cell types, enhancing infection of these cells (Halstead and O'Rourke, 1977). The presence of these cross-reactive and non-neutralizing antibodies has also been shown to correlate with severe disease outcome (DHF/DSS) in several studies (Halstead, 1998; Kliks et al., 1989; Vaughn et al., 2000). This antibody dependent enhancement (ADE) effect may also explain the sequential nature of epidemic outbreaks as well as the severe disease seen in infants being weaned from protective maternal antibodies (Halstead, 1998; Kliks et al., 1988). Concerns regarding incomplete protection and predisposition towards ADE have complicated vaccine development. If ADE is one of the major risk factors for DHF/DSS, and a roadblock for the development and use of vaccines, then the investigation of strategies to block ADE should be considered.

As there is no well-accepted animal model that displays the effects of DENV ADE, we used a well-characterized, human cell-based, *in vitro* system for this study. We examined the ability of two distinct DENV entry inhibitors to block ADE in a cell culture system using Fc receptor II (FcRII) bearing human K562 cells. We show that these entry inhibitors are capable of fully blocking the ADE effect and reducing infection to levels equivalent to that found in the absence of anti-DENV serum. These results show promise for the development of specific treatments for DENV infection that might be useful for intervention in the process of ADE, whether induced by waning maternal antibodies, naturally occurring secondary infection, or as a consequence of incomplete or declining vaccine protection.

2. Materials and methods

2.1. Cells and virus

Human FcRII expressing K562 cells (ATCC #CCL-243) were grown in RPMI-1640 medium (HyClone, Logan, UT) containing 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamax, 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B at 37 °C with 5% (v/v) CO₂. DENV-2 strain NG-C was obtained from R. Tesh at the University of Texas, Galveston and propagated in the *Macaca mulata* epithelial cell line, LLC-MK2 (ATCC #CCL-7), grown in Dulbecco's modified Eagle's medium (DMEM) with the same supplements and conditions. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted.

2.2. Anti-DENV serum

Human serum was obtained from patients at Tan Tock Seng Hospital, Singapore, approximately two weeks post-recovery. Informed consent was obtained from all study participants, and full institutional review and approval was obtained at Tan Tock Seng Hospital (DSRB B/07/275) and Florida Gulf Coast University (IRB 2007-12) according to US Department of Health and Human Services guidelines. Patients were initially diagnosed by serology or PCR. Post-recovery serum was characterized by ELISA and neutralization assay as described (Schieffelin et al., 2010). Equal volumes from seven highly cross reactive samples were heat inactivated and combined.

2.3. Peptides

DN59 (MAILGDTAWDFGSLGGVFTSIGKALHQVFGAIY) (Hrobowski et al., 2005) and 1OAN1 (FWFTLIKTOAKQPARYRRFC) (Costin et al., 2010) were synthesized by solid-phase N- α -9-fluorenylmethyl-oxycarbonyl chemistry, purified by HPLC, and confirmed by mass spectrometry (Genemed Synthesis, San Antonio, TX or EZBiolab, Carmel, IN). Peptide stock solutions were prepared by dissolving in 20% (v/v) DMSO in water, adjusting with NaOH, and concentrations determined by absorbance at 280 nm.

2.4. Neutralization assays

Virus focus forming unit reduction assays were carried out using LLC-MK2 cells as previously described (Costin et al., 2010; Hrobowski et al., 2005) with the exception that a 1.2% solution of Avicel in complete medium (FMC, Philadelphia, PA) was used in place of agarose. Neutralization assays using quantitative reverse transcriptase PCR (qRT-PCR) were carried out by infecting monolayers of LLC-MK2 in 12 well plates with approximately 4000 focus forming units (FFU) at 37 °C with 5% CO₂. After 1 h, the media was removed and 1 ml of complete culture media was added to each well. Plates were incubated for 72 h at

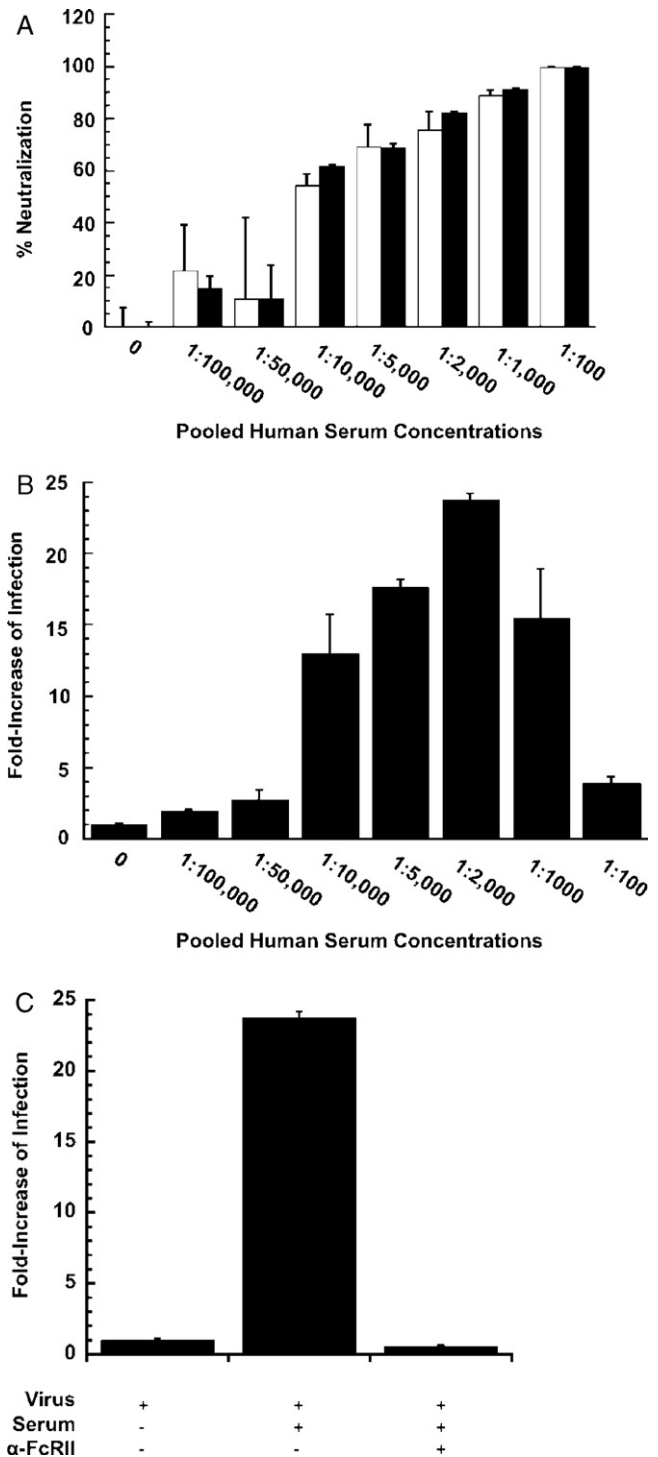


Fig. 1. Pooled anti-DENV serum neutralizes infection in LLC-MK2 epithelial cells. (A) Neutralization was measured either by focus forming unit reduction (white bars), or qRT-PCR (black bars), reaching approximately 80% inhibition at 1:2000 dilution. (B) Pooled anti-DENV serum enhances infection in FcRII expressing K562 cells with a maximum at 1:2000 dilution. (C) Addition of an anti-FcRII Ab blocks the enhancement effect. Graphs show the mean of three independent replicates \pm SD.

37 °C with 5% CO₂ and medium was collected and RNA isolated using a QiampViral RNA mini kit (Qiagen, Valencia, CA). qRT-PCR was carried out with primers 10503F and 10599R (Chutinimitkul et al., 2005), using a LightCycler 480 II (Roche, Indianapolis, IN) and a one step LightCycler RNA Master SYBR Green I kit (Roche). Amplification conditions were 61 °C for 30 min, 95 °C for

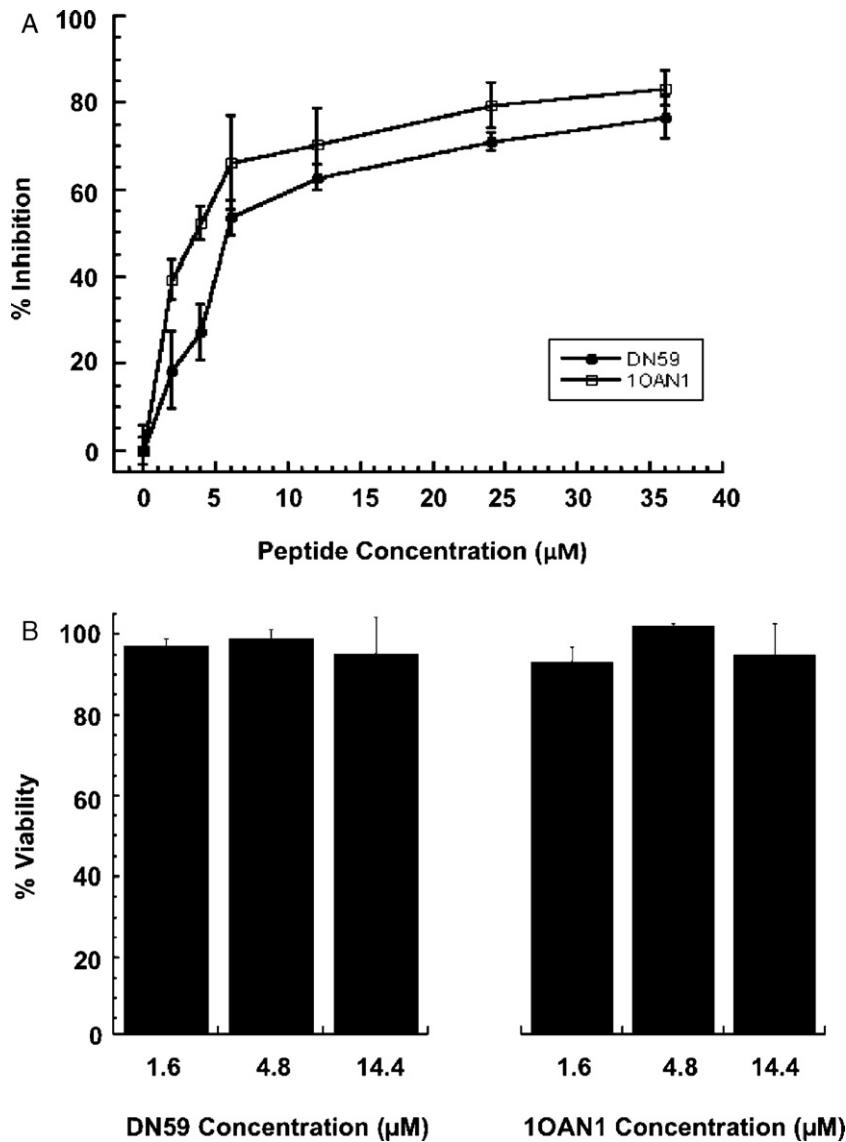


Fig. 2. Entry inhibitors block ADE in Fc bearing cells. (A) Two different DENV entry-inhibitory peptides inhibit infection of FcRII expressing human K562 cells in the presence of the maximally enhancing dilution (1:2000) of anti-DENV serum. (B) Neither peptide shows evidence of cellular toxicity over the concentration range used to inhibit enhancement. Dilution of the peptide inhibitors with virus and medium during infection results in the cells being exposed to the lower concentrations shown in (B). Graphs show the mean of three independent replicates \pm SD.

30 s, and 45 cycles of 95 °C for 5 s, 61 °C for 20 s, and 72 °C for 30 s.

2.5. Enhancement and ADE-inhibition assays

Dilutions of pooled serum were first incubated with 4000 FFU of virus for 1 h at 37 °C in 700 μ l of serum free RPMI-1640. 200 μ l of virus and serum was then incubated with either peptide for an additional 1 h. The addition of antibodies to the virus prior to addition of peptides was done to mimic what would be expected to occur in a hypothetical *in vivo* intervention. The mixtures were added to 80,000 K562 cells in 300 μ l of complete growth medium in a 24-well plate and incubated at 37 °C with 5% CO₂ for 72 h. RNA was extracted from cell lysates using the RNeasy Mini kit (Qiagen, Valencia, CA). qRT-PCR was performed as above. ADE-inhibition assays were performed identically using a 1:2000 serum dilution. Control experiments were performed with DMSO and NaOH, as well as in the presence of 1 μ g/ml mouse anti-human FcRII (anti-CD32) (Biolegend, San Diego, CA).

2.6. Toxicity assay

The effect of peptides on K562 cells was tested by measuring mitochondrial reductase activity using the TACS™ MTT cell proliferation assay (R&D Systems Inc., Minneapolis, MN) after 72 h of exposure, as per manufacturer's instructions. Peptide concentrations were adjusted by 0.4 to account for the dilution of the peptide/virus mixtures that occurred during the ADE inhibition assays.

3. Results

Pooled human anti-DENV serum was able to neutralize infection in LLC-MK2 epithelial cells, as shown by both a focus forming unit reduction assay and by qRT-PCR (Fig. 1A). This serum was also able to enhance infection in FcRII expressing K562 cells, with a maximum enhancement at 1:2000 dilution (Fig. 1B). This dilution showed an approximately 80% inhibition of infection in LLC-MK2 cells. Addition of an anti-FcRII Ab to the K562 cells blocked enhancement completely (Fig. 1C). Incubation of virus-serum mixtures

with inhibitory peptides showed a dose responsive inhibition of ADE, with a 50% inhibitory concentration of 3 μM for 1OAN1 and 6 μM for DN59 (Fig. 2A). The inhibition of ADE did not reach 100% for either peptide, but reduced the amount of viral genome present to a level equivalent to control infection in the absence of anti-DENV serum. No inhibitory effect was seen in either the LLC-MK2 cells or the K562 cells using buffers containing control amounts of DMSO or NaOH, but without peptides (data not shown). Both peptides were previously shown to be non-toxic in LLC-MK2 cells (Costin et al., 2010; Hrobowski et al., 2005), and neither peptide displayed evidence of toxicity to the K562 cells (Fig. 2B).

4. Discussion

We have shown that two different peptides are able to inhibit ADE-mediated DENV infection of FcRII expressing cells *in vitro*. These two peptides target the DENV surface E glycoprotein, but are distinct in their mechanisms of action. 1OAN1 is a computationally optimized mimic of the first beta sheet strand connecting E protein domains I and II. It interacts with purified, monomeric E protein, alters the surface structure of DENV virions, and has been shown to inhibit DENV entry and block virus:cell binding (Costin et al., 2010). The DN59 inhibitor is a direct mimic of a portion of the E protein pre-membrane stem region (Hrobowski et al., 2005). DENV E stem region peptides similar to DN59 interact with lipid bilayers and bind to post fusion E protein trimers to inhibit the fusion process (Schmidt et al., 2010). It is perhaps surprising that we observed inhibition of ADE, since antibodies are large and could sterically hinder peptide binding. Additionally, both peptides have relatively lower affinities for the E protein, with dissociation constants in the range of 10^{-7} M (Costin et al., 2010; Schmidt et al., 2010), compared to some neutralizing human monoclonal antibodies that bind with dissociation constants in the range of 10^{-9} M (Schieffelin et al., 2010).

The K562 cell line was chosen as a simple and easily interpretable model system for the study of ADE and ADE inhibition as it expresses only the FcRII gamma antibody receptor (Littau et al., 1990). Other immortalized cell lines or primary macrophage cultures express different combinations of distinct Fc receptors in variable amounts. It remains to be shown if these peptides will block ADE in other cell culture systems. We made no attempt to separate out the possible differences in ADE-mediated entry, ADE-mediated signaling changes, or ADE-mediated upregulation of viral replication that have been noted to occur in some systems (Boonak et al., 2008).

ADE is a phenomenon that is thought to be a major cause of DHF/DSS, and fear of inducing ADE has hampered the development of a DENV vaccine. We show here that entry inhibitors can effectively block ADE *in vitro*. Based on these observations, entry inhibitors like DN59 or 1OAN1 may serve as lead compounds for the development of clinically useful DENV treatments. It may also be important to evaluate other potential DENV treatment strategies for the ability to block ADE.

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