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Broadly Neutralizing Human Antibodies Inhibit Dengue Virus Infection In Aedes Mosquitoes

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ABSTRACT

Introduction

Dengue virus is the leading cause of morbidity and mortality among mosquito-borne viruses globally. There are four Dengue serotypes; immunity to one serotype provides protective immunity to that serotype but increases the risk of severe disease upon infection with a second serotype. Dengue virus, like all *Flaviviruses*, utilizes class II fusion proteins to gain entry into the host cytoplasm. The fusion loop, which is responsible for viral-host fusion, lies within the envelope (E) glycoprotein and has nearly 100% amino acid identity among all *Flaviviruses*. Two anti-Dengue virus human monoclonal antibodies (hMAbs), 1.6D and D11C, were isolated from Dengue infected patients, and determined to target the fusion loop (1). These antibodies not only neutralize all four Dengue serotypes, but also have broadly anti-*Flavivirus* cross-neutralizing capabilities in mammalian cells. While these antibodies could contribute to antibody enhancement of infection in humans, their neutralizing capabilities could be employed within a transgenic mosquito line serving as a novel control strategy to block arbovirus transmission.

Methods

The neutralization capacity of purified hMAbs 1.6D and D11C were tested against Dengue virus 2, 3, and 4, and Zika virus in C6/36 *Aedes albopictus* mosquito cells using a Fluorescent Focal Unit Assay. Additionally, the inhibitory capacity of 1.6D was evaluated in *Aedes aegypti* mosquitoes. Mosquitoes were intrathoracically inoculated with Dengue virus 2 and either 1.6D or C11, a hMAb anti-Gp41 HIV1, used as an antibody control. Viral RNA was extracted from mosquitoes two and four days post infection and viral loads were quantified by qRT-PCR.

Results

Antibodies 1.6D and D11C reduced viral infections in C6/36 cells for all Dengue serotypes tested, in a dose-dependent manner. Both antibodies inhibited 50% of each Dengue virus at less than 2μg/ml, comparable to what was reported in mammalian cells, although inhibition was not observed for Zika virus. Dengue 2 viral loads were significantly decreased in *Aedes aegypti* mosquitoes treated with 1.6D compared to the control antibody.

Conclusion

This study demonstrates the efficacy of broadly neutralizing human antibodies to reduce *Flavivirus* infections in mosquitoes. Additional research should assess the efficacy of this approach in a biologically relevant context and determine the impact of this strategy to reduce mosquito vector competence for *Flaviviruses*.

INTRODUCTION

Epidemiology and Burden of Disease

Dengue virus is transmitted by *Aedes* mosquitoes in tropical and sub-tropical climates globally. There are an estimated 390 million cases of Dengue virus infections per year including about 100 million cases of dengue fever and 21,000 deaths (2). Dengue fever is a flu-like illness, which can be fatal when it progresses to dengue hemorrhagic fever or dengue shock syndrome. There are four dengue virus serotypes (DENV1, DENV2, DENV3, DENV4); infection with one provides sterilizing immunity from that serotype but only partial protection to other serotypes with subsequent infections having increased risk of severe manifestations (3).

Currently there is no specific treatment for dengue viral infections. Hospitalization and fluid replacement therapy can provide effective support for the symptoms of severe disease (4). Phase III clinical trials have concluded for a tetravalent vaccine developed by Sanofi-Pasteur. While promising, vaccine efficacy was only 35-80% depending on the dengue serotype. It is not recommended for children under 9 years old as it has been shown to increase the severity of disease in subsequent infections (2).

In addition to the four Dengue serotypes, other notable mosquito-borne *Flaviviruses* cause significant morbidity and mortality globally. Yellow Fever virus (YFV), also transmitted by *Aedes* mosquitoes, infects an estimated 100,000 people causing 60,000 deaths per year in Africa and Latin America (5). An effective vaccine is available but does not always reach the populations with the greatest need as evidenced by an ongoing outbreak in Angola that

started in December 2015 and includes 1,132 suspected or confirmed cases and 168 deaths (6). Zika virus (ZIKV), also transmitted by *Aedes* mosquitoes, spread out of Africa and Asia, caused epidemics in the Pacific in 2007, and has now spread to the majority of countries in Central and South America. Zika is typically associated with minor disease in adults but is being investigated as a causative agent of microcephaly and Guillain-Barré syndrome, among others (7). West Nile Virus (WNV), which is transmitted by *Culex* mosquitoes, is found in Asia, Africa, the Middle East, and since 1999 North America. While birds are the reservoir host, humans can become infected through the bite of an infected mosquito. Approximately 80% of infected people remain asymptomatic. However, in 20% of infected individuals disease ranges from fever, headache and vomiting to a more severe neuroinvasive disease that can include coma, muscle weakness, and paralysis. No vaccine or specific treatment is available (8).

Viral Structure and Function

Dengue, Yellow Fever, Zika and West Nile viruses are all members of the *Flavivirus* genus; a genera that includes over 70 viruses. *Flaviviruses* are single-stranded positive-sense RNA viruses approximately 10.8kb in length, comprising a single open reading frame that encodes three structural proteins: capsid (C), membrane (prM is precursor to M), and envelope (E), and seven additional non-structural proteins for replication and transmission (Figure 1A)(9). The genome is packaged into a capsid protein, surrounded by a host-derived lipid bilayer, and further encased by 180 copies of two glycoproteins (prM and E). The E protein is responsible for receptor-binding and fusion (10). After the E protein binds the cell receptor the virus is internalized in an endosome and the pH is lowered triggering a

conformational change that exposes the fusion loops of each of the E monomers. The fusion loops penetrate partway through the host membrane causing the fusion protein monomers to collect into trimers. Once in this conformational shape there is a build up of energy that is released when the fusion protein folds back on itself bringing the fusion loop (inserted in the host membrane) towards the fusion protein anchor (inserted in the viral membrane) close together until fusion occurs (11).

The fusion process uses class II fusion proteins in order to fuse the viral membrane and host cell membrane and deliver the viral genome into the host cytoplasm. It is vital for virus entry and eventual replication. The essential protein sequences and structures are conserved among *Flaviviruses* (11). However, structural analysis of different Dengue serotypes suggests that there are differences in the distribution of electric charge across the virus surface, which impacts host cell receptor binding. There are also differences in the amino acid interactions between the E proteins and the membrane proteins that are at the virus surface (12). Furthermore, the virus structure changes throughout the infection cycle, and changes in temperature can affect the exposure of structural epitopes that are bound by antibodies (12, 13). The fusion-loop of WNV was identified as a region that had variable accessibility to antibodies. Neutralization with a fusion-loop binding antibody was dependent on time and temperature. Because the virus structure is dynamic, given time, antibodies could inhibit infection despite binding epitopes that are not constantly exposed (14). Additionally, there is some evidence that the virus structure changes when infecting a vector or mammalian host. The structure of DENV was determined to change when

incubated at 28°C compared to 37°C, the temperature of mosquitoes and humans respectively (2, 12, 15, 16).

Broadly Neutralizing Antibodies

The E protein is well conserved with about 40% amino acid identity within the *Flavivirus* genus. Mosquito-borne *Flaviviruses* are even more closely related to one another than to tick-borne or *Flaviviruses* not transmitted by a vector (Figure 1B)(10). But within the fusion loop (amino acid residues 98-110 of the E protein) there is near 100% amino acid identity. There have been frequent reports of cross-reactive antibodies that bind the fusion loop, but only a few studies have reported characterizing antibodies that are cross-reactive and strongly neutralizing (1, 2, 17-23). Characterization of these antibodies and further development is being pursued for therapeutic purposes. However, poorly neutralizing antibodies may contribute to Antibody-dependent enhancement (ADE) of dengue hemorrhagic fever through enhanced infection of macrophage cells (2, 12, 21). Additionally, research suggests that human neutralizing antibodies typically recognize quaternary structure that requires intact virus rather than a recombinant protein that might be achieved in a vaccine (2). The importance of quaternary structure may explain why antibodies that are mapped to a highly conserved amino acid sequence, such as the fusion loop, are not necessarily cross-reactive or cross neutralizing.

Human antibodies that neutralize all four Dengue serotypes and are active against other *Flaviviruses* have previously been characterized. Costin et al. (1) determined that two related broadly neutralizing human monoclonal antibodies 1.6D and D11C both bound to a

well conserved region of the E protein fusion loop, specifically W101 and G109 (Figure 1C). Antibody 1.6D was isolated from a patient's blood sample 3 months after an infection acquired in Jamaica of an unknown Dengue serotype. The patient reported symptoms including fever, headache, retro-orbital pain, and blood in sputum. A molecular clone was constructed from stimulated peripheral blood mononuclear cells (PBMCs). Antibody D11C was isolated from a patient's blood sample 4 weeks after recovering from a Dengue infection acquired in Singapore that was determined to be a secondary Dengue infection. A molecular clone of D11C was produced by Epstein-Barr virus transformation of B cells. Both antibodies were grown in PBMCs and purified by protein A affinity chromatography. They are made up of IgG1 heavy chains and kappa light chains. In addition to neutralizing all four Dengue serotypes in mammalian cells, both 1.6D and D11C were shown to effectively neutralize two related *Flaviviruses*, WNV and YFV. High affinity to the fusion loop inhibited fusion of the virus membrane and human cell endosome membrane. It was also demonstrated that these antibodies could contribute to ADE at subneutralizing concentrations (1).

Mosquito Control Strategies

Vector control is a widely utilized method to block transmission of mosquito-borne diseases. There have been prior successes, such as for malaria control, utilizing insecticide-treated bed nets and indoor residual spraying. However, chemical tolerance and resistance threatens progress of mosquito-borne infection control (24-26). New strategies to manipulate and control mosquitoes are under development and field studies are already showing promise. Transgenic *Anopheles* mosquitoes were created that express proteins

and human antibodies that inhibit malaria transmission (27-29). Furthermore, mosquito midgut microbiota have been manipulated to express and secrete anti-*Plasmodium* effector proteins that decrease the development of parasites (30). It was shown that the *Aedes* microbiota is important for DENV infection. The presence of *Wolbachia* bacteria makes *Aedes aegypti* resistant to Dengue infections (31). Sterilized transgenic *Aedes aegypti* mosquitoes have been tested at field sites as a new vector control measure to decrease mosquito populations (32, 33). Additionally, a transgenic *Aedes aegypti* line has been created that expresses an inverted-repeat RNA in the mosquito midgut that is resistant to DENV2 infections (34).

Despite the advancement of technology to create transgenic mosquitoes, the plethora of research on anti-dengue human antibodies, and the unchecked threat of Dengue globally, there is no published research on the capacity of human antibodies to prevent DENV infections in mosquitoes. This study seeks to characterize the capacity of broadly cross-neutralizing anti-dengue virus antibodies (1.6D and D11C) to inhibit *Flavivirus* infections of mosquitoes. Development of this strategy could provide a targeted approach to decrease the burden of mosquito-borne viruses. The antibodies tested hold the potential to prevent transmission of all Dengue virus serotypes and other mosquito transmitted *Flaviviruses*, without disrupting the natural role mosquitoes play in the ecosystem.

MATERIALS AND METHODS

Phylogenetic Tree

A phylogenetic tree was generated on MegAlign by DNASTAR® Software for Life Sciences (Madison, WI). A panel of *Flavivirus* E protein nucleotide sequences were downloaded from GenBank. Viruses (accession numbers) include: Dengue 1 (KC741442.1, AB624554.1, KJ415098.1), Dengue 2 (FJ606704.1, AB545874.1, AB545873.1), Dengue 3 (EU617038.1, GQ357889.1, FJ606712.1), Dengue 4 (AY786201.1, AY786200.1, AY786202.1), Yellow Fever (U23580.1, U23577.1, U23578.1), Zika (KJ634273.1), West Nile (AF459403.3, JX442282.1, DQ823150.1), Tick-Borne Encephalitis Virus (TBEV) (GQ845440.1). TBEV is a tick-borne *Flavivirus*. Alignment was performed by Clustal W method and phylogenetic tree was constructed using 1000 Bootstrap trials, seed 111. Scale is percentage of genetic difference.

Cells and Viruses

C6/36 *Aedes albopictus* mosquito cells were used in Fluorescent Focus Unit (FFU) assays and to grow virus stocks. Vero (African green monkey kidney epithelial) cells were used to propagate and increase viral titers. Cells were maintained in complete growth media, containing 10% Fetal Bovine Serum (FBS), 0.28% Sodium Bicarbonate, 1% L-glutamine, 1% Penicillin/ Streptomycin Antibiotic, 1% Non-essential amino acids. C6/36 cells were incubated at 28°C, Vero cells at 37°C, with 5% CO_2. Viruses (see Table 1) were grown approximately one week in cells; aliquots were centrifuged at 1500rpm for 5 minutes and then stored in -80°C until use in experiments. Viral concentrations were estimated using the FFU assay, described below.

Fluorescent Focus Unit (FFU) Assay

FFU Assays were carried out in C6/36 cells as described previously (35). One day prior to infection $0.5x10^6$ cells per well were seeded into Lab-Tek II 4-chamber glass slides, ThermoScientific (Rochester, NY). Cells were infected with serial dilutions of virus stock in complete media, loading 150µL per well, and incubated for 1 hour shaking gently by hand about four times per hour. Virus media solution was removed and 750µL of 5% Methylcellulose in 5% FBS complete media was added per well. Cells were incubated for 3 days at 28°C. After 3 days Methylcellulose solution was removed, 500µL of sterile PBS was added per well and placed on ice for 5 minutes. PBS was removed; cells were fixed by adding 500µL ice cold 100% Methanol per well and incubated for 10 minutes at -20°C. Methanol was then removed and replaced with 500µL Permeabilization buffer (0.3% Triton in PBS) for about 10 minutes at room temperature. Buffer was removed and replaced with 500µL Blocking buffer (5%BSA, 0.1%Tween in PBS) and incubated for 30 minutes at room temperature on shaker, 5rpm. Next cells were stained with the primary antibody Pierce™ FlaviVs Envelope Antibody (FE1), Thermo Scientific Prod #MA1-71258, 1:100 in Blocking buffer, 100µL per well. Cells were incubated for 1 hour at room temperature on shaker, 5rpm. Cells were washed three times with 500µL per well 0.1%Tween in PBS for 5-10 minutes per wash. Cells were stained with secondary antibody Alexa Fluor 488 goat anti-mouse, Life Technologies, 1:200 in Blocking buffer, 100µL per well. Cells were incubated for 1 hour, room temperature on shaker, 5rpm. Cells were again washed three times with 500µL per well 0.1%Tween in PBS for 5-10 minutes per wash. All liquid was removed from wells, chambers were detached from the slides, and 30-50µL of

ProLong® Gold antifade reagent with DAPI from Life Technologies (Eugene, OR) was added to each well. Slides were covered with a glass coverslip and allowed to dry overnight. Microscopy was conducted using Zeiss AXIO Imager.M1 at 20X magnification. The entirety of each slide was scanned and FFUs were counted (Figure 2). Eight serial dilutions of virus stock were tested to estimate the dilution for a final concentration of 100 FFUs per well.

This process was repeated for neutralization assays using approximately 100 FFUs of virus per well with 0.1, 1.0, 10.0, or, 100.0μg/μL of neutralizing antibodies: 1.6D or D11C, described above. Neutralization by these antibodies was compared to a control antibody C11, a human monoclonal antibody that binds a conserved region of the HIV1 envelope protein, Gp41 (All antibodies were generously provided by Dr. John Schieffelin). Viruses and antibodies were mixed together and allowed to incubate at room temperature for 1 hour prior to infecting C6/36 cells. Cells were infected with 150μL of each solution and the procedures followed as described above. Three biological replicates were performed for each virus and antibody concentration.

Rearing Mosquitoes

Aedes aegypti mosquito eggs were provided by the Connecticut Agricultural Experiment Station. Eggs suspended on filter paper were allowed to hatch in a 1% solution of yeast liver extract in H_2O. Containers were kept in a 28°C incubator and fed with additional yeast liver extract solution every 1-3 days for a week. After one week pupae were transferred to emergence containers on a daily basis until few larvae remained. Pupae were provided

10% sucrose solution in deionized-H_2O on cotton balls during the emergence stage for mosquito feeding. Approximately 2-3 weeks after eggs were allowed to hatch mosquitoes were aspirated, knocked-down on ice, and females and males were separated. Approximately 70-100 females were held in each container with access to sucrose solution until experiments, males were sacrificed.

Mosquito Microinjections

Virus and antibody solutions comprised of 100 FFUs of DENV2 mixed with 0.05µg of antibody, either 1.6D or control antibody C11, brought up to 20µL volume with PBS. The solution was allowed to incubate at room temperature for 1 hour. Mosquitoes were knocked-down on ice and then injected in the thoracic cavity using the Nanoject II Drummond Scientific (Broomall, PA). Each mosquito was injected with 69nL at the fast speed setting, 46nL/second. After injection mosquitoes were placed back into containers with 10% sucrose and incubated for 2 days and 4 days. Mosquitoes were then knocked down on ice and individually placed in 2mL safety-lock tubes with a bead and 300µL of PBS. Tubes were shaken in a bead beater for 30 seconds, 25 frequency and stored at -80°C until RNA analysis.

Viral RNA Extraction and qRT-PCR

RNA extraction was carried out using OMEGA bio-tek Mag-Bind® Viral DNA/RNA 96 kit M6246-03 (Norcross, GA) solutions were made to manufacturer protocol. The 96-well plate extraction machine King Fischer Flex by Thermo Scientific carried out extraction automatically. BindIt Software 3.3 was programmed to: pick-up tip plate, mix sample plate

for Lysis binding for 10 minutes, bind beads, wash 1 minute in VHB buffer, bind beads, wash 1 minute in SPR wash #1, bind beads, wash 1 minute in SPR wash #2, dry for 45 seconds, mix 5 minutes for elution in 50µL nuclease-free H_2O, and collect beads.

Quantitative RT-PCR was carried using previously characterized PCR primers and probe that were verified for use on four Dengue virus serotypes (36). Solutions were made to manufacturers instructions using BioRAD 2x iTaq Universal Probes One-Step Reaction Mix, iScript™ Reverse Transcriptase, in a 20µL total volume. Primers purchased from IDT include DENV1-4 qPCR Forward 5'-GCA TAT TGA CGC TGG GAR AGA – 3' and DENV1-3 qPCR Reverse 5'-TTC TGT GCC TGG AAT GAT GCT G-3', 500nM of each primer was used per reaction. The RT-PCR probe was purchased from IDT: DEN1-4 qPCR 5'-6FAM-CAG AGA TCC TGC TGT C/3IABkFQ/-3', (6FAM is 6-carboxy fluorescein) 250nM of probe per reaction. A 96-well plate was loaded with 15µL of solution and 5µL of each RNA extracted sample, each run was completed with DENV2 standards at a concentration of 10^3-10^7 in duplicate as well as two RNA free wells. Plates were centrifuged to 1500rpm for 2 minutes. The BIORAD C1000 Touch Thermal Cycler CFX96 Real-Time System (Hercules, CA) ran SYBR/FAM; 50°C, 5 minutes; 95°C, 20 seconds; then 40 repeats of 95°C, 3 seconds and 60°C, 30 seconds with photo. Data was configured on BIORAD CFX Manager 3.1 software for estimation of viral load, genome equivalents per milliliter (GE/ml).

Data Analysis

Data were analyzed in SAS 9.3 Statistical software (Cary, NC). Graphs were created in Microsoft Excel (Redmond, WA) or GraphPad Prism 6.0 (La Jolla, CA). FFU assays were

analyzed for 1.6D and D11C as percent inhibition of the average C11 FFU for each replicate and then the average percent inhibition and standard deviation was calculated and graphed in Excel. 50% Inhibitory Concentration (IC50) was calculated from the linear regression model best-fit line in SAS constructed from the antibody log concentration as the predictor variable and the percent inhibition as the response variable.

The mean qRT-PCR viral loads (GE/ml) for 1.6D and C11 antibody microinjected mosquito groups were calculated and compared using a 2-sample 2-tailed t-test in SAS. A p-value less than 0.05 was considered statistically significant, the Cochran method, which assumes unequal variance, was employed.

RESULTS

Phylogenetic Tree

The *Flavivirus* E protein nucleotide sequence phylogenetic tree is rooted with TBEV, a tick-borne *Flavivirus*, as an outgroup (Figure 1B). YFV, which is transmitted by the same mosquitoes as DENV, is more distantly related to the DENV cluster than WNV, a virus transmitted by a different mosquito genus. Whereas the newly reemerging ZIKV, also transmitted by the same mosquitoes as DENV, is more closely related than WNV. This agrees with previous phylogenetic analyses (9, 37).

Fluorescent Focal Unit Assay

DENV2, DENV3, DENV4, and ZIKV readily infected C6/36 cells and FFUs could be visualized and counted as previously described (35)(Figure 2). Broadly neutralizing human antibodies 1.6D and D11C inhibited DENV2, DENV3, and DENV4 infections in C6/36 cells in a dose dependent manner (Figure 3A and B, respectively). Inhibition was similar for all three viruses and was similar between antibodies. Simple linear regression models were constructed for the relationship between inhibition and antibody concentration, enabling the calculation of the 50% inhibitory concentration (Figure 3C). IC50 was lowest for D11C against DENV3 at 0.07μg/ml and also low for 1.6D at 0.80μg/ml. For DENV2 IC50 of D11C and 1.6D were 0.56μg/ml and 0.77μg/ml respectively. DENV4 IC50 was slightly higher for D11C and 1.6D at 1.56μg/ml and 1.00μg/ml respectively. Neither antibody inhibited ZIKV well. D11C showed a slightly more linear relationship and IC50 of 140.47μg/ml, whereas 1.6D had an IC50 well above 1000.00μg/ml, too far out of range for our tested concentrations to accurately predict an inhibitory concentration.

Mosquito microinjections

Viral loads were statistically significantly lower in *Aedes aegypti* mosquitoes injected with a mixture of 1.6D antibody and DENV2, compared to mosquitoes injected with the control C11 antibody (Table 2). The log viral loads were compared because viral load ranged from 10 to 100,000 GE/ml. At day 2 the mean log viral load was 2.72 ± 0.54 GE/ml for antibody 1.6D (N=86) and 3.62 ± 0.58 GE/ml for the control antibody (N=89). At day 4 the mean log viral load was 4.49 ± 0.64 GE/ml for antibody 1.6D (N=32) and 5.00 ± 0.65 GE/ml for the control antibody (N=38). Day 2 viral loads are calculated from five independent replicates of 10-20 mosquitoes per replicate. Day 4 is calculated from three independent replicates of 7-20 mosquitoes per replicate. Statistical significance was maintained at day 2 and day 4 post infection although the difference between the antibody and control viral loads diminishes overtime ($p<0.0001$ and $p=0.0020$ respectively). There were negative samples within both antibody groups, determined by comparing viral loads to negative mosquito controls. Negatives among the control group may have been owing to physical injection error but overall the decreased viral load consistently found for 1.6D treated mosquitoes compared to the control suggests a potent neutralizing capability.

DISCUSSION

Antibodies 1.6D and D11C are both promising candidates to inhibit Dengue virus infection in *Aedes* mosquitoes. Results were consistent for DENV2, DENV3, and DENV4 with 50% inhibitory concentration in C6/36 cells ranging from 0.07 to 1.56μg/ml. This is similar to Costin et al.'s findings in mammalian cells for Dengue virus serotypes 1-4, where IC50 was 0.2-1.5μg/ml for antibody 1.6D. However, while IC50 of D11C ranged from 1-1.6μg/ml for DENV1, DENV2, and DENV4 they found weaker inhibition of DENV3, IC50 of 10.2μg/ml (1). We found the greatest inhibition of DENV3 with antibody D11C with an IC50 of 0.07μg/ml. Perhaps this is indicative of conformational changes that allow greater antibody binding of the target epitope in DENV3 at lower temperatures.

ZIKV infection was not inhibited by antibody 1.6D or D11C in our FFU assay. This is somewhat surprising given the relatedness between the ZIKV E protein, particularly the fusion loop, and that of DENV. These antibodies were previously shown to weakly inhibit YFV and more robustly inhibit WNV in mammalian cells. WNV is more closely related to DENV than YFV, and ZIKV is more closely related still. Potentially other differences in the ZIKV E protein amino acid sequence results in a quaternary structure that the antibody does not recognize or bind as effectively. The conserved residues 101W and 109G in DENV have a homologous region in ZIKV although there are other stretches of amino acids missing in the ZIKV E protein. The truncation of this protein may form a structure dissimilar enough to make binding less efficient. Given the current public health importance of ZIKV an alternative antibody may be more appropriate for use in areas endemic with both Dengue and Zika. Nevertheless, DENV remains the leading cause of

mosquito-borne viral disease and this technology is still very valuable for preventing transmission. Within the mosquito, human antibodies in combination with natural mosquito immunity may be sufficient to significantly reduce mosquito infection and transmission.

Results from our microinjection experiments suggest that the 1.6D antibody consistently neutralizes DENV2 and inhibits the infection of *Aedes aegypti* mosquitoes. C6/36 cells provided valuable information about neutralization and inhibitory concentration in insect cells. However, these results were strengthened by the additional evidence that neutralization is maintained within living mosquitoes. By examining the effects at day 2 and day 4 we are confident that mosquitoes were appropriately injected with virus and antibody solutions for both the 1.6D antibody and the control and the decrease in viral load is a demonstration of neutralization. Overtime the viruses that are able to infect the mosquito replicate and diminish the difference in viral loads. If an appropriate concentration of antibody can be secreted into the mosquito midgut at the time of taking an infected blood meal, antibody neutralization in combination with mosquito immunity could suppress mosquito cell infection. Additionally, virus must spread from the midgut to the salivary glands in order to be transmitted to a new human host. Antibodies could be secreted throughout this pathway as was achieved in mosquitoes with anti-malaria antibodies. This strategy successfully prevented the infectious form of malaria parasites from reaching the mosquito salivary glands (27). The true test of this new technology will not simply be a reduction in mosquito infections but a reduction in mosquito competence as a vector for lethal pathogens.

Limitations

Unfortunately we were unable to verify inhibition of DENV1 in our FFU assay owing to challenges faced in our assay development, and not related to the antibody-virus interactions. The strain of DENV1 tested caused so much C6/36 cell death it was impossible to visualize fluorescent foci or count foci accurately. Previous studies of these antibodies in human cells showed strong inhibition of DENV1 at par or better than other serotypes.

The eventual goal of this research is to demonstrate the efficacy of inhibiting mosquito infection that ultimately reduces viral transmission. We showed antibodies could reduce viral load in mosquito cells and live mosquitoes but further work needs to show that this translates to reduced viral infection following a blood meal and that the reduction is sufficient to reduce viral transmission. Upon membrane blood feeding of *Aedes aegypti* mosquitoes with DENV2 less than 40% of mosquitoes became infected. With limited antibody stock and with antibody at a concentration of approximately 1mg/ml it was not feasible to produce solutions of virus and antibody where both the control mosquitoes became infected and the antibody concentration reflected the inhibitory concentrations determined in FFU assays.

Future Directions

This study demonstrates that human antibodies can neutralize viruses at lower temperatures typical of mosquitoes and neutralization significantly decreases mosquito infection. Further development of a more sensitive assay to detect virus in mosquitoes

could help determine whether this strategy would be biologically feasible and impactful in a mosquito population.

The development of a transgenic *Aedes aegypti* line could then be created that allows for viral-antibody interactions to occur within the mosquito over the course of a natural virus infection. Given the advancement of gene drive, these antibodies could be propagated throughout *Aedes* populations and reduce the population of Dengue virus transmitting mosquitoes (38, 39). Additional work should be considered that would extend neutralization to Zika and Yellow Fever virus, two prominent and life-threatening viral diseases that are transmitted by the same mosquitoes and within the same geographical regions.

ACKNOWLEDGEMENTS

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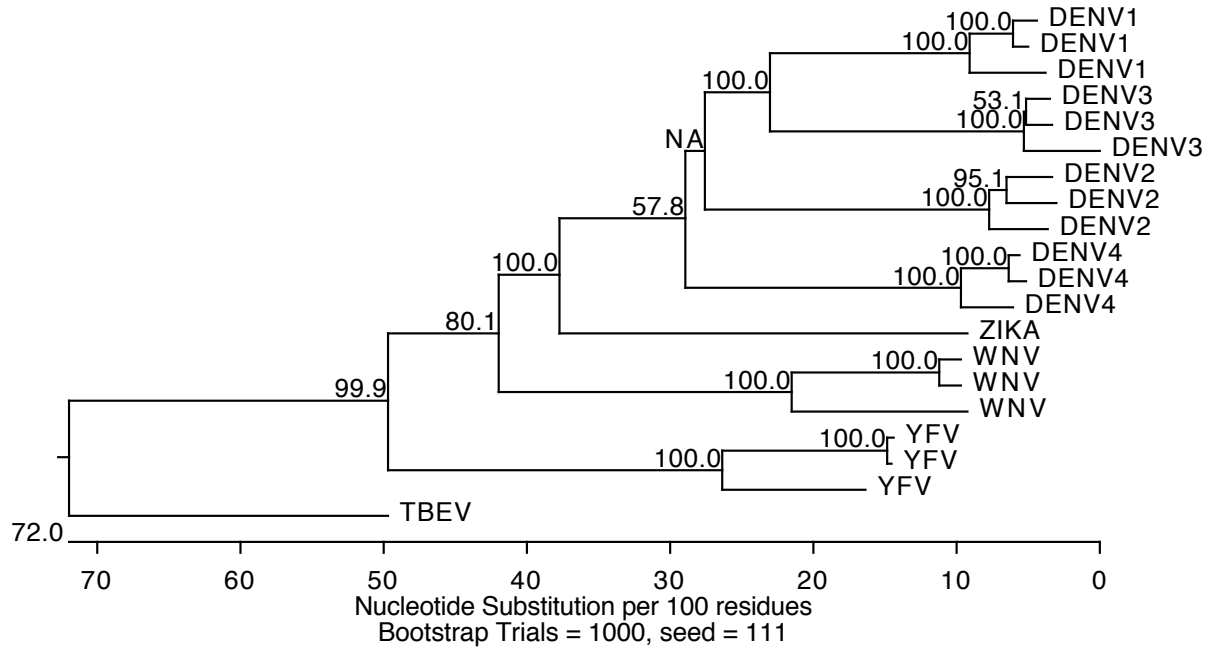
Figure 1. (A) Genome Organization of *Flaviviruses* (9). Composed of the structural proteins: capsid (C), premembrane (PrM), and envelope (E) proteins followed by enzymes utilized for virus replication and transmission (1). **(B)** *Flavivirus* phylogenetic tree of E protein nucleotide sequences accessed from GenBank. Clustal W alignment conducted in MegAlign, scale is percentage of genetic difference; Tick-Borne Encephalitis Virus (TBEV) is a tick-borne *Flavivirus*. **(C)** Highly conserved amino acid sequence of the E protein fusion loop, identical in Dengue viruses and Zika virus used in phylogenetic alignment. Highlighted are two amino acids (101W and 109G) mapped to be important in 1.6D and D11C binding (1).

A

5'NC	C	PrM	E	NS1	NS2A/B	NS3	NS4A/B	NS5	3'NC

B.



C.

97	98	99	100	**101**	102	103	104	105	106	107	108	**109**	110	111	112	113
V	D	R	G	**W**	G	N	G	C	G	L	F	**G**	K	G	S	L

Table 1. *Flaviviruses* used in experiments. Emphasis was placed on viruses with low passage in either mammalian or insect cell lines. Dengue virus 3 and 4, and Zika virus came from the Yale Arbovirus Research Unit collection. C6/36 are an *Aedes albopictus* mosquito cell line, Vero are an African green monkey kidney epithelial cell line.

Virus	**Location/ Year**	**Cell Passage**	**Strain**	**GenBank Accession no.**
Dengue type 2	Jamaica/1983	NA	1409	M20558.1
Dengue type 3	Thailand/1989	C6/36 (X2)	-	-
Dengue type 4	El Salvador/1989	C6/36 (X2)	-	-
Zika	Senegal/1987	Mosquito homogenate in Vero/ C6/36/ Vero (X2)	41519	HQ234501

Figure 2. Fluorescent Focus Unit Assays, microscopy images taken on Zeiss AXIO Imager.M1, 20X magnification. Examples of Dengue virus 2 and Zika virus foci in C6/36 cells compared to a control that was not infected with virus. Virus stained with primary antibody, Pierce™ FlaviVs Envelope Antibody (FE1), Thermo Scientific, which binds the *Flavivirus* envelope, and was effective for all viruses tested in our experiments. Secondary antibody purchased from Life Technologies, goat anti-mouse Alexa Fluor 488, was visualized with FITC. The cell nucleus was stained with ProLong® Gold antifade reagent with DAPI.

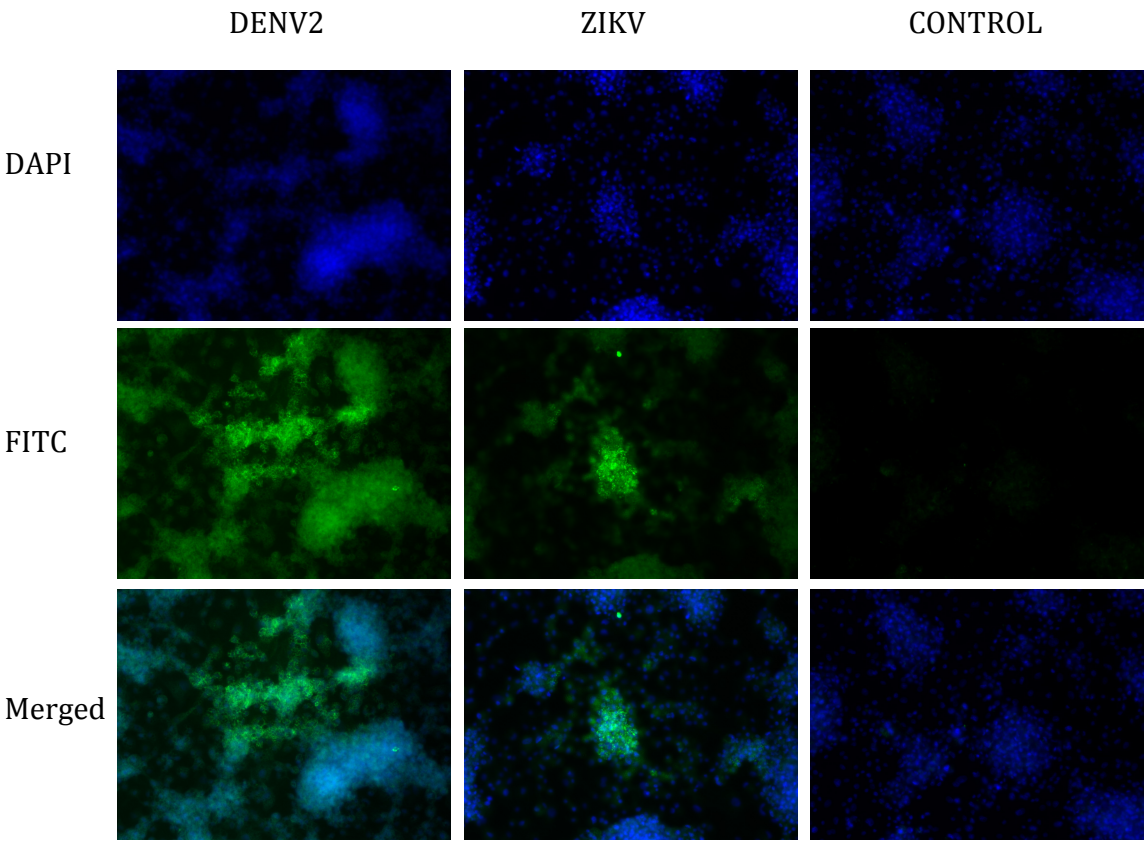
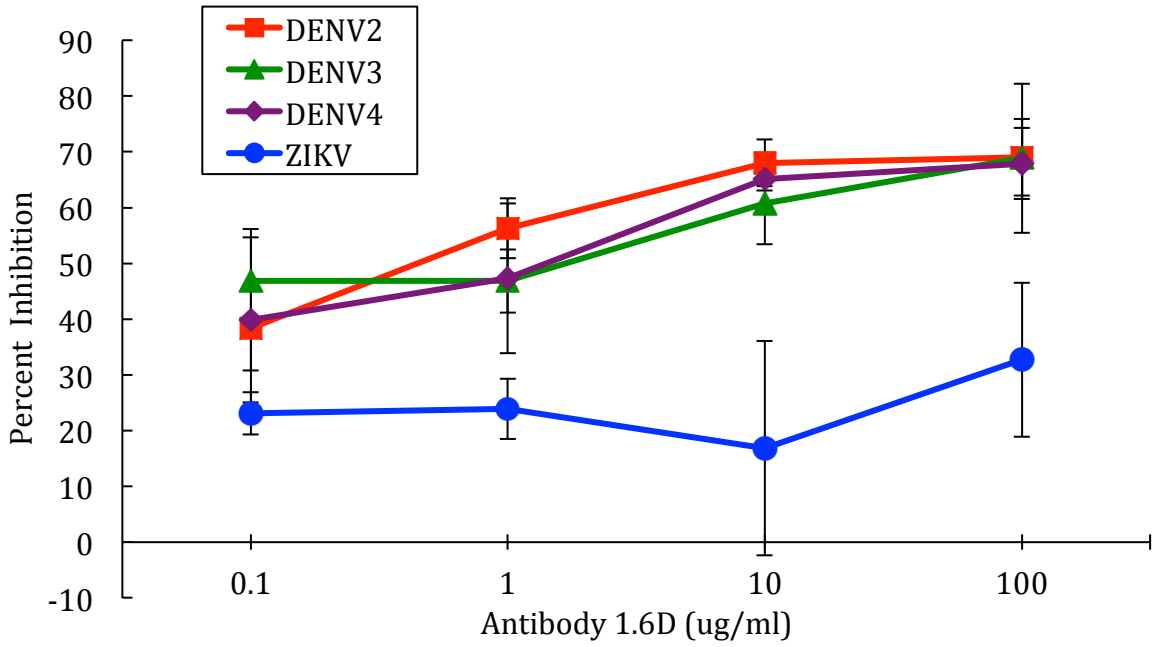
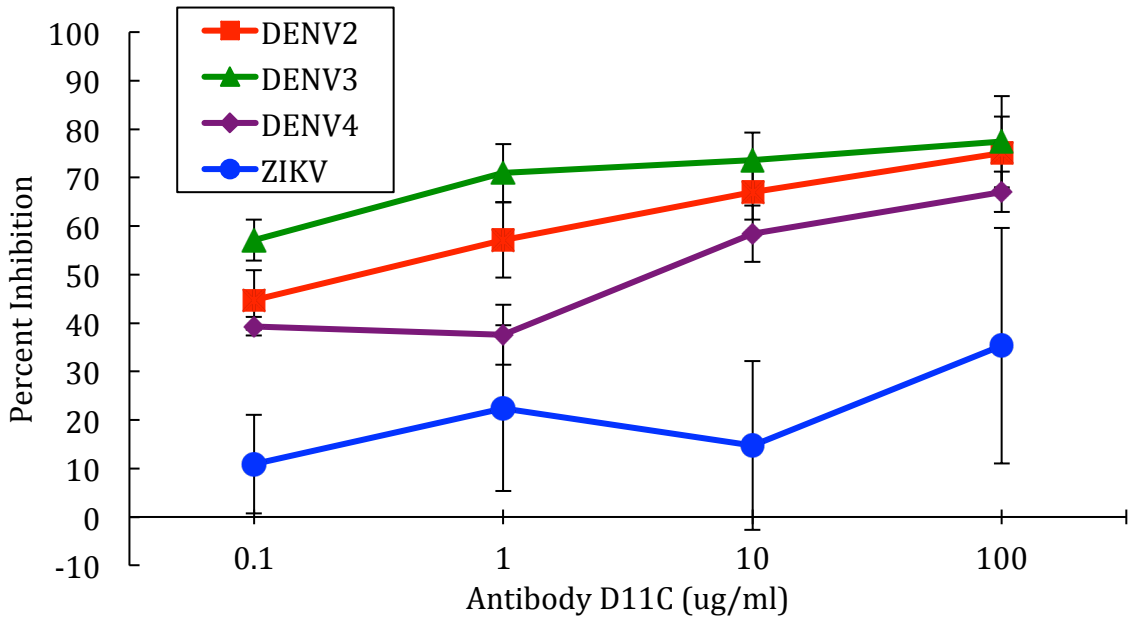


Figure 3. Dengue virus infection percent inhibition, fluorescent focus unit assays in C6/36 *Aedes albopictus* cells. **(A)** Antibody 1.6D. **(B)** Antibody D11C. The error bars signify standard deviations. **(C)** Linear regression best-fit model with log base 10 transformed antibody concentration. 50% inhibitory antibody concentration (IC50) calculated from the best-fit line presented as µg/ml.

A.



B

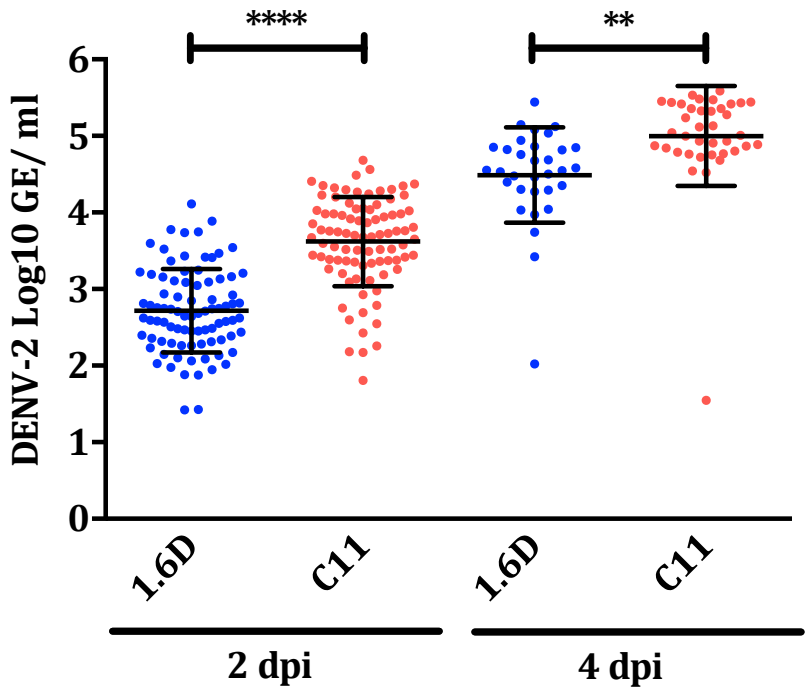


C.

Antibody	Virus	Linear Regression Best-fit line	IC50 (μg/ml)
1.6D	**DENV2**	Y = 10.36x + 52.74	0.77
	DENV3	Y = 8.00x + 51.82	0.80
	DENV4	Y = 10.21x + 49.95	1.00
	ZIKV	Y = 2.19x + 23.02	>1000.00
D11C	**DENV2**	Y = 10.09x + 55.94	0.56
	DENV3	Y = 6.36x + 66.58	0.07
	DENV4	Y = 10.40x + 45.40	1.56
	ZIKV	Y = 6.56x + 17.57	140.27

Figure 4. (A) Decreased DENV2 viral load with the DENV neutralizing antibody 1.6D compared to the C11 control antibody. Infection in *Aedes aegypti* mosquitoes carried out through 69nL microinjections of the thoracic cavity, 100 FFUs of DENV2 and 0.05μg antibody per injection. Mosquitoes sacrificed 2 days (five biological replicates of 10-20 mosquitoes per replicate) and 4 days (3 biological replicates of 7-20 mosquitoes per replicate) post infection (dpi). Mean and Standard Deviation (SD) of the log base 10 viral load (GE/ml) were calculated and compared for each day. P-values were calculated as two-tailed probabilities of a t distribution in SAS; Cochran method assumes unequal variance. **(B)** Table of Sample Sizes (N), Means, Standard Deviations (SD), Minimums, Maximums and P-values.

A.



B.

	Antibody	N	Mean ± SD	Minimum	Maximum	P-value
Day 2	**1.6D**	86	2.72 ± 0.54	1.43	4.11	<0.0001
	C11	89	3.62 ± 0.58	1.81	4.68	
Day 4	**1.6D**	32	4.49 ± 0.64	2.02	5.44	0.0020
	C11	38	5.00 ± 0.65	1.55	5.59	

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