Antibody Prophylaxis against Dengue Virus 2 Infection in Non-Human Primates

Monika Simmons,^{1*} Robert Putnak,² Peifang Sun,¹ Timothy Burgess,¹ and Wayne A. Marasco³

¹*Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, Maryland;* ²*Division of Viral Diseases, Walter Reed Army Institute of Research, Silver Spring, Maryland;* ³*Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts*

Abstract. Passive immunization with anti-dengue virus (DENV) immune serum globulin (ISG) or monoclonal antibodies (Mabs) may serve to supplement or replace vaccination for short-term dengue immune prophylaxis. In the present study, we sought to establish proof-of-concept by evaluating several DENV-neutralizing antibodies for their ability to protect rhesus macaques against viremia following live virus challenge, including human anti-dengue ISG, and a human Mab (Mab11/wt) and its genetically engineered variant (Mab11/mutFc) that is unable to bind to cells with Fc gamma receptors (FcγR) and potentiate antibody-dependent enhancement (ADE). In the first experiment, groups of animals received ISG or Mab11/wt at low doses (3–10 mg/kg) or a saline control followed by challenge with DENV-2 at day 10 or 30. After passive immunization, only low-titered circulating virus-neutralizing antibody titers were measured in both groups, which were undetectable by day 30. After challenge at day 10, a reduction in viremia duration compared with the control was seen only in the ISG group (75%). However, after a day 30 challenge, no reduction in viremia was observed in both immunized groups. In a second experiment to test the effect of higher antibody doses on short-term protection, groups received either ISG, Mab11/wt, Mab11/mutFc (each at 25 mg/kg) or saline followed by challenge with DENV-2 on day 10. Increased virus-neutralizing antibody titers were detected in all groups at day 5 postinjection, with geometric mean titers (GMTs) of 464 (ISG), 313 (Mab11/wt), and 309 (Mab11/mutFc). After challenge, there was complete protection against viremia in the group that received ISG, and a reduction in viremia duration of 89% and 83% in groups that received Mab11/wt and Mab11/mutFc, respectively. An *in vitro* ADE assay in Fcγ receptor-bearing K562 cells with sera collected immediately before challenge showed increased DENV-2 infection levels in the presence of both ISG and Mab11/wt, which peaked at a serum dilution of 1:90, but not in Mab11/mutFc containing sera. The results suggest that antibody prophylaxis for dengue might be beneficial in eliminating or reducing viral loads thereby minimizing disease progression. Our results also suggest that blocking FcγR interactions through Mab11 Fc engineering may further prevent ADE.

BACKGROUND

The dengue viruses (DENVs), which consist of four antigenically distinct serotypes, DENV 1, 2, 3, and 4, in the family *Flaviviridae* are estimated to cause up to 100 million symptomatic infections each year and 50,000 deaths due to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).^{1–3} There are currently no antiviral drugs or methods for dengue disease prophylaxis. Tetravalent live attenuated vaccine (TLAV) candidates currently in clinical trials may require three doses administered over 9–12 months to confer protective immunity.^{4–6} The leading TLAV (CYD; Sanofi Pasteur, Lyon, France), although only partially protective, is now licensed in several countries.⁷ There is uncertainty of its long-term efficacy as a higher incidence of hospitalization for DENV was observed in year 3 in regions of endemic disease among children younger than 9 years of age, indicating possible antibody-dependent enhancement (ADE) of infection, although the risk among children 2–16 years of age was lower in the vaccine group than in the control group. Arguably, a vaccine may not be an ideal prophylaxis strategy for travelers or military personnel deployed on short notice to dengue-endemic areas until longer follow-up studies showing their safety and efficacy are completed.

An alternative strategy is to use passively administered anti-DENV immune serum globulin (ISG) or monoclonal antibodies (Mabs) to protect at-risk individuals. Although ISG has

been used successfully against other pathogens (e.g., hepatitis, rabies) for postexposure prophylaxis, it has never been tried for the prevention of DENV infections.^{8–10} Nevertheless, passive antibody-mediated protection against dengue still faces significant safety concerns. A major concern is that it could lead to ADE of infection and increased risk for severe disease, DHF/DSS. This is thought to occur when anti-dengue antibodies at subneutralizing concentrations bind to virus thereby enabling infection of Fcγ receptor-bearing DENV target cells (i.e., monocytes, macrophages, dendritic cells, and other immune cells).^{11–13} In practice, this risk might be mitigated by using higher initial doses of ISG or repeated administration so as to maintain antibody titers at protective levels. Moreover, some human IgG subclasses have *in vivo* half-lives greater than 30 days, and passively administered antibodies have been shown to provide immunity against some viral infections for several months.¹⁴ In other examples, human Mab Fc engineering has been used to extend the *in vivo* half-lives¹⁵ and to decrease effector functions.^{16,17}

Passive antibody transfer could also be used to provide temporary or bridging immunity between the time a vaccine is first administered and active immunity is acquired. Haigwood and others¹⁸ found that simian immunodeficiency virus (SIV)-infected macaques that received polyclonal ISG with a high neutralizing antibody titer against SIV early in infection not only had significantly improved outcomes but also exhibited accelerated production of neutralizing antibodies compared with controls that did not receive SIV immune globulin. In another experiment, rhesus macaques challenged with simian/human immunodeficiency virus (HIV) after being given anti-HIV Mabs and ISG had viral loads 7 days postinfection that were 700-fold lower than those in non-antibody-treated controls.¹⁹ Peak viral replication rates in acute infection were also

*Address correspondence to Monika Simmons, Viral and Rickettsial Diseases Department, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500. E-mail: monika.simmons.civ@mail.mil

reduced by 25%, leading the authors to conclude that passively administered antibody early in infection leads to disease attenuation by reducing the initial viral load and the number of infected cells, which allows time for the adaptive immune response to better control the infection over the long term. There is also evidence that passively administered antibodies may prevent or ameliorate disease caused by other viral infections.^{20–22} For example, anti-Ebola virus (EBOV) antibodies administered to mice and EBOV hyperimmune goat serum administered to guinea pigs protect against lethal EBOV challenge, and DENV envelope (E), pre-membrane (prM), and nonstructural protein 1 (NS1) Mabs have been shown to protect mice against lethal DENV challenge.^{20,23–26} ISG pools are currently available for hepatitis B, respiratory syncytial virus, rabies, tetanus, cytomegalovirus, vaccinia, and varicella zoster.²⁷ Polyclonal ISG preparations have certain advantages in that they consist of diverse immunoglobulins (Igs) varying in subclasses, abundances, and viral epitope specificities, which enable them to have broad biological activity, as well as being relatively easy to produce given sufficient immune donors. However, the abundance of virus-specific antibody represents only a fraction of total serum globulin, so dosing must be based on the specific antibody titer against the antigen of interest. In contrast, Mab preparations have a single isotype/subclass and epitope specificity, and can be produced from hybridoma, mammalian, insect, or plant cells in virtually unlimited amounts. This may permit the use of lower doses of Ig; however, it might be necessary to combine individual Mabs into a “cocktail” to achieve maximum protective efficacy and target nonoverlapping epitopes to minimize selection of escape mutants. The inclusion of Mabs directed against epitopes resistant to neutralization escape may mitigate this risk.^{22,28}

In the studies presented here, we used a rhesus macaque DENV infection model, with viremia (presence of infectious virus in sera) as a surrogate for disease, to evaluate the protective efficacy of passively administered, pooled, human polyclonal anti-DENV ISG, and a recombinant human Mab, Mab11, directed against the West Nile virus fusion loop at the distal end of E domain II. Mab11 was previously described and was shown to cross-neutralize all four DENV serotypes, and protect mice against challenge with West Nile virus, DENV-2, and DENV-4.²⁹ In a follow-up experiment in rhesus macaques, we also included a genetically altered form of Mab11, that is, Mab11/mutFc, containing two leucine-to-alanine mutations in the CH2 domain. This mutation known as the “LALA” mutation, abolishes antibody binding to FcγR1, FcγR2, and FcγR3 thereby abrogating its ability to induce ADE via the Fcγ-receptor-mediated route.^{30,31} In this proof-of-concept study, we identified antibody preparations that eliminated or reduced the duration of viremia, providing a short window of protection when administered before DENV-2 challenge. We also demonstrated that only serum from Mab11/mutFc treated, but not from ISG or Mab11wt treated, macaques showed no ADE in an in vitro assay.

METHODS

Ethics statement. The research protocol using animals in this study was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center's Institutional Animal Care and Use Committee according

to the principles set forth in the *Guide for the Care and Use of Laboratory Animal Research*, Institute of Laboratory Animals, National Research Council, NIH Publication no. 92-3415 (protocol license numbers D005-10 and 13-IDD-40L). The experiments reported herein were conducted in compliance with all applicable Federal regulations governing the protection of animals in research. The procedures used in this study were non-painful.

For virus injection and blood collection, animals were sedated with ketamine/acepromazine (11 mg/kg)/(0.55 mg/kg) administered intramuscularly; if necessary, additional anesthesia was administered to achieve the desired level of sedation. All animals in this study weighed between 4 and 10 kg, and were housed individually in stainless steel squeeze-back cages, approximately 25" (w) × 27" (d) × 30" (h) in a study room with a 12-hour day/12-hour night cycle, 10 room air changes per hour, and negative pressure to the corridor. Cages were washed with a low-pressure water hose two times daily and sanitized once every 2 weeks. Animals were fed Old World Primate Chow (5038; Quality Lab Products, Elkridge, MD) twice daily, fresh fruit at least three times a week, and water ad lib. Animals received routine veterinary care in accordance with (IAW) WRAIR SOP UWN-622, VSP SOP UWN-626, and VSP SOP UWN-012 *Husbandry of Old World Non-human Primates*. Environmental Enrichment including TV, treats, and toys was provided IAW VSP SOP UWN-626, *Enrichment for Non-Human Primates*. Animals were observed daily by a veterinary technician IAW VSP SOP UWN-012, *Daily Animal Medical Rounds*. Treatment, if required, was administered by a study veterinarian after consultation with the principal investigator. The studies performed under this protocol were nonterminal.

Cells and viruses. African green monkey kidney cells (Vero 81) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cell culture supernatant harvested from Vero cells infected with DENV-2 (S16803) was used as virus stock for the plaque reduction neutralization test (PRNT) and to prepare enzyme-linked immunosorbent assay (ELISA) antigen. DENV-2 (S16803) challenge virus stock was low passaged, near wild-type virus grown in Vero cells.

Plaque assay and PRNT. Plaque assays were performed as previously described.³² PRNT modified from the method originally described,³³ was used for measuring DENV-neutralizing antibodies. In brief, Vero cell monolayers were seeded in six-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ) and incubated at 37°C in a 5% CO₂ incubator. Sera from immunized rhesus macaques were tested using serial 2-fold dilutions starting at 1:10 until a 50% plaque reduction endpoint was reached. The serum dilutions were mixed with DENV-2 to obtain approximately 50 plaque forming units (PFU) per 0.2 mL serum, incubated at 37°C for 30 minutes, then inoculated onto duplicate wells overlaid with nutrient agarose (eagle's minimal essential medium, 2% fetal bovine serum, 1% agarose). Plaques were visualized on day 4 by staining with 0.02% neutral red in Hank's balanced salt solution. The number of plaques reported for each serum dilution was the average of the duplicate wells. The percent reduction in plaques was calculated by comparison of the results obtained with control sera from unimmunized rhesus macaques. The neutralization titer was the test serum dilution at which 50% plaque reduction occurred (PRNT₅₀ titer) determined by probit analysis.

Enzyme-linked immunosorbent assay. Virus antigen was prepared by polyethylene glycol (PEG 8000) precipitation of DENV-2-infected and DENV-2-uninfected Vero cell supernatants. PEG-adsorbed virus preparations were centrifuged at 10,000 rpm for 30 minutes, and pellets were resuspended in tris-ethylenediaminetetraacetic acid. The partially purified virion and control antigens were stored at -80°C until use. The analysis of sera from immunized rhesus macaques for DENV-2 antibodies was carried out as previously described.³² In brief, microtiter plates were coated with DENV-2 virions in phosphate-buffered saline (PBS) at 4°C overnight followed by blocking with 5% nonfat dry milk in PBS/0.01% Tween 20 for 1 hour at 37°C . Plates were then incubated with the test sera at 2-fold serial dilutions starting at 1:100 in blocking buffer for 1 hour at 37°C . The secondary antibody was peroxidase-conjugated goat antihuman IgG (Kirkegaard and Perry, Gaithersburg, MD) diluted in blocking solution and incubated for 1 hour at 37°C . Plates were incubated with 2,2'-azino(3-ethylbenzthiazoline-6-sulfonate) peroxidase substrate system (Kirkegaard and Perry) to detect DENV-specific antibody binding. Net optical density (OD) values were determined by subtracting the absorbance of test serum with negative control antigen from the absorbance of test serum with DENV-2 antigen. Endpoint dilution titers were determined by the serum dilution at which the OD value was ≥ 0.10 .

ADE assay. An ADE assay was performed using Fc γ receptor-bearing cells K562 (ATCC), which are nonpermissive for direct DENV infection but permissive for antibody-dependent DENV infection as previously described.³⁴ Briefly, serum samples were serially diluted in culture medium in 96-well microtiter plates (Costar, Thermo Fisher Scientific, Rockville, MD). Of titrated serum samples, 30 μL was added in duplicate to a 96-well U-bottom plate (Costar). Wells containing only culture medium were used as a negative control. DENV-2 (S16803) at a previously determined dilution, which allows 50% infection in Raji-DC-SIGN cells within a 24-hour period, was added to the wells containing diluted serum and the control wells. The plate was then incubated for 30 minutes in a humidified 37°C 5% CO_2 incubator to allow for antibody-virus complex formation. K562 cells, harvested and washed once with culture medium, were counted using a cell counter (Nexcelom Cellometer Auto T4, Nexcelom Bioscience, Lawrence, MA) then adjusted to 2×10^6 cells/mL, and 60 μL of the cell suspension was added to each well of the 96-well plate. Plates were incubated in a 37°C , 5% CO_2 incubator for 20 hours to allow ADE to occur, then washed twice with PBS (Thermo Fisher Scientific) and fixed using a cell fixing solution (1 $\times$ Perm-Fix; Becton Dickinson Biosciences [BD], Lincoln Park, NJ) for 10 minutes at room temperature. Cells were washed with a cell permeabilization solution (Perm/Wash; BD) twice, and then stained with a fluorescein labeled anti-DENV Mab 2H2 for 30 minutes at room temperature. Cells were washed twice in 1 $\times$ Perm/Wash solution, and then PBS was added to each well. The samples were evaluated using a FACS array (BD), and the percentage of cells stained with 2H2 was measured and analyzed.

Viremia assay. The presence of virus in serum samples collected daily for 10 consecutive days after virus challenge was detected by amplification in Vero cell cultures followed by immunofluorescent antibody (IFA) assay on the cells.

Briefly, Vero cells were propagated in 25-cm² flasks (T25) with EMEM, 10% heat-inactivated FBS, and penicillin/streptomycin. Duplicate flasks were inoculated with 0.3 mL of a 1:2 dilution of each postchallenge serum sample and incubated at 37°C for 8 days. Cells from each flask were harvested by scraping, washed with PBS, and spotted in duplicate onto immunofluorescent slides. DENV-infected cells were detected by staining with anti-DENV-2 serotype-specific hyperimmune mouse ascitic fluid followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig. The cell fluorescence was scored as (+) or negative (−) compared with uninfected control cells. For the samples found positive by IFA, a fresh serum sample was used to quantify the amount of virus by direct plaque assay on Vero cell monolayers. Direct plaque assays were carried out as previously described.³⁵

Ig preparation. Total IgG was prepared from a human dengue hyperimmune plasma pool from blood samples collected for medical reasons from patients hospitalized at the Mahidol University Hospital, Bangkok, Thailand, in the 1970s, where individual plasma samples were combined to produce an anonymized plasma pool. No institutional review board approvals were required at the time the plasma was collected and the pool prepared. The plasma pools were heat inactivated and screened for the presence of neutralizing antibody to DENV by PRNT. For the purification of ISG, plasma was diluted 1:10 in Melon Gel purification buffer (Thermo Scientific, Rockford, IL) and applied to a column packed with gel support. The flow-through fractions were further purified with Protein A/G agarose (Thermo Scientific) per manufacturer's instructions. IgG containing fractions were pooled, concentrated, and dialyzed extensively in PBS. The concentration was determined using the Bradford assay (BioRad, Hercules, CA) and the purity was determined by nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to be $> 96\%$. The preparations were tested for the presence of endotoxin using Toxinsensor Chromogenic LAL Endotoxin Assay (Genscript, Piscataway, NJ). Endotoxin levels for the IgG used in the first and second animal studies were 0.09 and 0.072 EU per mg of protein, respectively. The Mab11wt Clone A-A9 for the first experiment was grown in Chinese hamster ovary (CHO) cells with serum-free media, concentrated and purified by Harlan Laboratories (Indianapolis, IN) with an endotoxin level of 0.05 EU/mg and purity of $> 96\%$ by SDS PAGE. The Fc γ receptor-mutated Mab11 (Mab11/mutFc) was grown in CHO cells, concentrated and purified by another commercial vendor SELEXIS (Plan-Les-Quates, Switzerland) with a reported endotoxin level of 0.033 EU/mg of protein and a purity of $> 98\%$ by SDS PAGE. Antigen binding and specificity of all preparations were evaluated by ELISA, western blot, and PRNT₅₀.³³ Table 1 shows PRNT₅₀ titers for all antibody preparations at their appropriate protein concentrations for both studies.

Animal passive immunization and virus challenge. For study 1 (low Ig doses), passive immunization experiments were carried out in 24 adult, flavivirus naive rhesus macaques (*Macaca mulatta*, Indian strain) randomly assigned to three groups of eight animal each using Ig doses of either 3 or 10 mg/kg. Antibody preparations in PBS were administered to the animals by intramuscular (IM) injection in the upper arm: group I (ISG, 3 mg/kg), group II (Mab11wt, 10 mg/kg),

TABLE 1
PRNT of purified DENV ISG, mosquito-borne flavivirus, and dengue cross-reactive human Mab11/wt and Mab11/mutFc

50% PRNT titers*				
Experiment 1				
Original concentration	Virus	ISG 11 mg/mL	Mab11/wt 20 mg/mL	
Dose administered	DEN-2	19456 3 mg/kg	24576 10 mg/kg	
Experiment 2				
Original concentration	Virus	ISG 69 mg/mL	Mab11/wt 78 mg/mL	Mab11/mutFc 65 mg/mL
Dose administered	DEN-2	60800 25 mg/kg	30400 25 mg/kg	30400 25 mg/kg

DENV = dengue virus; ISG = immune serum globulin; Mab11/mutFc = Fcγ receptor-mutated Mab11; Mab11/wt = monoclonal antibody (wild type); PRNT = plaque reduction neutralization titers. *PRNT titers are performed in Vero cells.

and group III (PBS, control). Blood was drawn for antibody measurements on days 0, 5, 10, 20, 30, 40, and 60. Ten days after antibody administration, four animals from each group were challenged with low Vero cell passaged DENV-2 (S16803), 1.3×10^5 PFU, administered subcutaneously in the skin of the upper back to measure short-term protection. The remaining four animals per group were similarly challenged on day 30 to measure intermediate-term protection. Following challenge, the animals were bled for 10 consecutive days, and sera were assayed for infectious virus (viremia) by amplification in Vero cell culture followed by detection of viral antigen by IFA. For study 2 (high Ig dose), 16 adult flavivirus naive rhesus macaques were randomly assigned to four groups of four animals each. Antibody preparations in PBS were administered at 25 mg/kg to the animals by IM injection in the upper arm. Groups were inoculated with ISG, Mab11/wt, Mab11/mutFc, or saline (control). Blood was drawn for antibody measurements on days 0, 5, 10, and 40. On day 10 postvaccination, all animals were challenged with low passage DENV-2 (S16803), 1.3×10^5 PFU, subcutaneously in the skin of the upper back to measure short-term protection. Sera from daily bleeds for 10 days after challenge were assayed for the presence of infectious virus as described. Because of a non-study-related incident at the animal facility, one animal in the ISG group had to be removed from the protocol after the first dose.

Statistics. Primary analysis included descriptive statistics of each animal's antibody responses (e.g., geometric mean and standard deviation). All titers were log transformed to

stabilize variance. Statistical tests were used in the data analysis; however, *P* values obtained were considered to represent descriptive measure of strength of evidence rather than formal statistical inference. Statistical significance was defined as *P* < 0.05.

RESULTS

Responses to passively administered antibody. In the first experiment, groups of rhesus macaques (*N* = 8/group) were passively immunized with the antibody preparations shown in Table 1 (uppermost rows) at 3 or 10 mg/kg or with a PBS control. Ten days after antibody administration, four animals from each group were challenged with a low passage, near wild-type strain of DENV-2 to measure short-term protection, and the remaining four animals in each group were challenged on day 30 to measure intermediate-term protection.

As shown in Figure 1A and B, the group that received the human Mab11 exhibited higher total serum IgG antibody titers measured by ELISA on day 5 than the group that received ISG, which correlated with the higher administered Ig dose for this group (10 mg/kg) compared with the ISG group (3 mg/kg). Antibodies were detectable by ELISA in both groups on day 10. Although both groups exhibited a moderate decline in ELISA titers through day 20, antibodies were still measurable at day 30 (Figure 1B). Neutralizing antibody titers, however, did not differ significantly between both groups through day 10, regardless of the difference in

FIGURE 1. Serum IgG antibody responses in rhesus macaques. Animals passively immunized with (low dose) purified dengue virus immune serum globulin (ISG), mosquito-borne flavivirus, and dengue cross-reactive human monoclonal antibody 11 wild type (Mab11/wt) evaluated in the enzyme-linked immunosorbent assay (ELISA) using purified virions. (A) Immunized animals challenged with live virus on day 10 and (B) challenged on day 30. Bars indicate geometric mean reciprocal serum dilution endpoint titers (> 0.1 optical density) by ELISA. Numbers above bars represent geometric mean 50% plaque reduction neutralization titers.

FIGURE 2. Serum IgG antibody responses in rhesus macaques. Animals were passively immunized with (high dose) purified dengue virus immune serum globulin (ISG), mosquito-borne flavivirus, dengue cross-reactive human monoclonal antibody 11 wild type (Mab11/wt) and Fc γ receptor-mutated Mab11/mutFc in the enzyme-linked immunosorbent assay (ELISA) using purified virions. Bars indicate geometric mean reciprocal serum dilution endpoint titers (> 0.1 optical density) by ELISA. Numbers above bars represent geometric mean 50% plaque reduction neutralization titers.

total anti-DENV IgG measured by ELISA. By day 20, only the ISG group had detectable neutralizing antibody with a PRNT₅₀ titer of 10, the assay cutoff, which declined to < 10 by day 30. By 30 days postchallenge, all groups developed high total and neutralizing antibody responses to the challenge virus.

A second experiment was performed to test higher antibody doses including a new Mab11 construct in which the Fc coding region of Mab11/wt was mutated to produce Mab11/mutFc, thereby rendering it incapable of participating in ADE of infection. In this study, 16 flavivirus naive animals were randomly assigned to four groups ($N = 4/\text{group}$), and inoculated with purified ISG, Mab11/wt, or Mab11/mutFc, each at a dose of 25 mg/kg. A control group received PBS only. Ten days after antibody administration, all animals were challenged with the same strain of DENV-2, and sera were collected daily for 10 days to measure viremia. The results (Figure 2) show that the higher administered antibody dose compared with the first experiment resulted in increased total antibody titers measured by ELISA as well as higher neutralizing antibody titers measured by PRNT₅₀ in all immunized groups on days 5 and 10. The highest neutralizing antibody GMTs were seen on day 5 in the ISG group (464), followed by Mab11/wt (313) and Mab11/mutFc (309). By the time of challenge on day 10, neutralizing antibody GMTs were still high but had declined by about 50%.

Responses to virus challenge. After the day 10 challenge in the low-dose experiment (Figure 1A and B), partial protection against viremia was seen only in animals passively immunized with ISG, with a 75% reduction compared with controls (Table 2). However, after the day 30 challenge (Figure 1B), none of the groups exhibited any reduction in viremia although the onset appeared to have been delayed in the group that received ISG (data not shown). By 30 days postchallenge, all groups had developed significant total and neutralizing antibody to the challenge virus (Figure 1A and B).

As shown in Table 3, after challenge in the high-dose experiment, the PBS control group exhibited 4.5 mean days of viremia with a peak titer of 2.34 log₁₀ PFU/mL. There was no detectable viremia in the group that received ISG. The Mab11/wt group exhibited a reduction in the duration of viremia of 89% versus the PBS control, with 1 day of viremia in two of the four animals (0.5 mean days), which could only be detected by virus amplification assay but not by direct plaque assay ($P = 0.001$). Similarly, the Mab11/mutFc group exhibited an 83% reduction in viremia duration with 1 day of viremia in three of our animals (0.75 mean days) and quantifiable virus titers by direct plaque assay in two animals ($P = 0.001$).

Thirty days after challenge, the Mab11/wt, Mab11/mutFc, and PBS control groups all exhibited greater than 4-fold increases in DENV-2 PRNT₅₀ titers consistent with seroconversion to the challenge virus (Figure 2). In contrast, PRNT₅₀ titers for the ISG group were changed little with a

TABLE 2
Viremia in rhesus monkeys 10 days after immunization with low-dose purified DENV ISG, mosquito-borne flavivirus, dengue cross-reactive human Mab11/wt

Group	Monkey	Days of viremia* after virus challenge										Mean days of viremia
		1	2	3	4	5	6	7	8	9	10	
ISG	DB1G	-	-	-	-	-	-	+	+	-	-	1.5
	07U012	-	-	-	-	+	+	-	-	-	-	
	6-221	-	-	-	-	+	+	-	-	-	-	
Mab11/wt	07U024	-	-	-	-	-	-	-	-	-	-	6.25
	DBE9	-	+	+	+	+	+	+	+	-	-	
	07U025	-	+	-	-	+	+	+	+	-	-	
	6-217	-	+	+	+	+	+	+	+	+	-	
Saline	7U021	-	+	+	-	+	+	+	+	-	-	6.0
	DBE4	-	+	+	+	+	+	+	-	-	-	
	07C021	-	+	+	+	+	+	-	-	-	-	
	07C022	-	-	+	+	+	+	+	+	-	-	
	6-187	+	+	+	+	+	+	+	-	-	-	

DENV = dengue virus; ISG = immune serum globulin; Mab11/wt = monoclonal antibody (wild type). Immunofluorescence antibody (IFA) assay results are presented as + or -. *Isolation of virus by amplification in Vero cells followed by IFA.

TABLE 3

Viremia in rhesus monkeys 10 days after immunization with high-dose purified DENV ISG, mosquito-borne flavivirus, and dengue cross-reactive human Mab11/wt and Mab11/mutFc

Group	Monkey	Days of viremia* after challenge										Mean days of viremia
		1	2	3	4	5	6	7	8	9	10	
ISG	08D247	—	—	—	—	—	—	—	—	—	—	0
	08U015	—	—	—	—	—	—	—	—	—	—	
	M226	—	—	—	—	—	—	—	—	—	—	
Mab11/wt	08D302	—	—	—	—	—	—	—	—	—	—	0.5
	08C105	—	—	—	—	—	—	—	+	—	—	
	09U011	—	—	—	—	—	—	—	+	—	—	
Mab11/mutFc	09U014	—	—	—	—	—	—	—	—	—	—	0.75
	08D278	—	—	—	—	—	—	—	—	—	—	
	08U020	—	—	—	—	—	—	—	+1.65	—	—	
Saline	08D301	—	—	—	—	—	—	—	+	—	—	4.5
	M233	—	—	—	—	—	—	—	+0.9	—	—	
	09U016	—	+2.34	+2.02	+1.35	+1.18	—	—	—	—	—	
	09U021	—	+1.78	+1.35	—	+	+1.48	+1.48	+1.18	—	—	4.5
	09U028	—	+1.48	+	—	+1.18	+1.35	—	+0.9	—	—	
	09U035	—	+1.88	+1.35	—	—	—	—	—	—	—	

DENV = dengue virus; ISG = immune serum globulin; Mab11/mutFc = Fcγ receptor–mutated Mab11; Mab11/wt = monoclonal antibody (wild type). Results are presented as + or – (immunofluorescence antibody [IFA])/results on direct plaque assay of virus from serum (as log₁₀ PFU/mL).

*Isolation of virus by amplification in Vero cells followed by IFA assay.

GMT of 265 on the day of challenge versus a GMT of 166 30 days later, which suggests a state of sterile immunity. While total IgG titers measured by ELISA were maintained for the Mab11/wt and Mab11/mutFc groups, titers for the ISG group exhibited a significant decline ($P < 0.00001$).

Antibody-dependent infection enhancement. To determine if the passively transferred antibodies could potentiate ADE of infection, an in vitro viral enhancement assay was performed by preincubating equivalent Vero cell PFU of DENV-2 with serially diluted rhesus sera collected on day 10 immediately before challenge and then adding it to Fcγ-bearing K562 target cells. As shown in Figure 3, ADE peaked at a serum dilution of 1:90 for the ISG and Mab11/wt groups with 10% and 4% infection rates, respectively. In contrast, the

infection rate for both the Mab11/mutFc and PBS control groups was $< 0.25\%$ (Figure 3).

DISCUSSION

Our results demonstrate that anti-DENV antibodies administered intramuscularly to rhesus macaques conferred short-term protection against viremia following DENV-2 challenge. The best protection was seen with pooled, human polyclonal anti-dengue ISG. In the first experiment, an ISG dose of 3 mg/kg resulted in a moderate prechallenge neutralizing antibody GMT of 16 and a 75% reduction in the duration of viremia after challenge on day 10, but by day 30 the GMT had fallen to < 10 , and there was no reduction in viremia

FIGURE 3. Antibody-dependent enhancement. Infection enhancement of K562 cells with dengue virus 2 (DENV-2) in the presence of serially diluted sera (day of challenge) from animals passively immunized with 25 mg/kg purified DENV immune serum globulin (ISG), mosquito-borne flavivirus, dengue cross-reactive human monoclonal antibody 11 wild type (Mab11/wt) and Fcγ receptor–mutated Mab11/mutFc. Infected K562 cells were detected by intracellular immunofluorescent staining with anti-dengue Mab 2H2 20 hours postinfection. The percent infection of K562 cells is shown. Background staining of cells infected with DENV-2 in the absence of serum was subtracted. Error bars represent 1 standard deviation.

compared with PBS controls. Therefore, not unexpected, protection with ISG appeared to correlate with measurable virus-neutralizing antibody on the day of challenge. Interestingly, however, no protection was seen in the group that received a 10 mg/kg dose of Mab11/wt even though the group still had neutralizing antibody GMTs of 19 at the time of challenge.

In a second experiment, an ISG dose of 25 mg/kg conferred complete protection against day 10 challenge (prechallenge GMT 265), as demonstrated by the absence of both viremia and seroconversion to the challenge virus (i.e., sterile immunity). Partial protection (> 80% reduction in duration of viremia) was also seen in this experiment with the human pan-flavivirus neutralizing Mab, Mab11/wt, and its mutated variant, Mab11/mutFc, administered at a dose of 25 mg/kg (prechallenge GMTs of 171 and 155, respectively).

In the first experiment, total serum antibody levels of ISG and Mab11/wt declined gradually between days 5 and 10, with less than a 2-fold decrease in ELISA titer through day 20. Similar to ELISA titers, serum-neutralizing antibody GMTs were low in the first experiment but higher in the second consistent with a higher administered antibody dose. Although a day 30 challenge was not performed in the second experiment, assuming approximately linear antibody decay kinetics, it is possible that we would have seen some residual protection beyond day 10, particularly in the ISG group, which exhibited partial protection with a day of challenge GMT of only 16 in the first experiment (see above).

We previously reported that virus-neutralizing antibodies as well as total antibody titers and high antibody avidity are correlates of protection against DENV challenge in rhesus macaques vaccinated with tetravalent DENV vaccines.^{36,37} The present studies also indicate the importance of measurable antibodies, especially virus-neutralizing antibodies, on the day of challenge. This is especially relevant in the case of passive protection, since there is no immunological memory to enable the host to mount a rapid anamnestic response to the infecting virus. Before passive protection can be a safe and effective strategy for dengue prophylaxis, methods for maintaining antibody at protective levels must be demonstrated, such as repeat immunizations or higher initial dosages as suggested earlier. This is especially important for preventing ADE of infection as protective antibody titers decline. Although we saw no evidence for ADE as demonstrated by longer viremia durations or higher virus titers in passively immunized animals versus saline controls, we could not rule it out because of the breakthrough viremia seen in the first experiment. These results prompted us to evaluate a genetically modified version of Mab11/wt (Mab11/mutFc) that is unable to bind Fc γ receptors and potentiate ADE. An *in vitro* ADE assay in Fc γ receptor-bearing K562 cells demonstrated that Mab11/mutFc did not increase the number of infected cells above background. In contrast, both Mab11/wt and ISG enhanced viral infection rates by about 20- to 50-fold, respectively, at a serum dilution of 1:90. Nevertheless, the results of virus challenge in rhesus macaques showed that both Mab11/wt and Mab11/mutFc, administered at 25 mg/kg, induced partial protection at similar levels against DENV-2 viremia.

As was discussed earlier, in our experiments ISG was found to be more protective overall than Mabs at similar doses and serum antibody levels, although the reasons for

this are not clear. It is possible that Mabs are less efficient than polyclonal ISG at neutralizing virus *in vivo*, perhaps due to differences in the number and variety of epitopes targeted, the stoichiometry of virus neutralization, antibody avidity, or antibody effector functions. The possibility for selection of antibody escape variants must also be considered with Mabs unless they target highly conserved epitopes required structurally or functionally by the virus. Indeed, Mab11 is a pan-reactive Mab against a highly conserved epitope expressed on E protein of many mosquito-borne flaviviruses. In addition, an escape mutant of West Nile virus from Mab11 showed marked attenuation of neuropathology and viral fitness *in vitro* and *in vivo*.³⁰ To determine whether DENV-2 neutralization escape variants generated after virus challenge could have been responsible for the breakthrough viremia seen in our first experiment, viremic virus isolated from animals passively immunized with Mab11/wt was compared with the original challenge virus using PRNT. Both viruses exhibited similar neutralization profiles against Mab11, which argues against an antibody escape mutant. Engineering Mab11 to be even higher affinity may be another way to further improve its *in vivo* performance. For Mab-based therapeutics, a cocktail of Mabs targeting at least two non-overlapping epitopes, which neutralizes all four serotypes of DENV while failing to enhance infection may provide an alternative for the prevention or treatment of dengue.

In summary, this study demonstrated the potential utility of passive immunization for protection against dengue. Complete protection against live viral challenge was observed in nonhuman primates vaccinated with ISG. It was also shown that reduction in viral load could be achieved with a human Mab containing a genetically modified Fc portion, potentially attenuating disease progression and mitigating risk for ADE of infection. In addition, removing the Fc portion of antibodies in the ISG may lead to a safer product to afford short-term protection against dengue infection, which will be studied in a future experiment. The availability of monoclonal or polyclonal antibody preparations able to significantly reduce viremia without participating in ADE of infection would greatly improve the safety and feasibility of antibody therapy against DENV infection.

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Authors' addresses: Monika Simmons and Peifang Sun, Naval Medical Research Center (NMRC), Silver Spring, MD, E-mails: monika.simmons.civ@mail.mil and peifang.sun2.ctr@mail.mil. Robert Putnak, Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD, E-mail: j.r.putnak.ctr@mail.mil. Timothy Burgess, Infectious Diseases Service, Walter Reed National Military Medical Center (WRNMMC), Bethesda, MD, E-mail: timothy.burgess@usuh.edu. Wayne A. Marasco, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, E-mail: wayne_Marasco@dfci.harvard.edu.

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