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Bispecific Monoclonal Antibodies Mediate Binding of Dengue Virus to Erythrocytes in a Monkey Model of Passive Viremia

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Dengue viruses (DEN), causative agents of dengue fever (DF) and more severe dengue hemorrhagic fever (DHF)/dengue shock syndrome, infect over 100 million people every year. Among those infected, up to one-half million people develop DHF, which requires an extensive hospital stay. Recent reports indicate that there is a significant correlation between virus titer in the bloodstream of infected individuals and the severity of the disease, especially the development of DHF. This suggests that if there is a procedure to reduce viremia in infected subjects, then the severity of the disease may be controlled during the critical early stages of the disease before it progresses to DHF. We have generated bispecific mAb complexes (heteropolymer(s), HP), which contain a mAb specific for the DEN envelope glycoprotein cross-linked with a second mAb specific for the primate E complement receptor 1. These HP facilitate rapid binding of DEN to human and monkey E in vitro, with ~90% bound within 5 min. Furthermore, in a passive viremia monkey model established by continuous steady state infusion of DEN, injection of HP during the steady state promoted rapid binding of DEN to the E, followed by subsequent clearance from the vascular system. Moreover, HP previously infused into the circulation is capable of efficiently capturing a subsequent challenge dose of DEN and binding it to E. These data suggest that HP potentially can be useful for alleviating DEN infection-associated symptoms by reducing titers of free virus in the vascular system. The Journal of Immunology, 2001, 166: 1057–1065.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by one of four closely related, but antigenically distinct, dengue virus (DEN) serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) of the genus flavivirus family Flaviviridae. Infection with one of these serotypes does not provide lasting cross-protective immunity. In addition, preexisting Ab titers against different DEN serotypes can enhance the severity of the disease during subsequent exposure to different serotypes (5–7). Dengue is primarily an urban disease of the tropics, and the viruses that cause it are mainly maintained in a cycle that involves humans and mosquito vectors, Aedes aegypti and Aedes albopictus, domestic day-biting mosquitoes that prefer to feed on humans (8–10). Infection with DEN can produce a spectrum of clinical illness, ranging from a nonspecific viral syndrome to severe and fatal hemorrhagic disease. Important risk factors for DHF include the strain and serotype of the virus involved, as well as the age, immune status, and genetic predisposition of the patient (4, 11, 12).

Currently, DF/DHF is the most important mosquito-borne viral disease affecting humans; its global distribution is comparable with that of malaria, and an estimated 2.5 billion people are living in areas at risk for epidemic transmission (3, 13–17). Each year, tens of millions of cases of DF occur along with hundreds of thousands of cases of DHF. The case-fatality rate of DHF in most countries is about 5%; most fatal cases are among children. Furthermore, the southern part of the United States is experiencing increased numbers of DEN infections in recent years, especially in Texas and Florida (18). In 1999, the Texas Department of Health confirmed 51 cases of DF with one fatality due to the DHF (19, 20).

Recent clinical investigations involving a prospective study in Thailand found that increased dengue disease severity correlated with high levels of viremia, secondary DEN infection, and the DEN-2 virus type (21). This study reported that the maximum virus titer increased 9-fold from DF to DHF in DEN-2 infection and another 7-fold from DHF to more severe grade 3 DHF (dengue shock syndrome). Furthermore, the disease severity correlated not only with the maximum titer of virus in the bloodstream, but also with the duration of high titer viremia during disease progression. These data suggest that if DEN infection can be diagnosed rapidly and the viral load subsequently reduced, then it should be possible to alleviate disease severity before an individual develops DHF.

In previous investigations, we have reported that it is possible to use cross-linked bispecific mAb complexes (heteropolymer(s), HP) to bind prototype pathogens to primate E and to facilitate the clearance of these substrates from the circulation in monkey models without E destruction (22–24). The HP are composed of a mAb, specific for the primate E complement receptor 1 (CR1), which is covalently linked to a second mAb specific for the target pathogen (22, 25, 26). In this study, we report that HP specific for the envelope glycoprotein of DEN-2 (anti-CR1 × anti-DEN-2) can effectively bind this virus to primate E in vitro and can substantially increase the amount of virus bound to E in the circulation of cynomolgus monkeys (n = 6) in a passive model of DEN-2 viremia. After DEN-2 is bound to E via HP, the majority of the E-bound virus (≥90%) is cleared from the circulation in 2 h or less.
Materials and Methods

Cells, virus, and Abs

A. albopictus larva cell line C6/36, CV-1. Baby Chinese hamster kidney fibroblast (BHK-21, clone 13), African green monkey kidney fibroblast (CV-1), and hybridoma lines 3H5 (IgG1) and 4G2 (IgG2a), specific for DEN-2 (27–29), were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained according to the ATCC specification. Anti DEN-2 mAb 9D12 was kindly provided by Dr. Alan King (U.S. Army Medical Research Institute of Infectious Diseases) (29). Anti-CR1 mAbs 7G9 (IgG2a), HB8592 (IgG1), and 9H3 (IgG1) have been previously reported (30, 31), and the irrelevant control mAbs (7B7, anti-dx174, IgG2a (22); 7C12, anti-C3b, IgG1; 23D1, anti-DNP, IgG1 (32) have been described previously. DEN (type 2) live attenuated virus strain PR-159 (S1), taken directly from Lot 1 of the vaccine prepared by the Department of Biopolitics Research, Walter Reed Army Institute of Research, and dated January 1976 (33), was kindly provided by Dr. J. Strauss (Cal Tech, Pasadena, CA). The virus was passed twice in C6/36 cells at low multiplicity of infection of <0.1 PFU/cell and as the infectious inoculum for all virion propagation (34). For generation of large quantities of viruses, 10–20 150-cm² flasks of subconfluent C6/36 cells were infected at low multiplicity of infection (0.01 PFU/cell), and culture fluids were harvested following 5 days of incubation at 27°C with two media changes and one culture dilution. Selected cultures were radiolabeled by adding [35S]methionine (1 μCi/ml; ICN, Irvine, CA) at 5 days postinfection. Infected culture fluids were clarified by centrifugation at 3000 × g for 30 min, and virus was concentrated by precipitation using polyethylene glycol and additional NaCl. 35S-labeled virions were further purified by potassium bromide-activated Sepharose 4B following the manufacturer’s instructions.

Preliminary in vitro binding experiments were performed with the 35S-labeled DEN preparation after it was purified by gradient centrifugation and titered. We used this reagent because it allowed for a relatively simple initial screen for mAb binding (immunoprecipitation with mAb-coupled Sepharose) and for HP-mediated binding of DEN to E. However, the particle to infectious unit ratio of the 35S-labeled DEN was higher than the ratio determined for nonradioactive samples that were simply purified from culture supernatants by removal of cell debris and precipitation with polyethylene glycol. In addition, approximately one-third of the radioactive counts in this preparation could not be immunoprecipitated by specific mAbs (see below). This fraction may represent denatured protein or adsorbed radioactive impurities. Therefore, most in vitro binding assays as well as the passive infusion experiments in monkeys used a less pure but highly infectious nonradioactive DEN preparation that was isolated by low speed clarification and concentration by polyethylene glycol. This preparation had a particle to infectious unit ratio of ~3.

Generation of anti-DEN × anti-CR1 HP

HP were prepared using chemical cross-linking procedures, as previously reported (23, 36, 37). In all cases, the HP were purified by FPLC to eliminate high m.w. material. All mAbs and HPs used are described in Table I.

In vitro binding of DEN to Sepharose 4B-conjugated mAbs

mAbs 9D12, 3H5, 7B7, and 23D1 were covalently coupled to cyanogen bromide-activated Sepharose 4B following the manufacturer’s instructions (Pharmacia, Piscataway, NJ). The Sepharose-conjugated mAbs were washed with PBS several times, diluted to a mAb concentration of 100 μg/ml, and mixed with 2.5–5 × 109 particles of 35S DEN (~500–1000 cpm) in a 100-μl vol. Samples were incubated at 37°C for 5–20 min in the presence of either 10% plasma or 1% BSA, and supernatants and pellets were separated by centrifugation at 1000 × g for 5 min. Pellets were washed twice with PBS. Both supernatants and pellets were assayed for 35S using scintillation counting.

In vitro binding of DEN to E via HPs

The in vitro HP-mediated binding of DEN to E via HP was studied using two different protocols. First, E were charged with HP before exposure to DEN (“franked” (38)). Charging was performed by adding a 5-fold molar excess of HP to the E (calculated based on the number of CR1 epitopes per E), followed by a 15-min incubation and two washes with PBS. Subsequently, ~1 × 1010 franked E resuspended in homologous plasma (containing EDTA) were incubated with 2–5 × 1010 particles of DEN in a final volume of 30 μl for a period of 5–20 min at 37°C. The sample was then pelleted, and both the supernatant and cell pellet were examined for DEN particles by competitive RT-PCR (see below), or in some cases, the amount of 35S in the supernatants was determined by scintillation counting. In a second pass protocol, the resulting supernatant was incubated with fresh franked E, and the sample was processed again. Alternatively, to measure solution phase binding, ~1 × 1010 E were mixed with 2–5 × 1010 particles of DEN in a final volume of 30 μl and then 0.2- to 0.5-fold molar ratio of HP to E epitopes was added, and the samples were similarly processed.

Ex vivo whole blood cell fractionation after DEN binding to cells via HP

Fresh whole blood was obtained from a monkey (~2500 CR1/E, see below) and after anticoagulation in EDTA, 90 μl were incubated with 3 × 1010 particles of DEN and 174 ng of the 7G9 × 9D12 HP to give a HP to E CR1 molar ratio of ~1/2. After a 15-min incubation at 37°C, the sample were chilled to 4°C and overlayed on 400 μl of 60% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS (d = 1.090) and centrifuged for 10 min at 700 × g. Under these conditions (32, 39–41), more than 99.9% of E were pelleted through the Percoll, and almost all of the leukocytes were retained at the interface. Alternatively, to measure in vitro HP-mediated binding of DEN to E via HPs was studied using

Table I. mAbs and HPs used in this study

<table>
<thead>
<tr>
<th>mAb</th>
<th>Ag</th>
<th>Ig IsoType</th>
<th>HP</th>
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<tbody>
<tr>
<td>7G9</td>
<td>CR1</td>
<td>IgG 2a</td>
<td>7G9 × 9D12</td>
</tr>
<tr>
<td>HB8592</td>
<td>CR1</td>
<td>IgG 1</td>
<td>HB8592 × 9D12</td>
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<tr>
<td>9H3</td>
<td>CR1</td>
<td>IgG 1</td>
<td>9H3 × 9D12</td>
</tr>
<tr>
<td>9D12</td>
<td>DEN envelope</td>
<td>IgG 1</td>
<td>7G9 × 4G2</td>
</tr>
<tr>
<td>4G2</td>
<td>DEN envelope</td>
<td>IgG 2a</td>
<td>7G9 × 3H5</td>
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<tr>
<td>3H5</td>
<td>DEN envelope</td>
<td>IgG 1</td>
<td>HB8592 × 3H5</td>
</tr>
<tr>
<td>7B7</td>
<td>dx174</td>
<td>IgG 2a</td>
<td>7G9 × 7B7</td>
</tr>
<tr>
<td>23D1</td>
<td>DNP</td>
<td>IgG 1</td>
<td></td>
</tr>
<tr>
<td>7C12</td>
<td>C3b</td>
<td>IgG 1</td>
<td></td>
</tr>
</tbody>
</table>

Virus particle quantitation by RT-PCR

DEN RNAs were isolated from samples (both in vitro and in vivo) containing DEN particle using RNA-STAT (Tel-Test, Friendswood, TX) solution, followed by ethanol precipitation in the presence of 20 μg carrier RNA, and resuspended in 20–50 μl RNA-free Tris-EDTA buffer. RT-PCR of DEN RNA was performed in the presence of defined amounts of cDNA, 250–500 ng of each primer set for 35S primer set, and in the presence of 10–5 copies of competitor RNA in the same tube. For each RNA sample, at least three to four RT-PCR were performed in the presence of different amounts of competitor RNAs varying over a 100-fold range. The PCR...
condition was 95°C for 30 s, 58°C for 45 s, and 72°C for 40 cycles, followed by 10-min extension at 72°C. This is an optimized condition for the primer set we used (see above for sequences), and the detection limit for this condition was 25 RNA copies per reaction. Resulting RT-PCR products were separated by agarose gel electrophoresis using an agarose gel containing a mixture of 2% biogel (Bio 101, Vista, CA) and 0.5% LE agarose (FMC Bioproducts, Rockland, ME) to maximize separation between 225 and 308 bp. DNA bands were visualized by ethidium bromide staining, and the ratio between PCR product bands of competitor and viral RNA was examined visually as well as by using a densitometer.

\textbf{In vivo reduction of passive DEN viremia by HP injection}

Experiments were performed in cynomolgus macaques (\textit{Macaca fascicularis}) having E CR1 levels (defined by RIA with mAb 7G9) in the range of 1600–5000 epitopes per E. E CR1 levels are relatively stable in healthy humans and nonhuman primates (37, 42, 43), and E CR1 was determined on each monkey within less than 1 mo of the experiment. In most cases, we measured E CR1 levels on separate blood samples taken from a monkey two or more times over a period of 4 mo or more, and the value remained constant. All monkeys were tested for seroconversion when they were arrived and were first quarantined at the University of Virginia animal facility. All monkeys were seronegative for SIV, seropositive for rhesus CMV, all except Cy-2 were seronegative for simian retroviruses, and all except Cy-6 were seropositive for monkey herpes B virus. Important details of each experiment and pertinent information about each animal are listed in Table II. On the day of the experiment, the monkey was anesthetized with ketamine 10 mg/kg i.m., administered atropine 0.05 mg/kg, and intubated under sodium pentothal i.v. as needed. Anesthesia was maintained using isoflurane and oxygen. Both i.v. and intraarterial catheters were placed. Blood pressure, heart rate, respiratory rate, body temperature, and oxygen saturation were monitored continuously, and stability was established for at least 15 min before any reagents were infused. Lactated Ringers Solution was infused i.v. at a rate of 10 ml/kg/h to maintain hydration. A high titer DEN stock was diluted in saline solution containing 10% monkey plasma and infused i.v. at a rate of 5–6 ml/h, and HP was infused at defined time points(s), as described in Table II. All infusion experiments used aliquots (stored frozen at −80°C) of a single DEN preparation. At designated time points (marked with symbols in each figure, including two independent samples taken before each experiment as controls), blood was drawn and placed into tubes (anticoagulated with a final concentration of 10 mM EDTA) and immediately placed on ice. At the end of the experiments, all animals were humanely euthanized under anesthesia. All animal experiments were supervised by a qualified veterinarian and in accordance with approved protocols by the University of Virginia Animal Care and Use Committee and the Institutional Biosafety Committee.

\textbf{DEN RNA preparation and quantitation from blood samples}

Blood samples were processed by centrifugation at 1000 \times g for 5 min, and the plasma was separated and aliquoted and used for RNA isolation, as described above. Cell pellets were washed three times with PBS containing 1 mM EDTA and aliquoted and used for RNA isolation. In some experiments (see Fig. 4A below), after three washes, the cell pellets were treated with 0.5 M ammonium chloride to lyse the E, and after centrifugation the supernatants were collected to obtain virion particles associated with E (44). All samples were stored at −70°C until quantitation by competitive RT-PCR. Each time point sample (plasma, total cells, E) was examined in the presence of at least three different defined copy numbers of competitor RNA ranging over a range of 100–1000-fold. Each sample was examined (as a set) at least twice with RT-PCR independently, and two or three independent RNA isolations followed by quantitative RT-PCR were performed for each time point sample (both plasma and cells). The lowest copy number of competitor RNA without addition of viral RNA as the RNA source for RT-PCR was used as a control to detect potential viral RNA contamination in each set of RT-PCR. A typical monkey experiment contains data collected from between 1000 and 1500 RT-PCR.

\textbf{Results}

\textit{Binding of 35S-labeled DEN to anti-DEN Abs or HPs in vitro}

We first sought to examine the ability of the anti-DEN mAbs to bind the virus. DEN virion particles were prepared from infected C6/36 cells that were continuously labeled with 35S during the last 2 days of infection. Radiolabeled enriched virion particles free of cell debris were further purified by a tartarate/glycerol step gradient centrifugation followed by high speed centrifugation to precipitate virion particles. Aliquots of fractions isolated from the gradient were titered by plaque assay and RT-PCR, and the active fraction was used to identify DEN.

We used the radiolabeled virions to measure the ability of Sepharose 4B-conjugated anti-DEN mAbs 9D12 and 3H5 to bind to DEN-2. DEN-2 in 35% plasma was incubated with the Sepharose 4B-conjugated mAbs for 5 min and then pelleted at 1000 \times g for 5 min. Both supernatants and pellets were measured for radioactivity to determine the efficiency of binding of DEN-2 to Sepharose 4B-conjugated anti-DEN mAbs. As shown in Fig. 1A, conjugates specific for DEN showed higher radioactivity in the pellet when compared with Sepharose 4B alone or an irrelevant mAb (7B7) conjugate. A similar result was obtained when the experiment was conducted with another irrelevant mAb (23D1) conjugate (data not shown). However, there was some nonspecific binding to the Sepharose beads and, in addition, up to one-third of the total label remained in the supernatant. Additional incubation with DEN-specific Sepharose-mAb conjugates failed to increase specific binding. This suggests that up to one-third of the total counts may not be recognized by the specific anti-DEN mAb, and such counts may represent either partially denatured DEN particles (e.g., containing altered epitopes) or perhaps non-DEN radiolabeled contamination. Despite these uncertainties, the data suggest that both anti-DEN mAbs can efficiently form immune complexes with DEN particles sufficient to bind and immunoprecipitate DEN from the plasma.

We next examined the ability of E to bind DEN in the plasma via HPs. For these experiments, we either preincubated HP with E (franked; see Materials and Methods) or coincubated HP, E, and DEN together (solution phase) in plasma for 5 min. Due to the E

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Weight (kg)</th>
<th>CR1/E</th>
<th>HP Infused, μg/kg</th>
<th>Estimated % CR1 Occupied</th>
<th>DEN Particles Infused</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy-1</td>
<td>4.00</td>
<td>3000</td>
<td>180</td>
<td>40</td>
<td>1.50 \times 10^9 particles(e)</td>
<td>4A</td>
</tr>
<tr>
<td>Cy-2</td>
<td>2.87</td>
<td>5089</td>
<td>188</td>
<td>24</td>
<td>1.13 \times 10^9 particles(e)</td>
<td>4B</td>
</tr>
<tr>
<td>Cy-3</td>
<td>2.85</td>
<td>2579</td>
<td>214</td>
<td>53</td>
<td>1.25 \times 10^9 particles(e)</td>
<td>4C</td>
</tr>
<tr>
<td>Cy-4</td>
<td>3.10</td>
<td>1661</td>
<td>210</td>
<td>78</td>
<td>1.25 \times 10^9 particles(e)</td>
<td>5A</td>
</tr>
<tr>
<td>Cy-5</td>
<td>4.42</td>
<td>2575</td>
<td>201</td>
<td>50</td>
<td>1.79 \times 10^9 particles(e)</td>
<td>5B</td>
</tr>
<tr>
<td>Cy-6</td>
<td>4.32</td>
<td>2506</td>
<td>125; 101(f)</td>
<td>30; 56</td>
<td>1.75 \times 10^9 particles(e)</td>
<td>5C</td>
</tr>
</tbody>
</table>

\(a\) In the naive animal, E CR1 levels were constant over an extended time period before infusion.

\(b\) In each case the HP infused was 7G9 \times 9D12, except for Cy-1, in which a mixture containing equal amounts of this HP and 9H3 \times 9D12 was used.

\(c\) Presumes high avidity (approximately stoichiometric) binding of HP to RBC CR1 due to high avidity binding of anti-CR1 mAbs to RBC CR1.

\(d\) Number of DEN particles infused during two 1-h periods of continuous infusion.

\(e\) Number of DEN particles infused during two 1-h periods of continuous infusion.

\(f\) Two HP injections were performed at the defined times shown in the figure.

\(g\) Number of DEN particles infused during two 1-h periods of continuous infusion.

\(h\) Presumes high avidity (approximately stoichiometric) binding of HP to RBC CR1 due to high avidity binding of anti-CR1 mAbs to RBC CR1.

\(i\) Summary of experimental protocols used for each monkey

| Number of DEN particles infused during two 1-h periods of continuous infusion. | Two HP injections were performed at the defined times shown in the figure. |

\(j\) Number of DEN particles infused during two 1-h periods of continuous infusion.
quenching of scintillation counting, we cannot measure radioactive counts accurately in E pellets. As shown in Fig. 1B, DEN-specific HP can clear DEN more efficiently from the plasma when compared with E without HP or E charged with an irrelevant HP. When the incubation time was increased to 10 or 20 min, binding did not increase significantly, suggesting that equilibrium is reached within 5 min (Fig. 1C).

**DEN RNA quantitation by competitive RT-PCR**

Although the above data showed that DEN-specific HP potentially can be effective reagents for promoting binding of DEN to E in plasma, the method we used for measuring the efficacy of binding was clearly suboptimal due to nonspecific binding and also probably due to contamination of the DEN by antigenically unrelated material. To overcome this problem, we have developed an RT-PCR-based DEN quantitation method. This method is based on the presence of defined amounts of competitor RNA that use the same primer set for amplification in the same tube with sample RNA. For each given experimental RNA sample, at least three different competitor RNA input concentrations were used in side by side RT-PCR. By comparing the band intensity between competitor and viral origin DNA bands, we can calculate the absolute RNA copy number in the tube within a factor of 2. Our detection limit using this method is 25 RNA copies per RNA sample in RT-PCR.

**Binding efficacy of DEN to E via HPs in vitro: determination by RT-PCR**

We examined three anti-DEN/CR1 HP for their ability to remove DEN from the plasma and bind it to E. DEN binding to E was performed using E from 11 different monkeys (2 rhesus and 9 cynomolgus monkeys) whose E CR1 epitope levels (defined by mAb 7G9) varied between 1600 and 5000. As described above, nonspecific binding of DEN to E franked with an irrelevant HP (7G9 × 7B7) or to E alone was 35–50% of the input virus. However, compared with these controls, specific binding of DEN to E franked with HP in plasma averaged 80% for a single pass binding experiment, and averaged ~90% for a double pass in which the unbound supernatant was added to fresh HP-franked E (Fig. 2A). Moreover, when solution phase binding in plasma was measured, specific binding, after correction for background, averaged 75% for a single pass and 85% for a double pass experiment (Fig. 2B).

In all of these measurements, specific binding is calculated based on the amount of material removed from the plasma and transferred to E compared with the baseline values observed for either naive E or E treated with irrelevant HP.

We performed similar experiments to measure the specific HP-mediated binding of DEN to human E in the presence of plasma (350–500 CR1 epitopes per E). Although there was less binding compared with the results with monkey E (which have more CR1), the data clearly demonstrate that a relatively high level of binding of DEN to human E in plasma, especially with HP 7G9 × 9D12, can be achieved in the presence of HP for both franked and solution phase binding conditions (Fig. 3, A and B). In addition, when E were franked with E that recognize two different CR1 epitopes (7G9 × 9D12 and HB8592 × 9D12), a significant increase in specific binding was observed, suggesting that by increasing the number of HP bound per E, binding of DEN may be enhanced (Fig. 3A). The in vitro binding studies were conducted in plasma containing EDTA, and thus clearly demonstrate that HP-mediated binding of DEN to E does not require complement activation.

**Clearance of monkey DEN passive viremia by HP infusion**

We next examined the efficacy of E/HP-dependent clearance of passive DEN viremia in a cynomolgus monkey model system. A passive viremia model was chosen with the following rationale: 1) It is relatively straightforward to establish a steady state viremia by continuous infusion; 2) this paradigm will allow examination of several different DEN-HP/E models. These conditions include the binding of E to preexisting vascular DEN, the interaction of newly introduced DEN in the presence of the HP/E complex, and clearance of the DEN-HP complex from the vascular system. Based on the above in vitro binding data, we chose HP 7G9 × 9D12 for these experiments.

In the first series of experiments, conducted on three cynomolgus monkeys, DEN was i.v. infused continuously for 4 h at an input of ~1–2 × 10⁹ virion particles/kg/h. Two hours after initiation of infusion, over 1 min, a bolus of HP (200 µg/kg) was injected into a vein different from that used for DEN. Throughout
the procedure, blood samples were periodically collected and immediately placed on ice. Competitive RT-PCR was used to measure DEN particles in the plasma- and blood cell-associated virion particles, as described in Materials and Methods.

As is demonstrated in Fig. 4, both plasma- and cell-associated virion particles remain relatively constant during the first 2 h of infusion. Based on the rate of infusion of DEN and the estimated blood volume of the monkeys (~60 ml/kg), only a fraction (less than ~10%) of the infused DEN was recoverable in the circulation. However, upon injection of a bolus of HP, cell-associated DEN increases by 5- to 20-fold even though the rate of DEN infusion is held constant before and after HP injection. Moreover, DEN particles in the plasma either remain constant or gradually decrease during the remaining 2 h of DEN infusion. When DEN infusion is terminated, both plasma DEN and cell-associated DEN decreased. Interestingly, we find the kinetics of disappearance of DEN from blood samples (both cell associated and in the plasma) upon termination of DEN infusion varies substantially between individual monkeys even though the same HP (7G9 × 9D12) is used at similar concentrations in all three monkeys. Finally, in one experiment, E-associated DEN was compared with total cell-associated DEN (see Materials and Methods), and after HP infusion these values, which represent a substantial increase in cell-bound DEN, were approximately equal (Fig. 4A).

We also developed an in vitro protocol in whole blood to confirm that HP-mediated cellular binding was indeed to E (see Materials and Methods). HP and DEN were added to whole blood, and then the amount of cell-bound DEN was determined by RT-PCR. Binding to a complete cell pellet (simple centrifugation) gave 1.1 ± 0.5 × 10^6 bound DEN particles per 90 μl of whole blood. When the blood was separated into leukocyte and E fractions, the respective cellular binding was 4 ± 2 × 10^5 bound DEN (leukocytes) and 2 ± 1 × 10^5 bound DEN (E). Thus, after Percoll separation, more than 99% of the recoverable cellular bound DEN is E associated, and this finding is in agreement with our previous reports that HP principally facilitate binding to E, and not white cells, in whole blood (32, 41). The reduction, after Percoll separation, of net E-bound DEN compared with the whole cell pellet is most likely due to problems associated with processing and recovery of the sample as well as a potential effect of small amounts of residual Percoll on the RT-PCR assay.

Three findings from these experiments are worth noting. First, there is a significant increase of viremia (total DEN particles in the bloodstream) upon injection of HP compared with the level before HP injection. Second, upon injection of HP, the vast majority of circulating DEN particles become associated with blood cells, which are most likely E. The ratio between cell-associated DEN and free DEN upon injection of HP varies between 15 and 50 to 1. These data demonstrate that HP can efficiently promote binding of DEN to E in vivo. Third, the results obtained before HP injection, when recoverable virus is quite low, strongly suggest that the majority of infused DEN is either rapidly cleared from the blood by specific organs or can still be recovered in the bloodstream because it may be at least partially associated with other cell types in the vascular system such as endothelial cells.

In the next series of experiments conducted on three cynomolgus monkeys, DEN was infused continuously for 1 h at a rate of ~2–4 × 10^9 virion particles/kg/h. One hour after termination of DEN infusion, a bolus of HP (200 μg/kg) was injected. Between 1.5 and 2 h later, a second DEN infusion was performed for 1 h using the same dose as in the first infusion. As described above, DEN particles in the plasma and blood cell-associated DEN particles were determined at regular time intervals throughout the experiment.

The results of these experiments, illustrated in Fig. 5, indicate that upon termination of the first virus infusion after 1 h, both cell-associated and DEN particles in the plasma decrease substantially. In addition, after HP injection, there is large increase in cell-associated DEN particles (approximately a 20-fold increase compared with that before HP injection), but there is no change, or a slight decrease in plasma-associated particles. The quantitative changes and the associated kinetics vary between the monkeys, but the overall trend, that is, recovery of DEN virus as cell associated upon HP injection, followed by a variable rate of clearance, is evident in all three experiments (Fig. 5).

The second infusion of DEN (initiated between 210 and 240 min) leads up to a 100-fold increase in cell-associated DEN particles. Similar to the first set of experiments, upon termination of this second DEN infusion in the presence of HP, there is a gradual decrease of cell-associated DEN, and the rate of decrease of cell-associated virus varies between the monkeys. However, the decrease in plasma DEN levels is rapid. Injection of a second bolus
respectively) with DEN passive viremia. DEN was infused continuously for 4 h at a rate of 1–2 × 10⁶ DEN particles/kg/h. Two hours after initiation of infusion, a bolus of HP (∼200 μg/kg) was injected. DEN particles in the plasma (B and C) or E-associated (A) and total blood cell-associated (Cell; A–C) DEN particles were determined at regular time intervals (30 min) shown in the figures, as described in Materials and Methods. The horizontal bar denotes the duration of DEN infusion, and the arrow shows HP injection. Time zero represents pre-DEN infusion blood samples that denote the detection limit of each experiment. Each point represents a mean value and SD from at least four independent RT-PCR quantitation reactions from three independent RNA isolations.

of HP during this time period did not significantly alter the kinetics of decrease of cell-associated DEN particles (Fig. 5C).

Discussion
Although development of a live attenuated vaccine might be the best approach to controlling DEN epidemics, a number of factors may limit broad applications of a vaccine. These include: 1) protective Abs against all four serotypes must be generated simultaneously so that the vaccine does not serve to introduce a primary mild infection that can be followed by DEN infection with a different serotype, thus promoting a high probability of DHF/dengue shock syndrome; 2) poor understanding of massive T cell and macrophage activation upon secondary infection that can be potentially mimicked by either vaccination or DEN infection after vaccination. This led us to focus on a therapeutic modality which can attenuate viremia by HP injection. There are available mAbs that recognize all four DEN serotypes, and so in principle it may be possible to generate a HP with broad application for treating DEN infections. In this study, we first tested mAbs specific for DEN envelope protein that are, respectively, type specific (4G2) (27). For in vivo passive viremia clearance studies, we used HP containing 9D12 because this mAb and the corresponding HP gave the highest level of E binding. In addition, as mAb 9D12 is subcomplex specific, it has the potential for targeting all four DEN types in the circulation.

Our results indicate that HP have the potential to redirect DEN in the bloodstream by binding it to E (Figs. 2–5). As reported previously (25, 41, 45), HP-mediated binding of substrates to E reaches equilibrium within 5 min both in vitro and in vivo, and this is clearly the case with DEN (Fig. 1C). In fact there is more than a 5-fold increase of cell-associated DEN within 5 min of HP injection (see Figs. 4 and 5), and under conditions in which DEN was not continuously infused, within 5 min of HP injection the ratio between cell-associated and plasma DEN reached a value of over 20 to 1. That is, at least 95% of virus recoverable in the blood vessel is in association with cells (most likely E) within 5 min of HP injection (see Fig. 5, B and C, at times shortly after 120 min).

Clearance of DEN from the plasma in vitro was also efficient; within 5 min of incubation, when monkey E were used, E franked with HP could bind ∼80% of DEN compared with naive E or to E charged with an irrelevant HP, and solution phase binding was only slightly lower. When human E were used for in vitro binding under similar conditions, the efficiency of clearance was lower than that seen with monkey E. This may be partly due to a lower number of available CR1 epitopes on human E (∼350–500/E) when compared with monkey E (∼1700–5000/E). In fact, when human E are franked with two different HP that recognize different CR1 epitopes (7G9 and HB8592), the binding efficacy of DEN is almost as high as that of a double pass experiment with human E franked with a single HP. The combination of two different HP that bind to different sites on CR1 (7G9 × 9D12 and HB8592 × 9D12) should allow for loading of a larger number of HP per E, which would be expected to increase the net efficiency of binding of DEN to E. This enhancement of binding was not observed in solution phase studies, but in these experiments less HP was added per E than in the franking experiments in which excess HP was used to saturate available CR1 binding sites. Therefore, in the solution phase experiments, the local concentration of the two different HP on individual CR1 clusters may not have been sufficiently high to allow for enhanced ligation of DEN.

In the absence of HP, during passive DEN viremia established by continuous infusion of DEN, the turnover of circulating DEN was rapid. Less than 10% of the infused virus can be recovered from blood samples, based on the amount of virus infused and a presumed blood volume of 60 ml/kg (Fig. 4, 30–120 min, and Fig. 5, 30–60 min). Furthermore, upon termination of DEN infusion, there is a rapid decrease of DEN in the blood sample. These data are consistent with findings with Langat virus, another member of the flavivirus genus, for passive viremia in cynomolgus and spider monkeys (46) and for SIV clearance studies in rhesus monkeys (47). In fact, passive viremias investigated in mouse models also are manifest by rapid clearance kinetics (48–52). Interestingly, dX174 bacteriophage infused into experimental animals is cleared very slowly from the circulation (36, 53).
During passive viremia, 30–75% of DEN particles recovered from blood samples were associated with cells. These data are consistent with the in vitro binding studies of DEN to E, as described in Results. This is different from a previous report using Langat virus, in which in a spider monkey model ~5–10% of recovered virus was in association with cell fractions (46). However, in the present system, we find that upon infusion of HP, between 95 and 99% of DEN particles are associated with cells in the blood samples. In addition, infusion of HP led to a large increase in DEN that could be recovered from the blood sample during continuous DEN infusion. We found that between 5 and 30 times more DEN particles can be recovered in the bloodstream after infusion of HP (Table III), under conditions of constant virus infusion.

When virus infusion was terminated after injection of HP, a large fraction of both plasma-associated and cell-bound DEN was cleared from the circulation. Plasma-associated virus appeared to clear faster than cell-bound DEN, but because there is much more cell-associated DEN in the presence of HP (up to 30-fold higher), it is difficult to compare these two fractions directly. In addition, it is likely that plasma-associated DEN continued to reequilibrate and bind to E after DEN infusion was terminated, and this reaction would be manifest in apparently a more rapid clearance of plasma-associated virus. Moreover, as noted above, cell-associated virus was also cleared rapidly in the absence of HP, and the results suggest that the kinetics of clearance of DEN virus bound to E via HP were slower than the clearance of cell-bound DEN in the absence of HP.

The fact that much more DEN was demonstrable in the circulation after HP infusion, and that putatively cleared virus could be recovered after HP infusion (Fig. 5) is particularly interesting. Several reports suggest that DEN may bind directly to endothelial cells (1, 54–58), and it is certainly possible that the E-HP complex can retrieve DEN initially bound to endothelial cells in the vasculature. Therefore, the specific organs and tissues that take up DEN may be different in the presence and absence of HP. Based on a substantial literature, it is certainly reasonable to anticipate that a substantial fraction of DEN is taken up by the liver and spleen even in the absence of HP (1, 54). However, the fact that some of the cleared virus is recoverable upon injection of HP suggests that it is either deposited in other sites (endothelial cells) or is perhaps handled in a very different fashion when its clearance is mediated by HP. Based on our previous studies, it is reasonable to suggest that the rate-determining step that presumably slows down the clearance of the E-bound DEN-HP complex is the proteolysis of E CR1 after recognition by Fc receptors on fixed tissue macrophages (59, 60). After this step, the complex of DEN, HP, and released CR1 should be phagocytosed by the macrophages. It is obviously of critical importance that this phagocytosed material be destroyed when it is taken up by a macrophage, and that the internalized DEN does not mediate infection. We intend to test this question in future in vitro and in vivo studies.

### Table III. Total recoverable DEN particles from blood sample before and after HP infusion

<table>
<thead>
<tr>
<th>Recoverable Virus without HP</th>
<th>Recoverable Virus with HP</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy-2 9.23 ± 1.92 × 10^7</td>
<td>7.27 ± 2.86 × 10^6</td>
<td>7.88</td>
</tr>
<tr>
<td>Cy-3 9.48 ± 5.55 × 10^7</td>
<td>4.55 ± 1.69 × 10^6</td>
<td>4.80</td>
</tr>
<tr>
<td>Cy-4 5.78 ± 1.04 × 10^7</td>
<td>3.20 ± 1.38 × 10^6</td>
<td>5.54</td>
</tr>
<tr>
<td>Cy-5 1.25 ± 0.32 × 10^6</td>
<td>3.71 ± 1.06 × 10^5</td>
<td>29.70</td>
</tr>
<tr>
<td>Cy-6 1.31 ± 0.34 × 10^6</td>
<td>2.80 ± 1.39 × 10^5</td>
<td>21.40</td>
</tr>
</tbody>
</table>

*Recoverable virus particles were calculated by adding plasma- and cell-associated virus particles per ml of blood after 30 min of steady-state infusion of DEN in the absence of HP.

*Recoverable virus in the presence of HP was calculated by adding plasma- and cell-associated virus particles per ml of blood either 30 min after injection of HP (for Cy-2 and Cy-3) or after 30 min of steady-state infusion of DEN in the presence of HP (for Cy-4, 5, and 6).

*Ratio of recoverable virus particles in the presence of HP over that in the absence of HP.

FIGURE 5. Effect of HP infusion and the HP-E complex on the levels of cell-associated and plasma-associated DEN in three monkeys (A–C; Cy-4, Cy-5, and Cy-6, respectively) with DEN passive viremia. DEN was infused for 1 h at a rate of 2–4 × 10^9 DEN particles/kg/h, and 1 h after termination of DEN infusion, a bolus of HP (~200 µg/kg) was injected. Ninety minutes to two hours after HP injection, a second DEN infusion was performed for 1 h using the same dose of DEN. DEN particles in plasma and blood cell-associated (Cell) DEN particles were determined at regular time intervals shown in the figures, as described in Materials and Methods. The horizontal bars denote the duration of DEN infusion, and the arrow shows HP injection. Time zero represents pre-DEN infusion blood samples that denote the detection limit of each experiment. Each point represents a mean value and SD from at least four independent RT-PCR quantitation reactions from three independent RNA isolations. In C, HP was injected one more time (101 µg/kg) at the 325-min mark (see Table II).
HETEROLIGOMER-MEDIATED CLEARANCE OF DENGGUE PASSIVE VIREMIA

It is also of interest that among individual monkeys, the rate of HP-mediated clearance of E-bound DEN was dramatically different. For instance, two monkeys (Figs. 4C and 5A) showed extremely rapid clearance of cell-associated DEN-HP when compared with the other monkeys. This is intriguing because in all six monkeys, the same HP (7G9 × 9D12) was used. Furthermore, injection of an additional bolus of HP did not accelerate clearance in the monkey in the experiment illustrated in Fig. 7C. The nature of this discrepancy is not clear, but might be related to polymorphisms in FcR in the monkeys. It should also be possible to investigate this question by preparing HP with a variety of IgG isotypes (61–65).

Many additional questions must be addressed, including the potential impact of secondary infections on HP effectiveness. However, our results suggest that with further development, HP may be effective in decreasing the DEN burden in the vascular system, which could in turn potentially decrease the severity of the disease associated with secondary DEN infection.

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References


