**Macaca nemestrina** and Dengue Virus Infection: a Potential Model for Evaluating Dengue Vaccine Candidates

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**Macaca nemestrina** has been shown to respond to infectious disease agents, such as HIV, and is more sensitive compared to other species of macaques such as rhesus (**M. mulatta**) and cynomolgus monkeys (**M. fascicularis**). To evaluate **M. nemestrina** for the ability to support dengue (DEN) viremia and serve potentially as an improved model for testing DEN vaccines, a series of experiments were conducted using primary viral isolates from individuals with DEN virus infections. This study shows that **M. nemestrina** develops consistent, measurable viremia with all four DEN serotypes and produces immune responses sufficient to protect against homologous virus. Anti-dengue antibodies generated after infection are predominately IgG1. This species of monkey therefore appears to be a suitable model for testing DEN virus vaccine candidates.

**Key words:** dengue infection, **Macaca nemestrina**

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most important arthropod-borne viral diseases worldwide. An estimated 100 million infections occur every year in dengue (DEN) endemic regions of the world (Halstead 1988). DHF, the more severe form of DEN infection, is associated with a mortality of 1% to 5% and may be as high as 30% to 40% in untreated patients. The tremendous public health impact of this disease emphasizes the need for an effective preventive DEN vaccine.

There are many DEN vaccine candidates in clinical and pre-clinical trials. Pre-clinical trials usually involve the evaluation of promising vaccine candidates in non-human primates (NHP). Since DHF manifests only in humans, the model for testing the efficacy of a DEN vaccine centers on the vaccine's ability to prevent, or significantly reduce, viremia after vaccinated animals are challenged with live DEN virus. Rhesus macaques (**Macaca mulatta**) have been used mostly as a model for pre-clinical trials, because this species of NHP supports DEN virus replication and manifests consistent detectable viremia (Angsubhakorn et al. 1988; Eckels et al. 1994; Bray et al. 1996; Raviprakash et al. 2000, 2006, 2008; Durbin et al. 2001; Putnak et al. 2003, 2005; Sun et al. 2005). Fifteen other species of NHP have been used as models for vaccine trials and dengue infections (Bente and Rico-Hesse 2006). However, the susceptibility of the pigtail macaque (**M. nemestrina**) to infection with DEN has not been tested.

Similar to rhesus, pigtail macaques possess dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) that has similar characteristics and functions as human DC-SIGN (Baribaud et al. 2001). This type II membrane protein with a C-type lectin functions as a receptor binding domain for dengue virus and several viral pathogens such as HIV-1 and influenza A/H5N1. This fact supports the possibility of pigtail macaques as a model for DEN infection. However, unlike other macaques, pigtailed macaques have been shown to be susceptible to HIV infection. Pigtail macaques possess the cytoplasmic body protein TRIM6, which is incapable of restricting HIV replication after viral entry to host cells. Other macaques possess TRIM5α, which inhibits reverse transcription of retrovirus (Stremiau et al. 2004; Brennan et al. 2007). The pigtail is the most potential macaque model in which HIV can cause as AIDS-like syndrome in non-human species (Agp et al. 1992; Baroncelli et al. 2008; Hatzioannou et al. 2009).

In this study, we evaluated the ability of dengue to replicate in pigtail macaques. A series of experiments where pigtail macaques were inoculated with all four serotypes of dengue virus were conducted. Viremia and anti-dengue antibody responses were studied and revealed that this monkey species may serve as a suitable model for evaluating experimental dengue vaccines.

**MATERIALS AND METHODS**

**Animals.** Thirty specific pathogen free (free of SRV, SIV, STLV and TB) pigtail macaques (**M. nemestrina**), between the ages of six months and one year, were screened for anti-flavivirus antibodies by hemaglutination inhibition assay, IgG and IgM ELISA (Focus Diagnostics, Cypress, CA) and plaque reduction neutralization tests (DEN and Japanese Encephalitis). The monkeys were obtained from the Primate Research Center, Bogor, West Java. Seventeen flavivirus naïve monkeys were selected and housed in mosquito-proof rooms at the Naval Medical Research Unit #2 AAALAC International-accredited animal facility. The animals ranged in weight from 2 kg to 3.5 kg and were pair caged with another animal inoculated with the same dengue...
virus serotype. Animal care was administered according to the Guide for the Care and Use of Laboratory Animals (NRC 1996). The study was conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Naval Medical Research Unit #2 number 98AUC02.

**Virus Inoculation and Blood Samples.** The animals were separated into four groups of four animals each. One monkey was used as an alternate to replace any animal that needed to be excluded during the study for any reason. Each group was assigned to receive either DEN-1, DEN-2, DEN-3 or DEN-4 virus. Each group received two inoculations of virus. For the first inoculation, two animals in each group received virus and two received phosphate buffered saline (PBS). For the second inoculation, all animals in the group received live virus. Each group (DEN virus serotype) therefore consisted of four examples of primary infection and two of secondary infection. The extra monkey was later included in the DEN-3 group.

The DEN-1 virus used was isolated from a DEN fever patient hospitalized in Jakarta, Indonesia and passaged five times in C6/36 cell culture. DEN-2, DEN-3, and DEN-4 isolates were derived from patients hospitalized in Palembang, Bandung and Yogyakarta, Indonesia, respectively. The DEN-2 isolate was passaged five times and the DEN-3 and DEN-4 isolates were passaged 4 times in C6/36 cell culture. All isolates were obtained in 1998 with the exception of the DEN-4 isolate that was obtained in 1996. Virus stocks were prepared from clarified cell culture supernatant and stored at 70°C until used. The cells were used to confirm DEN serotype and to rule out a possibility of other related viruses contamination by indirect fluorescence assay using monoclonal antibodies to DEN-1 through DEN-4, polyclonal antibodies to Flavivirus and Alphavirus. For each inoculation, approximately 10^4 plaque-forming units (PFU) were administered subcutaneously in the lateral chest area. Prior to inoculation, the site was shaved and cleaned with 70% alcohol.

Prior to virus injection, a 3 mL anti-coagulated blood sample was obtained from the femoral vein of each monkey and used for collecting plasma and archiving peripheral blood mononuclear cells (PBMC). Following the inoculation, 1 mL blood samples were obtained daily for 10 days for virus isolation in C6/36 cells and mosquito inoculation and virus detection by RT-PCR. The NAMRU-2 veterinarian observed the animals daily for ten days post-inoculation recording pulse, respiration, rectal temperature, body weight, food and water intake. At 14 and 28 days post-inoculation, additional blood samples were obtained for anti-dengue antibody profile and antibody avidity analysis.

**Dengue Virus Detection by RT-PCR.** Qiaamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) was used to extract viral RNA from 140 µL of serum sample following manufacturer's instruction. A total of 50 µL RNA was obtained and 5 µL used in the RT-PCR reaction. The methods of Lanciotti were used for the RT-PCR and semi-nested PCR (Lanciotti et al. 1997). PCR products were resolved by electrophoresis using a 2% agarose gel and ethidium bromide staining. Dengue viremic serum and negative serum were used as control positive and negative.

**Dengue Virus Isolation.** Serum samples obtained post-infection for 10 days were analyzed for the presence of virus by standard tissue culture in C6/36 cells and by mosquito inoculation. Virus isolation in cell culture was performed as described by Graham et al. (1999). For mosquito inoculation, Toxorhynchites mosquitoes were used following the method of Yamamoto et al. (1987).

**IgM and IgG Analysis.** Anti-dengue IgM and IgG antibodies were detected using a commercially available antibody capture ELISA kit (Focus Diagnostic, Cypress, CA). Assays were performed following manufacturer's procedures. A numerical index was calculated by dividing the OD of sample with OD of the cutoff control. A sample with an index greater than or equal to 1 was considered DEN antibody positive. Serum samples drawn on day 0, day 14, and day 28 were tested by IgM ELISA, while samples drawn on day 0, day 14, day 28, and day 87 were tested by IgG ELISA.

**Antibody Avidity Assay.** The method of Gassmann and Baner (1997) was used with some modifications, to evaluate the avidity of anti-DEN IgG antibody. To determine the appropriate urea concentrations to use in the test, high avidity and low avidity positive control sera, diluted 1:100, were tested at different urea concentrations that ranged from 6 M to 9 M in 0.5 M increments. Using the best urea concentration, 6.5 M, the samples were tested in duplicate for IgG as indicated above. After the initial sample incubation, one duplicate plate was incubated for 3 min with 6.5 M urea in PBS and then washed three times. The plates were further processed according to the usual procedure. For samples giving an OD value >2.0, the assay was repeated using two-fold serial dilutions starting at a 1:100 dilution. For samples giving an OD less than 0.6, samples were retested at two-fold dilutions starting at 1:10.

For samples tested at a single 1:100 dilution, the avidity index was calculated by dividing the OD from the urea treated sample by the OD of the untreated control. For samples requiring serial dilutions, fine determinations of the avidity indexes were calculated by dividing the dilution of the urea-treated curve necessary for a defined OD by the respective dilution of the control curve at the same OD. The defined OD was selected in the range 0.25-0.60 fold of the maximal OD.

**Antibody Subclass Analysis.** The subclasses of anti-DEN IgG produced in response to live virus infection were studied. The method of Shearer et al. (1999) was used for this analysis. The distribution of IgG subclasses was examined with the use of indirect ELISA. Briefly, dengue cell lysate antigen (DEN ag) and Vero-76 cell lysate antigen (mock ag) in carbonate-bicarbonate buffer pH 9.6 were coated onto five U8 Maxisorb microtiter plates (Nunc, Roskilde, Denmark). The plates were incubated overnight at 4°C. The plates were washed with phosphate buffer saline pH 7.4 containing 0.1% Tween 20 (PBS-T) six times and 100 µL of serum (diluted
1/100 in PBS with 0.1% Tween 20 and 5% defatted milk powder) was added into each well. After 1 h serum incubation in 37°C, plates were washed. Horseradish peroxidase labelled sheep anti-human IgG1 to IgG4 (The Binding Site, Birmingham, UK) at 1/50 dilution and horseradish peroxidase labelled goat anti-human IgG (Accurate, Westbury, USA) at 1/1000 dilution were added into each plate, so plate 1 was incubated with anti-IgG1, plate 2 with anti-IgG2, and so on. The plates were incubated 1 h in 37°C. The 2,2’-azino-di(3-ethylbenzthiazoline sulfonate (6)) (ABTS) substrate (Kirkegaard and Perry, Gaithersburg, USA) was added after the plates were washed. Following another one hour incubation at 37°C, the absorbance was measured at 415 nm. The specific absorbance for each serum sample was calculated as the mean A\text{415 nm} \text{DEN} \text{ag} - the \text{mean} A\text{415 nm} \text{mock} \text{ag}.

**Plaque Reduction Neutralization Test and Plaque Assay.** Neutralizing antibody titers at day 0, 14, and 28 were determined by standard plaque reduction neutralization as performed by Morens et al. (1985) and the results expressed as the reciprocal dilution that produces a 50% reduction in plaque count. Plaque assay was performed to determine the virus titer during viremia by inoculating 1:10 diluted serum in PBS/BA into BHK12 Clone-15 suspension.

**RESULTS**

**Clinical Signs and Symptoms.** Following infection, the animals were monitored daily for any signs and symptoms of DEN infection. There were no abnormal changes in temperature or respirations following live virus injection. The animals' weights remained stable before, during, and after viremia. No obvious decreases in food consumption were noted.

**DEN Viremia Following Infection.** There were 16 episodes of experimental primary infection among the animals. Fig 1 shows the days of viremia for each DEN serotype according to the different virus detection methods. Most of all the animals became viremic within 1 to 2 days after DEN injections. As expected, RT-PCR was the most sensitive method for detecting viremia. However, consistent viremia was detected in each of the animals by all three methods. DEN-2 resulted in the greatest average number of days viremia at 7.8±0.5, 6.8±1, and 5.8±1 days as measured by RT PCR, isolation in C6/36, and mosquito inoculation, respectively. If going by RT-PCR and mosquito inoculation methods, the least number of viremia days occurred with DEN-4 (5±1.4 and 3.3±1). By isolation in C6/36, DEN-3 produced the least (4±0.8).

Animals that were infected with each DEN serotype were re-challenged with the homologous virus. None of the animals re-challenged with DEN-1, DEN-2, or DEN-3 developed viremia. Two animals with secondary DEN-4 infections both developed breakthrough viremia as detected by RT-PCR. DEN-4 was detected in one animal on days 6 and 7 and in the other only on day 7.

Plaque assays were performed to quantify the amount of virus present. All attempts failed to produce plaques and it was concluded that the level of viremia was probably too low for detection by this method.

**Fig 1** The days of viremia in pigtail challenged with DEN viruses. Viral detection methods were C6/36 isolation, mosquito inoculation, and RT-PCR. Each bar represents days of viremia from individual animal.
Anti-DEN Antibody Responses. Anti-DEN IgG and IgM antibody responses following infection with the four DEN serotypes are shown in Fig 2 and expressed as the mean ELISA index value. In primary infection (Fig 2a), the highest mean anti-DEN IgM antibody index was detected on day 14. Some monkeys showed no IgM antibody at day 28. Anti-DEN IgG was detectable on day 28 and showed a steady increase up to day 87. In animals that received secondary infections, the highest anti-DEN IgG ELISA index was detected earlier on day 14, while anti-DEN IgM did not increase at all (Fig 2b). The avidity of anti-DEN IgG antibodies steadily increased after primary infection (Fig 2a). Following secondary infection there was a further increase in antibody avidity indicating additional maturation of anti-DEN IgG (Fig 2b).

Fig 3 shows the anti-DEN IgG subclass antibody response to DEN-4 virus. After primary infection, anti-DEN IgG1 was detected primarily and increased through day 87. The other IgG subclasses were either undetectable or detectable at low levels. Following secondary infection, IgG1 continued to predominate at higher levels. Increases in the proportions of the other IgG subclasses were also noted (data not shown). By 87 days post secondary infection, all levels showed a decrease but were still detectable. Similar patterns were observed with DEN-1 and DEN-2 (data not shown). Anti-DEN-3 IgG subclass analysis was not performed.

Table 1 Homologous anti-DEN neutralizing antibody responses after primary and secondary infections

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Primary</th>
<th>Secondary</th>
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<tr>
<td></td>
<td>Day 14</td>
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Table 1 shows the anti-DEN neutralizing antibody responses to infection with each of the serotypes. Primary and secondary infections with each serotype elicited extremely high levels of neutralizing antibodies by day 14. Reciprocal homologous neutralizing antibody titers prior to challenge (day 0, secondary infections) were 160 to >640. Neutralizing antibodies to other serotypes were lower than homologous antibody in primary and secondary infections (data not shown).

DISCUSSION

This study demonstrated that pigtail macaques are susceptible to infection with all four wild type DEN viruses, giving rise to consistent viremia for several days as detected by three different viral detection methods. Since macaques do not manifest obvious symptoms of dengue disease, vaccine developers gauge the protective efficacy of experimental dengue vaccines by evaluating the vaccine’s
ability to reduce the number of days of viremia following live virus challenge. *Macaca mulatta* and *M. fascicularis* are currently the species most frequently used in vaccine development because of the consistent viremia produced after live virus injection. The viremia results obtained with pigtail macaques suggests that this species is comparable to other species for use as a model for dengue vaccine developers. Due to the lack of standardized methods for DEN virus titration and detection, making comparisons between different macaque species regarding their ability to produce viremia following live dengue virus infection may be inappropriate. Nevertheless, the days of viremia caused by DEN-1 and DEN-4 primary infections in this study were slightly longer than the days of viremia seen in cynomolgus monkeys infected with these serotypes (Koraka et al. 2007). In addition, the overall length of viremia in pigtailed was longer than that seen in earlier rhesus monkey studies (Halstead et al. 1973; Freire et al. 2007). One prior study with rhesus monkeys showed that several strains of DEN viruses failed to produce viremia.

The current study utilized only a single wild type dengue viral isolate for each serotype. It remains to be determined if the viremia characteristics seen with this set of viruses is characteristic of the viremia that would occur if other viruses were used. While in theory, the same level of viremia should occur with other viruses, further evaluation of the pigtail macaque with other isolates is warranted.

Daily observations during the viremic period did not detect any overt signs of clinical illness. The animals maintained their weight and food intake and no obvious elevations in temperature were noted. This lack of clinical illness was consistent with observations made in earlier studies of chimpanzees (Price et al. 1974; Scherer et al. 1978), owl monkeys (Schiavetta et al. 2003), rhesus monkeys (Price et al. 1974; Freire et al. 2007) and cynomolgus monkeys (Price et al. 1974; Koraka et al. 2007) as well as in other studies reviewed by Bente and Rico-Hesse (2006).

The anti-DEN IgM and IgG antibody responses to each of the serotypes were similar to primary and secondary responses seen in human dengue virus infections (Innis 1997). Anti-dengue IgM was detected early and peaked within 2 weeks (2-4 weeks) after primary infection. Anti-DEN IgG was detected later and increased slowly. Following secondary infection, anti-DEN IgM was low or undetectable in some instances, as seen in dengue endemic areas where multiple serotypes circulate. Anti-DEN IgG rapidly increased over the next 2 weeks. Antibody avidity assay demonstrated the progressive increase in affinity in these antibodies over time after primary infection. Secondary infection induced only a slight increase in anti-DEN IgG antibody maturation. Avidity responses were also similar with the response in human (unpublished data).

There was no anti-pigtail IgG subclass available commercially, when IgG subclass assay was set. Anti-human IgG subclasses were chosen, since these reagents bound more anti-pigtail IgG compared with anti-macaque IgG subclasses (data not shown). Anti-DEN IgG1 was primarily detected and predominant IgG subclass following primary and secondary infections in pigtailed. In humans, IgG1 and IgG3 were the predominant IgG subclasses throughout the course of illness regardless of whether the illness was characterized as DF, DHF, or DSS (Thein et al. 1993; Koraka et al. 2001). In this study, it is not further determined whether this different due to different species response or less cross reaction of anti-human IgG3 than anti-human IgG2.

High-titer anti-DEN neutralizing antibodies were produced in response to dengue virus infection in each animal and demonstrated cross-reactivity against the dengue serotypes used in the plaque reduction neutralization assay. These antibodies resulted in solid protection against challenge with the homologous dengue virus serotype, although minimal break-through viremia occurred in two DEN 4 animals as detected by RT-PCR. Because no live virus was detected in these animals, this could have just represented residual RNA from non-viable virus.

The ability of pigtail monkeys to support the replication of all four serotypes of dengue raises the question of whether this species is suitable for evaluating the efficacy of experimental dengue vaccines and anti-dengue therapeutic drugs. Further studies are planned to evaluate the efficacy of DNA-based vaccines using the pigtail model.

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