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# Monkeys Immunized with Intertypic Chimeric Dengue Viruses Are Protected against Wild-Type Virus Challenge

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**Dengue epidemics caused by the four dengue virus serotypes continue to pose a major public health problem in most tropical and subtropical regions. A safe and effective vaccine against dengue is still not available. The current strategy for dengue immunization favors the use of a vaccine containing each of the four serotypes. We previously employed full-length dengue type 4 virus (DEN4) cDNA to construct a viable intertypic dengue virus of type 1 or type 2 antigenic specificity that contained the genes for the capsid-premembrane-envelope (C–pre-M–E) structural proteins of DEN1 or pre-M and E structural proteins of DEN2 substituting for the corresponding DEN4 genes. Chimeras DEN1/DEN4 and DEN2/DEN4, which express the nonstructural proteins of DEN4 and the C–pre-M–E structural proteins of DEN1 or the pre-M–E structural proteins of DEN2, and therefore the antigenicity of type 1 or type 2, were used to immunize rhesus monkeys. Other monkeys were inoculated with parental DEN1, DEN2, or cDNA-derived DEN4. Three of four monkeys immunized with DEN1/DEN4 developed neutralizing antibodies against DEN1 and were protected against subsequent DEN1 challenge. All four monkeys immunized with DEN2/DEN4 developed antibodies against DEN2 and were protected against subsequent DEN2 challenge. DEN1- and DEN2-immunized monkeys were protected against homologous virus challenge, but DEN4-immunized animals became viremic on cross-challenge with DEN1 or DEN2. In a second experiment, eight monkeys were immunized with equal mixtures of DEN1/DEN4 and DEN2/DEN4. Each of these monkeys developed neutralizing antibodies against both DEN1 and DEN2 and were protected against subsequent challenge with DEN1 or DEN2. Chimeric dengue viruses similar to those described here could be used to express serotype-specific antigens in a live attenuated tetravalent human vaccine.**

The four dengue virus serotypes (DEN1 to DEN4) form a subgroup within the *Flavivirus* genus. Dengue virus causes more human illness than any other flavivirus. The virus is transmitted from human to human by *Aedes* mosquitos and has spread widely in tropical regions of the Americas in the last decade. Millions of cases now occur annually around the world (13, 24). Large dengue virus outbreaks occur in densely populated areas, due to the urban range of the vector *Aedes aegypti*, but the recent spread of the vector species *Aedes albopictus* has extended the dengue threat (5, 17). Dengue virus produces a spectrum of disease, ranging from fever, headache, and prostration to severe or fatal illness, dengue hemorrhagic fever with shock syndrome. Recovery from dengue is accompanied by the development of serum neutralizing antibodies. Available evidence indicates that immunity against other serotypes (heterotypic immunity) following dengue virus infection is brief, lasting 2 to 3 months. On the other hand, resistance to the same serotype (homotypic immunity) is life-long (23). However, epidemiologic studies indicate that individuals who have recovered from infection with one dengue virus serotype may be at greater risk of developing dengue hemorrhagic fever with shock syndrome if reinfected with another serotype. This severe illness appears to result from immune enhancement of viral replication, caused by the uptake by monocytes/macrophages of virus complexed to nonneutralizing antibodies or to low-titer neutralizing antibodies (8). Alternatively, the severe dengue illness may be caused by virulent dengue virus strains that have emerged during an epidemic episode (20, 21). An ideal vaccine should therefore induce solid immunity against all four serotypes.

Recently, we described the construction of intertypic chimeric cDNA which was prepared by substituting the C–pre-M–E structural protein genes of full-length DEN4 cDNA with the corresponding genes of the Western Pacific (WP) strain of DEN1 or a mouse-neurovirulent variant of the New Guinea C (NGC) strain of DEN2 (2). During construction of the chimeras, the DEN4 component of these chimeric constructs was not modified. Transfection of RNA transcripts yielded chimeric viruses which expressed the structural proteins of DEN1 or DEN2 against a background of DEN4 nonstructural proteins. The chimera expressing DEN2 structural proteins was neurovirulent for mice, as was its DEN2 parent. These studies established that chimeras were viable in tissue culture and infectious in an experimental animal and suggested that similar chimeras could be developed as vaccines, perhaps after introduction of attenuating mutations into their DEN4 component. The present study was designed to evaluate the replication, immunogenicity, and protective efficacy of two chimeric dengue viruses inoculated singly or simultaneously into rhesus monkeys.

**Constructs of dengue intertypic chimeric viruses.** Construction of the full-length cDNA copy of DEN4 strain 814669 in pBR322 (designated clone 2A) and transfection of LLC-$MK_2$ cells with the RNA transcripts derived from that template were described earlier (15). To facilitate chimeric cDNA construction, a silent mutation was introduced to create an *Xho*I site near the 3′ end of the DEN4 E gene (2, 28). Progeny virus obtained from this construct was designated DEN4 2A. The cDNA segment encoding the DEN4 C, pre-M, and E genes, extending from a *Bgl*II (nucleotide [nt] 88) to the *Xho*I site (nt 2342) was replaced with the corresponding genes of DEN1

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WP. The chimeric cDNA was used to obtain progeny chimeric virus, designated DEN1/DEN4 (2). A chimeric virus containing the pre-M and E genes of the prototype strain of DEN2 NGC, kindly given by L. Rosen (University of Hawaii), was constructed as follows. The DEN2 pre-M and E genes represented in the cDNA segment between the *Pst*I site of DEN2 at nt 391 and the *Xho*I site near the 3′ end of E were joined with the DEN4 vector in a similar construction of a chimera containing the pre-M and E genes of tick-borne encephalitis virus (18). RNA transcripts derived from the full-length DEN2 prototype PreM-E/DEN4 construct was used to transfect LLC-$MK_2$ cells. The progeny virus are designated DEN2/DEN4 in this report. The parental, non-mouse-adapted DEN4 strains 814669, DEN1 WP, and DEN2 NGC were also grown in LLC-$MK_2$ cells.

**Immunization with DEN1/DEN4 or DEN2/DEN4 chimeric virus. (i) Viremia following immunization.** Twenty young monkeys were prebled and shown to be seronegative for DEN1, DEN2, or DEN4 infection as determined by radioimmunoprecipitation of dengue virus-specific antigens. Groups of four rhesus monkeys were inoculated subcutaneously with $3 \times 10^5$ PFU of parental DEN4 (DEN4 2A), DEN1 (DEN1 WP), or DEN2 (DEN2 NGC), chimeric DEN1/DEN4, or chimeric DEN2/DEN4 virus per dose. Inoculated monkeys were bled daily for 12 days for detection of viremia. Specimens were passaged once in mosquito C6/36 cell culture to amplify any virus present. Briefly, freshly thawed aliquots of serum were diluted 1:3 in a 0.20-ml volume of minimum essential medium (MEM) and inoculated directly onto confluent monolayers of C6/36 cells in T-25 flasks maintained with MEM plus 2% fetal bovine serum (FBS). The cells were incubated at 28°C for 14 days, with a change of medium at 7 days. The culture medium was then removed, mixed 1:1 with heat-inactivated FBS, aliquoted, and stored in a liquid $N_2$ freezer. The medium harvested from cultures inoculated with serum collected from the first through the tenth day postinoculation was tested for the presence of virus by a plaque assay on C6/36 cells. The presence of dengue virus in the inoculated cell cultures was also sought by the immunoperoxidase (IP) or immunofluorescence (IFA) procedure.

For the plaque assay, confluent C6/36 monolayers in T-75 flasks were inoculated with freshly thawed culture medium diluted in MEM plus 0.5% human serum albumin (HSA). Negative controls were inoculated with MEM plus HSA only. After 1 h of agitation on a rotating table at room temperature, an agarose overlay was added, and the flasks were placed in a humidified incubator with 5% $CO_2$ at 35°C for 5 days (22). A buffered neutral red solution was added, and plaques were counted the next day. This assay was performed twice for specimens derived from monkeys inoculated with DEN4, DEN1/DEN4, or DEN2/DEN4. For IP/IFA detection of dengue virus in cultures inoculated with monkey serum, confluent C6/36 cells in 24-well plates were inoculated with freshly thawed supernatant diluted 1:10 in MEM. Positive controls were infected with DEN1/DEN4 or DEN2/DEN4; uninfected cells served as negative controls. The cells were incubated at 28° C in 5% $CO_2$ for 1 week and fixed in methanol. Viral antigens were visualized in the inoculated cultures by sequential incubation with a 1:100 dilution in phosphate-buffered saline of hyperimmune mouse ascitic fluid (HMAF) from mice immunized with DEN1, DEN2, or DEN4 and a 1:100 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and finally 4-chloro-1-naphthol and hydrogen peroxide. IP/IFA detection was also performed with DEN1-specific monoclonal antibody (MAb) 1F1, 13G9, or 8C2 and DEN2-specific MAb 3H5, kindly supplied by E. Henchal and J. R. Putnak (Walter Reed Army Institute of Research, Washington, D.C.) (12).

Any day on which viremia was detected by one or more of the three methods used to detect virus was scored as positive (Table 1). One of the four monkeys inoculated with DEN4 did not become viremic, while two other animals had a single day of viremia (day 5 or 6 postinoculation). The fourth monkey in the group was viremic on days 1 and 2, possibly representing detection of residual inoculum. In contrast, all four monkeys inoculated with DEN1 developed viremia that began on day 4 or 5 postimmunization and extended for an additional period of 2 to 5 days. All monkeys given DEN2 developed viremia that began on day 2, 3, or 4 and extended for an additional period of 4 to 7 days. The response to infection with the DEN1/DEN4 chimera was similar to that to DEN4 in that two monkeys did not become viremic, while the other two had only 1 day of viremia on postinoculation day 6 or 7. Three monkeys inoculated with the DEN2/DEN4 chimera were viremic for 1, 2, or 3 days, with virus first detected on day 3, 4, or 6. The fourth animal was positive for 6 days, with virus present beginning on the first postinoculation day.

**(ii) Seroresponse to immunization.** A plaque reduction assay was performed to detect and quantitate neutralizing antibodies in serum 8 weeks postimmunization (22). Two of four monkeys inoculated with DEN4 developed DEN4 neutralizing antibody (titers of 1:80 and 1:160), whereas the titer attained by the other two monkeys was lower or not detectable. There was no clear correlation between the pattern of viremia and the titer of serum neutralizing antibodies that developed. A single elevated cross-reactive DEN1 neutralizing titer of 1:160 was noted. Monkeys inoculated with DEN1 or DEN2 developed a high titer of type-specific neutralizing antibody responses, ranging from 1:640 to 1:1280. Lower levels of cross-reactive neutralizing antibodies were detected: the DEN2 and DEN4 titers of the DEN1-infected monkeys were in the range of 1:10 to 1:160, while, with one exception, few or no antibodies to DEN4 were detected. Three of four monkeys immunized with chimera DEN1/DEN4 developed a DEN1-neutralizing antibody titer of 1:640 to 1:1,280, which approximated the response of DEN1-infected monkeys. The fourth (monkey I), which did not become viremic, developed a low titer of DEN1-neutralizing antibodies, 1:20. All four animals immunized with DEN2/DEN4 developed neutralizing antibody titers of 1:640 against DEN2, which was only slightly lower than the 1:1,280 response of DEN2-immunized animals. The titers of cross-reactive antibodies in recipients of chimeric viruses were similar to those in DEN1- and DEN2-immunized monkeys. Immunoprecipitation analysis of postinfection sera revealed the expected serotype-specific response to E, NS1, and pre-M that correspond to the genotype of the parental and chimeric viruses (data not shown).

**(iii) Response to challenge.** Monkeys initially immunized with DEN1 or DEN2 were completely protected against subsequent challenge 66 days postimmunization with the same virus at a dose of $3 \times 10^5$ PFU. In contrast, each of the monkeys immunized with DEN4 became viremic after subsequent challenge with this dose of DEN1 or DEN2. The day of onset and duration of the viremia following DEN1 or DEN2 challenge of DEN4-immunized monkeys were similar to the response of naive monkeys to primary inoculation with DEN1 or DEN2 (see also Table 2). All four monkeys immunized with chimera DEN2/DEN4 were protected against DEN2, and three of four immunized with DEN1/DEN4 were protected against DEN1. The single immunization failure involved a monkey (monkey I), which had a postimmunization, prechal-

TABLE 1. Analysis of viremia and antibody response in monkeys following immunization with dengue virus or chimeras and evaluation of protection against challenge with parental virus from which the chimeras were derived[a]

| Monkey no. (designation) | Immunizing virus | Viremia (day postinoculation of onset, days of duration) | Reciprocal titer at 8 weeks[b] | | | Challenge virus | Viremia (day postchallenge of onset, days of duration) |
|---|---|---|---|---|---|---|---|
| | | | DEN1 | DEN2 | DEN4 | | |
| B684 (A) | DEN4 2A | 1, 2 | 20 | 20 | 160 | DEN1 WP | 4, 4 |
| E113 (B) | | — | 40 | 40 | 80 | | 4, 4 |
| E275 (C) | DEN4 2A | 5, 1 | 20 | <10 | 20 | DEN2 prototype | 3, 6 |
| E667 (D) | | 6, 1 | 160 | 40 | <10 | | 4, 3 |
| H187 (E) | DEN1 WP | 5, 3 | 640 | 40 | 160 | DEN1 WP | — |
| E831 (F) | | 5, 2 | 1,280 | 80 | 40 | | — |
| H242 (G) | | 4, 3 | 1,280 | 40 | 40 | | — |
| E909 (H) | | 4, 5 | 1,280 | 80 | 10 | | — |
| H243 (I) | DEN1/DEN4 chimera | — | 20 | 40 | 160 | DEN1 WP | 4, 4 |
| H021 (J) | | — | 640 | 40 | <10 | | —[c] |
| H076 (K) | | 7, 1 | 640 | 20 | 40 | | — |
| H107 (L) | | 6, 1 | 1,280 | 80 | 10 | | — |
| H118 (M) | DEN2 prototype | 2, 6 | 160 | 1,280 | <10 | DEN2 prototype | — |
| H119 (N) | | 2, 7 | 80 | 1,280 | 20 | | — |
| H120 (O) | | 4, 4 | 80 | 1,280 | 10 | | — |
| H145 (P) | | 3, 6 | 80 | 1,280 | <10 | | — |
| H147 (Q) | DEN2/DEN4 chimera | 3, 3 | 20 | 640 | <10 | DEN2 prototype | — |
| H160 (R) | | 6, 1 | 80 | 640 | <10 | | — |
| H168 (S) | | 1, 6 | 80 | 640 | 20 | | — |
| H174 (T) | | 4, 2 | 80 | 640 | 20 | | — |

[a] DEN4 2A was derived from DEN4 cDNA clone 2A; the DEN1/DEN4 chimera contains the C, pre-M, and E genes of DEN1 WP replacing the corresponding genes of DEN4; the DEN2/DEN4 chimera contains the pre-M and E genes of DEN2 prototype NGC replacing the corresponding genes of DEN4.
[b] The titer of neutralizing antibodies in serum samples collected 8 weeks after immunization was determined by the 50% plaque reduction neutralization test (22). DEN1 WP, DEN2 NGC prototype, or DEN4 strain 814669, approximately 50 PFU in duplicate, was used in the test.
[c] Viremia was detected on day 6 in the initial assay but not in the subsequent experiment, in which the serum samples for the group were retested.

lenge DEN1 serum neutralizing antibody titer of 1:20, which is 32- to 64-fold lower than that of the other monkeys in the DEN1/DEN4 group. Another monkey (monkey J) in the group was initially found to be viremic by plaque assay on the sixth day postchallenge. However, in a subsequent attempt in which all postchallenge serum samples from monkeys immunized with DEN1/DEN4 were tested a second time to confirm viremia, this animal was negative.

**Simultaneous immunization with two chimeric viruses.** The encouraging results described above led us to perform a sec-

TABLE 2. Analysis of viremia and antibody response in monkeys following simultaneous immunization with a mixture of chimeric viruses and evaluation of protection against challenge with parental virus[a]

| Monkey no. (designation) | Immunizing virus | Viremia (day postinoculation of onset, days of duration) | | | Reciprocal titer at 8 weeks | | | Challenge virus | Viremia (day postchallenge of onset, days of duration) |
|---|---|---|---|---|---|---|---|---|---|
| | | D1/4 | D2/4 | D4 | D1 | D2 | D4 | | |
| H290 (A) | DEN4 2A | | | 5, 3 | 80 | 40 | 40 | DEN1 WP | 5, 2 |
| H294 (B) | | | | 4, 4 | 40 | 40 | 640 | | 5, 2 |
| H301 (C) | DEN4 2A | | | 6, 1 | 40 | 40 | 400 | DEN2 prototype | 5, 2 |
| H288 (D) | | | | 5, 1 | 40 | 80 | 400 | | 4, 3 |
| H296 (E) | DEN1/DEN4 + DEN2/DEN4 | — | 5, 5 | | 320 | 640 | <10 | DEN1 WP | — |
| H195 (F) | | — | 6, 4 | | 320 | 640 | <10 | | — |
| H213 (G) | | — | — | 320 | 320 | 40 | | — | |
| H214 (H) | | — | 5, 4 | | 320 | 640 | 160 | | — |
| H271 (I) | DEN1/DEN4 + DEN2/DEN4 | — | 7, 2 | | 320 | 320 | 40 | DEN2 prototype | — |
| H300 (J) | | — | 6, 3 | | 320 | 320 | <10 | | — |
| H273 (K) | | — | 5, 4 | | 320 | 640 | <10 | | — |
| H214 (L) | | — | 8, 1 | | 160 | 320 | ND[b] | | — |

[a] D1/D4 and D2/D4, the DEN1/DEN4 and DEN2/DEN4 chimeras, respectively; D4, DEN4 2A. See also Table 1, footnotes *a* and *b*.
[b] ND, not determined.

ond experiment, in which monkeys were immunized simultaneously with equal mixtures of the chimeric DEN1/DEN4 and DEN2/DEN4 viruses at a total dose of $3 \times 10^5$ PFU, while the control monkeys were inoculated with DEN4. Nine weeks later, half of each group was challenged with DEN1 and the other half was challenged with DEN2.

**(i) Viremia following immunization.** Monkeys given DEN4 became viremic for a period of 1 to 4 days (Table 2). Seven of the eight monkeys inoculated with the DEN1/DEN4 and DEN2/DEN4 chimeric viruses became viremic with the DEN2/DEN4 virus. This was determined by using DEN2 HMAF and a DEN2-specific monoclonal antibody, 3H5. The DEN2/DEN4 viremia was of 1 to 5 days in duration. Viremia with chimeric DEN1/DEN4 was not detected. The absence of detectable DEN1/DEN4 viremia is consistent with the minimal viremia observed in the first experiment, in which two of four monkeys immunized with DEN1/DEN4 did not become viremic, while the other two were positive for only 1 day.

**(ii) Seroresponse to immunization.** Infection with DEN4 induced a higher serum neutralizing antibody response than observed in the first experiment. Three of four monkeys developed a DEN4 neutralizing antibody titer of 1:400 or higher. Low-level cross-reactive antibodies against DEN1 and DEN2 were also detected. Each of the monkeys inoculated with DEN1/DEN4 plus DEN2/DEN4 developed neutralizing antibodies against both DEN1 and DEN2. Regardless of the extent of viremia, each of the monkeys developed a DEN1 titer of 1:160 to 1:320 and a DEN2 titer of 1:320 to 1:640 (Table 2).

**(iii) Response to challenge.** Each of the four monkeys in the DEN4-immunized control group became viremic after challenge with DEN1 or DEN2 at $3 \times 10^5$ PFU per dose (Table 2). Two monkeys whose cross-reactive anti-DEN1 neutralizing antibody titers were measured at 1:80 and 1:40 nevertheless sustained 2 days of DEN1 viremia. The other two monkeys with anti-DEN2 titers of 1:40 and 1:80 were viremic for 2 and 3 days, respectively, following DEN2 challenge. Each of the eight monkeys in the group simultaneously immunized with chimeras DEN1/DEN4 and DEN2/DEN4 remained free of viremia following DEN1 or DEN2 challenge.

Overall, the pattern of viremia response in rhesus monkeys observed in this study is similar to that reported earlier (9, 10, 14, 19). One of these reports indicated that approximately 90% of 122 rhesus monkeys inoculated with DEN1, DEN2, DEN3, or DEN4 at a dose of $10^{3.7}$ to $10^{5.7}$ PFU became viremic within 2 to 6 days of inoculation. In humans, viremia generally commences 2 to 6 days after dengue virus infection and lasts 3 to 4 days (11, 26). There is evidence that reduced infectivity of dengue virus in monkeys, as determined by days of viremia or the peak serum virus titer, may be a marker of attenuation for humans (14, 25). Chimera DEN1/DEN4 caused little or no detectible viremia in both experiments, significantly less than the parental DEN1. DEN1/DEN4 may be attenuated for primates. DEN2/DEN4, on the other hand, gave uneven results. In five of eight monkeys, it produced 4 or more days of viremia, equal in duration to that of prototype DEN2, but in other monkeys, it produced little viremia. Chimeric viruses on the background of the parental DEN4 may be attenuated because of a mixed constellation of dengue virus genes. In our experience, the antibody response to infection with dengue virus has varied for different serotypes and in different experiments. For example, DEN4 induced a weak antibody response in the first experiment, while in the second, higher antibody titers were measured. Infection with DEN1 or DEN2, on the other hand, induced a neutralizing antibody titer of 1:160 or higher in 11 of 12 monkeys. During our previous studies, in which the mouse dengue virus encephalitis model was used, an antibody response to pre-M, E, or NS1 was shown to be sufficient to provide protection when mice were challenged intracerebrally with a homotypic neurovirulent dengue virus mutant (1, 3, 7, 27).

In the present study, we observed that chimeric dengue viruses infected rhesus monkeys and were highly immunogenic when given individually or simultaneously as a bivalent vaccine. These chimeras were effective in inducing protective immunity against challenge with dengue virus of the same serotype. Among 16 monkeys immunized with these viruses in two experiments, only 1 was not protected against homotypic virus challenge. Chimeric dengue viruses, suitably engineered, have a potential for use in a live attenuated virus vaccine. Recently, by using DEN4 cDNA, a viable DEN3/DEN4 chimera has been constructed (6). Also, viable DEN4 mutants containing deletions in the 3′ or 5′ noncoding region have been constructed, and some of these mutants are restricted in virus replication in vitro and in vivo without significant loss of immunogenicity (4, 16). Clearly, these deletion mutants could be used to construct attenuated DEN4 chimeras. The use of all four dengue virus serotypes on the same DEN4 background represents a novel dengue virus vaccine strategy.

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