REPLICATION OF DENGUE VIRUS TYPE 2 IN HUMAN MONOCYTE-DERIVED MACROPHAGES: COMPARISONS OF ISOLATES AND RECOMBINANT VIRUSES WITH SUBSTITUTIONS AT AMINO ACID 390 IN THE ENVELOPE GLYCOPROTEIN

MELINDA J. PRYOR, JILLIAN M. CARR, HELEN HOCKING, ANDREW D. DAVIDSON, PENG LI, AND PETER J. WRIGHT

Department of Microbiology, Monash University, Melbourne, Victoria, Australia; Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia

Abstract. The severity of dengue virus infection ranges from mild fever to dengue hemorrhagic fever and shock syndrome. The association of disease severity with virus replication in monocyte-derived macrophages (MDMs) was examined for dengue virus type 2 (DEN-2) isolates from Asia or America. Additionally, we constructed DEN-2 recombinant viruses with substitutions at residue 390 in the envelope glycoprotein (E390) because this residue is linked with the region of virus origin. Comparisons of virus yields of 3 isolates failed to show a correlation with clinical disease. However, the American strain did not replicate as well as the 2 Asian strains. For the recombinant viruses, substitution of Asn (Asian) at E390 with Asp (American) resulted in decreased ability to replicate in MDMs. These results are consistent with the proposal that the lack of association of native American DEN-2 strains with severe disease is linked to reduced ability to replicate in MDMs, and that Asp at E390 may contribute to this reduction.

INTRODUCTION

Dengue is widespread through tropical and subtropical regions of the world, with ~2.5 billion people at risk. Dengue fever (DF) is the more common and less severe form of disease, whereas dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) occur less frequently, but are associated with 1–5% mortality, depending on the availability of treatment. An increase in vascular permeability, hemorrhage, and involvement of the liver accompanies DHF/DSS. Basic studies on dengue and its transmission were completed before 1930. Work intensified after World War II and the occurrence of DHF in Southeast Asia. Although studies have continued for >50 years, there is still no commercially available vaccine, nor is there a consensus on the mechanisms by which dengue virus causes DHF/DSS. The lack of a suitable animal model for the disease has contributed to this slow progress, as have the complex interactions among the 4 serotypes of virus, strain variation within serotypes, infection history, and the interplay between the virus and the host immune responses. Several aspects of dengue disease, including replication, immunity, pathogenesis, epidemiology, and prevention, have been recently reviewed.

Examination of tissue from infected people detected dengue antigen in cells of the reticuloendothelial system, including macrophages and hepatocytes. Infectious virus has been detected in a variety of tissues, including peripheral blood mononuclear cells, and cultured monocytes or macrophages support replication of dengue virus. Cells of the monocyte lineage are central to the proposal of antibody-dependent enhancement of infectivity as the basis for the onset of DHF/DSS. Because the majority of patients with DHF/DSS have evidence of previous infection with a different serotype, it was proposed that enhanced infectivity via the binding of virus-antibody complexes to Fc receptors on monocytes or macrophages leads to increased virus yields and correspondingly more severe disease. In this scenario, the enhancing antibody is cross-reacting and nonneutralizing, and is produced after previous infection with a different serotype. An expanded model of dengue immunopathogenesis includes both antibody-dependent enhancement and activation of cross-reactive T lymphocytes, leading to elevated levels of cytokines and other mediators of vascular permeability as important contributors to the disease process.

Initially, dengue virus type 2 (DEN-2) was believed to be the predominant serotype associated with secondary infections and DHF/DSS. However, other serotypes have also been implicated. It is likely that genetic variation among serotypes results in strains that differ in their capacity to induce DHF/DSS. The existence of genetic diversity in dengue viruses is well documented for all serotypes, particularly DEN-2, and has been analyzed at the genomic level by T1 RNase fingerprinting (topotypes assigned) and nucleotide sequencing (genetic subtypes assigned). This variation may be reflected in the ability of a strain to replicate in leukocytes and monocyte or macrophages, either in the presence or absence of enhancing antibody. Epidemiological evidence for viruses differing in capacity to cause DHF comes from the comparison of strains isolated in Asia and the Americas. The detection of DHF/DSS in the Caribbean and the Americas is linked with the first isolations of Asian strains of DEN-2 in these regions.

Most recently, the analysis of full genomic sequences of DEN-2 strains has identified several nucleotide changes in translated and untranslated regions that distinguish strains of Asian and American origins—that is, strains associated with DF/DHF/DSS, or those exclusively associated with DF. With the availability of genomic-length cDNA for DEN-2 from which we can generate virus (an "infectious clone"), it is now possible to test the effects of these specific nucleotide differences on virus replication in relevant target cells: monocytes or macrophages.

Here we describe the replication in primary human monocyte-derived macrophages (MDMs) of DEN-2 strains that were isolated in Thailand or the Caribbean and that were associated with either DHF or DF. The aim of these studies was to seek possible correlations between virus growth, geographical origin of the viral strain, and disease severity. We also describe the replication of recombinant viruses prepared by use of an infectious clone on the basis of the DEN-2 New Guinea C strain. Because the Thai strains have Asn at ami-
no acid position 390 in the envelope glycoprotein (E390) and the native strains from the Americas have Asp, recombinant viruses were constructed to determine the effect of E390 substitutions on viral growth in primary human MDMs.

**MATERIALS AND METHODS**

**Cell lines and viruses.** We grew BHK-21 and *Aedes albopictus* C6/36 cells at 37°C and 28°C, respectively, in Eagle basal medium containing 7.5% heat-inactivated fetal calf serum. Stocks of all viruses were grown and titered by plaque assay in C6/36 cells at 28°C. Harvests from growth experiments that used MDMs were also titered in C6/36 cells.29

Cells for plaque assays were grown in 6-well trays, and assays were performed in duplicate. The 95% confidence limits for the mean plaque count n were calculated as $2\sqrt{n}$, where n is the number of experiments. The reduction in titer ($\log_{10}$) of a virus relative to MON601 was determined by subtraction of its titer ($\log_{10}$) from the MON601 titer ($\log_{10}$) in the same experiment. The statistical significance of pairwise comparisons of plaque counts and titer ($\log_{10}$) reductions was assessed by Student’s t-test. Results were considered significant at $P < 0.05$.

The strains of DEN-2 used are listed in Table 1. In this article, we refer to D80-100, PUO-312, and PR152 as natural isolates or strains and MON601 and its derivatives as recombinant viruses. The topotypes21 and subtypes22 of the viruses given in Table 1 are based on T1 RNase fingerprinting and genome sequencing, respectively.

**Preparation and infection of MDMs.** Peripheral blood mononuclear cells were prepared by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) of buffy coat blood packs from healthy donors provided by the Australian Red Cross Blood Bank. Monocytes were selected from peripheral blood mononuclear cells by adherence to tissue culture plastic for 1 hr.30 Nonadherent cells were further depleted of monocytes by a second round of adherence. Adherent cells were vigorously washed with warm Hanks balanced salt solution supplemented with calcium and magnesium (Gibco BRL, Gaithersburg, MD). Adherent monocytes from 2–4 different blood donors were detached, pooled, cultured in Dulbecco minimal essential medium (DMEM) containing 10% (v/v) fetal calf serum and 7.5% (v/v) human serum (MDM medium) and allowed to differentiate in culture into macrophages. On the fourth day after isolation, MDMs were detached by gentle scraping in Hanks balanced salt solution, and the purity of MDMs preparations was assessed by Wright-Giemsa staining and CD14 flow cytometry. Adherent MDMs were 85–90% CD14+ at the time of infection.

Cells were plated at $2 \times 10^5$ cells per well in a 48-well plate and allowed to adhere for 2 days before infection. On the day of infection (Day 6 after isolation), the culture medium was removed and the cells washed in serum-free DMEM. Cells were infected with DEN-2 at a multiplicity of infection of 1 in a volume of 100–400 µL for 90 min at 37°C with intermittent rocking of the plate. The infection volume varied among experiments because of differences in the infecting virus titer, but was constant for a given experiment. Mock infections were performed as above with an
equivalent volume of serum-free DMEM. After infection, the supernatant was removed, cells were washed 3 times with serum-free DMEM, 400 μL of fresh MDM medium was added, and a sample of 200 μL was taken (0 hr after infection) with replacement of 200 μL of medium. Further samples were taken at 8, 24, 48, and 72 hr and in some experiments also at Day 5 and Day 9. Culture medium was sampled at each time point, clarified by centrifugation, and immediately stored at −70°C. Total cell lysates were prepared by first washing cells in cold phosphate-buffered saline, scraping cells into 200 μL phosphate-buffered saline, and then freezing at −70°C.

**Insertion of mutations into genomic length cDNA.** The plasmid pDVWS501 containing genomic length DEN-2 cDNA (New Guinea C strain) and its sequence was described previously29 (GenBank accession number AFO38403). There are 22 nucleotide differences between the pDVWS501 sequence and the first published New Guinea C sequence.30 Two differences are in the 3′ untranslated region; nucleotide 10321 of pDVWS501 is T rather than C, and an extra G is present at nucleotide 10415 (original numbering31). To produce plasmid pDVWS601 from pDVWS501, the change T→C was made at nucleotide 10321 and the extra G at nucleotide 10415 was deleted. In addition, HpaI and MluI sites were introduced at nucleotide 7404 (T→G) and nucleotide 9732 (C→A), respectively, by overlap PCR32 that used appropriate subfragments of genomic cDNA (Butcher R, unpublished data). These sites were not used in the experiments reported here.

The virus derived from pDVWS601 was designated MON601, following our earlier nomenclature. To change the residue at E390 (encoded by nucleotides 2104 to 2106) from Asn (AAC) to Asp (GAT), Ser (TCC), or Ala (GCT), a fragment spanning the 1847SpH1 and 2427NheI sites was produced by overlap PCR that used suitable flanking and mutagenic primers. The fragment was cleaved with SpH1 and NheI and cloned into the corresponding sites of pDVWS601. Two bacterial clones containing modified plasmids were selected for each of the Asp and Ser mutations, but only one for Ala. The presence of the required mutations was confirmed by sequencing. The viruses derived from these clones were designated 601D#11, 601D#14, 601S#5, 601S#8, and 601A#1, respectively.

**Production of virus from genomic-length cDNA.** The procedures for transcription of RNA from plasmids, electroporation into BHK-21 cells, and passaging of virus in *Aedes albopictus* C6/36 cells were described previously.29,33 The culture medium from BHK-21 cells that had been electroporated with transcribed RNA and maintained for 5–7 days was passaged twice in C6/36 cells to prepare virus stocks for infection of MDMs. The stocks were assayed in C6/36 cells and analyzed by reverse transcriptase–polymerase chain reaction, and both strands of cDNA were sequenced by established procedures29 without cloning. The GenBank accession numbers are AF264053 and AF264054, respectively. The GenBank accession numbers of other viruses shown in Table 2 are M29095 (New Guinea C), AF038403 (New Guinea C/MON601), and M244445 (D80-100).

### RESULTS

It has been proposed that severe dengue disease (DHF) may result from increased virus replication in monocyte or macrophages compared with replication during DF.9,34 Antibody-dependent enhancement of infectivity may be an important factor,9 but viruses may also differ intrinsically in their ability to grow in these cells.20 Initial evidence suggesting such differences for DEN-2 replication in leukocytes both with and without enhancing antibody was obtained previously.23 We therefore set out to test directly the hypothesis that viruses associated with DHF may replicate to higher titers in primary monocytes or macrophages than viruses associated with DF. Three DEN-2 viruses were used (Table 1): 2 Thai strains (PUO-312 and D80-100) that were isolated during a DF/DHF/DSS epidemic in 198027 and one Caribbean strain (PR152) that was isolated before DHF/DSS was described in the Americas. The E genes of PUO-312 and PR152 were sequenced and the deduced amino acid sequences compared with published data22 (Table 2). Viruses PUO-312 and D80-100 both belong to genetic subtype III, and PR152 belongs to subtype V.

**Growth curves of viruses in MDMs.** The virus MON601 was used initially to determine the time course of infection. Adherent MDMs were infected 6 days after isolation. The culture medium and cells were harvested separately at various times after infection up to 72 hr; cells were lysed by freeze-thawing to release cell-associated virus, and virus yield was assayed by plaque titer in *A. albopictus* C6/36 cells. The virus titer in the supernatant medium peaked at 48 hr after infection (Figure 1A). The titer of residual cell-associated virus was less than in culture medium. Therefore,

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**Table 2**

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>NGC</th>
<th>NGC*</th>
<th>PUO-312</th>
<th>D80-100*</th>
<th>PR152</th>
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<tr>
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<td>E</td>
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<td>−</td>
<td>I</td>
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* NGC = New Guinea C strain.
† Residue identical to NGC (Irie and others31).

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Comparison of virus strains. To test whether virus from a patient with severe disease was associated with enhanced replication in human MDMs, the yields of DEN-2 strains D80-100, PUO-312, and PR152 were examined. The titer of virus released into the medium was measured at 48 hr after infection. A summary of the results is presented in Table 3 (experiments 1–5). A fresh preparation of primary MDMs was used in each numbered experiment. To account for potential donor variation in dengue susceptibility, each preparation of MDMs was derived from monocytes combined from multiple donors. Because the inhibition of dengue virus replication in monocytes by endotoxin has been described, virus stocks and culture media from each experiment were monitored for endotoxin. The concentration of endotoxin was always < 25 pg/mL, below the reported inhibitory level. In each experiment, some cells were infected with MON601 as a reference to facilitate comparison between experiments that used different preparations of primary cells. The titer of MON601 varied over a range of 1 log10 and was sufficiently consistent to engender confidence in methods of cell preparation and infection. The titer of MON601 was also the highest of any strain tested in each experiment.

To compare the replication of the natural isolates between experiments, titers (log10) were subtracted from the MON601 titer (log10) in the same experiment, and the differences are shown in Table 3 (in italics). The reductions in titer were consistent for each virus, with means of 1.87 (PUO-312), 2.23 (D80-100), and 2.75 (PR152) (upper part of Figure 2). These means were used in pairwise comparisons between the viruses (Table 4). The difference between the two 1980 Asian strains PUO-312 and D80-100 was not significant (P > 0.10). However, PR152 replicated significantly less than PUO-312 (P < 0.05), although PR152 compared with D80-100 did not meet the 5% significance level at P < 0.10 (Table 4). As for MON601, none of the primary isolates induced cytopathic effects in MDMs.

There was no correlation between virus growth in MDMs and the severity of disease in the patients from whom the isolates were obtained because D80-100 (DHF) did not replicate better than PUO-312 (DF) and PR152 (DF). However, there was a correlation between virus growth and region of origin, with both Asian strains giving higher yields than PR152. The Asian strains were associated with a DHF/DSS
TABLE 3
Replication of dengue virus type 2 strains and recombinant viruses in macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>MON601</th>
<th>PUO-312</th>
<th>D80-100</th>
<th>PR152</th>
<th>601D#11</th>
<th>601D#14</th>
<th>601S#5</th>
<th>601S#8</th>
<th>601A#1</th>
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<tbody>
<tr>
<td>1</td>
<td>7.2 ± 1.4</td>
<td>2.6 ± 0.8</td>
<td>9.0 ± 4.8</td>
<td>4.8</td>
<td>1.7</td>
<td>2.1</td>
<td>4.4</td>
<td>4.4</td>
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<tr>
<td>2</td>
<td>6.4 ± 1.4</td>
<td>6.5 ± 1.4</td>
<td>5.3 ± 3.6</td>
<td>5.3</td>
<td>5.6</td>
<td>0.6</td>
<td>1.2</td>
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</tr>
<tr>
<td>3</td>
<td>4.5 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>5.3 ± 3.6</td>
<td>2.5</td>
<td>0.6</td>
<td>1.6</td>
<td>0.6</td>
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<td>4</td>
<td>6.4 ± 1.4</td>
<td>6.5 ± 1.4</td>
<td>5.3 ± 3.6</td>
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<tr>
<td>5</td>
<td>9.6 ± 5.0</td>
<td>10^4</td>
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<tr>
<td>6A</td>
<td>9.3 ± 4.8</td>
<td>2.2 ± 0.8</td>
<td>4.3 ± 1.0</td>
<td>4.3 ± 1.0</td>
<td>4.3 ± 1.0</td>
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<tr>
<td>6B</td>
<td>2.1 ± 0.8</td>
<td>4.1 ± 1.0</td>
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* Each experiment used a different preparation of freshly prepared monocyte-derived macrophages. In experiments 6A and 6B the same cells were used, but were infected in 250 µL (A) or 400 µL (B). Data from experiments 1 and 5 are shown in Figure 1B and data from experiment 6A in Figure 1C.
† Reduction in titer compared with MON601 in the same experiment expressed as log10 (log10 MON601 titer minus log10 virus titer).
‡ Reduction in titer compared with MON601 in the same experiment expressed as log10 (log10 MON601 titer minus log10 virus titer).

Comparison of recombinant viruses. Leitmeyer and others have proposed that amino acid 390 in the E glycoprotein may be an important determinant of DHF. In the majority of sequenced DEN-2 strains, including the New Guinea C and Thailand topotypes (genetic subtypes I and III), Asn is located at this position; Ser is detected in some strains isolated in the Seychelles and Sri Lanka (subtype IV) and Asp in strains of the Americas topotype (subtype V). The residue His has been reported on 2 occasions. The strains used in these experiments contained either Asn (MON601/ New Guinea C, PUO-312, and D80-100) or Asp (PR152) (Table 2).

It was not possible to assess the role of the residue at E390 in determining growth in macrophages from the preceding experiments because of multiple genetic differences between the strains. The viruses tested were isolated at different times and varied in passage history. They contained other changes in genomic sequence—for example, in the 3’ untranslated region (Pryor M, unpublished data) and elsewhere in the E gene (Table 2), which may also have affected virus replication. As a direct test of the role of E390 in replication in human MDMs, the amino acid at this position was varied in a uniform genetic background (New Guinea C) by mutagenesis of MON601.

The Asn residue at E390 in MON601 was replaced by Asp, Ser, or Ala by mutagenesis of plasmid DNA and virus recovery as described previously. Asp and Ser are naturally occurring residues at this position whereas Ala is not, but is

**FIGURE 2.** Reduction in viral titer compared with MON601 (log10 MON601 titer − log10 virus titer). Each value in Table 3 is represented by a bar. Results for the duplicate clones of 601D and 601S are grouped. Mean values are indicated.
considered a nonconservative substitution. For the changes to Asp and Ser, plasmids from 2 clones (11 and 14, and 5 and 8, respectively) were used to produce recombinant viruses. The titers obtained at 48 hr after replication in human MDMs (experiments 5 to 7, Table 3) and log_{10} differences from the parental MON601 (Table 3 and Figure 2) are shown. All 601-derived viruses containing a substitution at E390 replicated less well than MON601 in every experiment.

The results were analyzed in 2 ways. First, the reductions in titers (log_{10}) (means of 0.55, 0.78, and 1.00 as shown in Figure 2) were assessed for significance in pairwise comparisons between the recombinant viruses with substitutions at E390; no significant differences were detected (P > 0.05 in each case; values of P not shown). Second, in order to make comparisons with the parental MON601 virus, the titers (expressed as plaque-forming units per milliliter [pfu/mL]) in experiments 5–7 were averaged for each virus, again pooling results for viruses derived from duplicate clones. The results of pairwise comparisons are shown in Table 5. This approach of using the experimental titers (pfu/mL) rather than the reductions in titer (log_{10}) bypassed the standardization relative to MON601 that was introduced to provide for any variation among the primary cultures of MDMs. However, again, no significant differences were detected between the viruses 601D, 601S, and 601A (P > 0.05), but the decreases in titer of 601D and 601S relative to MON601 were significant (P < 0.025 and P < 0.05, respectively). The titers of MON601 and 601A were not significantly different (P > 0.05). These results strongly suggest that Asn at E390 was optimal for replication in human MDMs.

**DISCUSSION**

Studies on the virulence of dengue viruses are problematic because of the lack of a suitable animal model. The induction of encephalitis in mice has been widely used, but encephalitis is extremely rare in humans. Infected nonhuman primates show no symptoms, viremia can be detected. An alternative approach is the use of virus replication in cultured primary human cells of the monocyte/macrophage lineage as a possible marker of viral virulence. It is a simplified in vitro system and has the advantage of using cells that are a major site of virus replication in the human host and are important in the models of immunopathogenesis of dengue.

The growth of DEN-2 in human monocytic cell lines has been previously documented. Dengue virus infection of monocytes is reportedly optimal with enhancing antibody, but as shown in this and other studies, antibody enhancement of infection in MDMs is not required to obtain high level virus production. In the current study, the yields (pfu/mL) obtained for MON601 (New Guinea C strain) were as high or higher than those reported previously for primary monocyte cultures and most myelomonocytic cell lines. The peaking of yields at 48 hr after infection was earlier than previously reported for low multiplicity of infection (0.05), but at the same time as previous studies that used higher multiplicity of infection (3 or 10). The production of intracellular virus paralleled that for secreted virus. The titer of MON601 was the highest achieved for all viruses in each experiment. The reason for this is unknown. The virus is essentially the New Guinea C strain, which has been extensively passaged in mice and cell culture since isolation in 1944, and yet it replicated better in primary human macrophages than the isolates PUO-312, D80-100, and PR152 of lower passage levels. This finding indicates that passage through mice and cultured cells does not necessarily reduce replication in primary human cells.

Previous studies that used DEN-2 and peripheral blood leukocytes or monocytes suggested a correlation between disease severity and virus growth. Viruses retained their relative growth properties in the presence or absence of enhancing antibodies. These studies used a number of virus strains, but in the first study, samples from only 2 cell donors were used. In our experiments, only 3 virus strains were used; however, the cells were prepared from multiple donors and were differentiated rather than freshly prepared blood monocytes. The MDMs were infected in the absence of enhancing antibodies to exclude variation in virus-antibody interactions as a possible explanation for any observed growth differences. In our studies, PUO-312 replicated similarly to D80-100 (Figure 2). This observation was not necessarily inconsistent with a link between disease severity and virus growth. Both viruses were isolated in 1980 in Thailand, and sequencing of the E gene (Table 2) showed that both belong to genetic subtype III. Each strain may well have been associated with cases of DHF and DF during the 1980 epidemi. However, our results are consistent with the proposal that strains from the Americas (genetic subtype V), which are not associated with severe dengue disease, (represented in this study by PR152) replicate less well in macrophages than Asian strains (subtype III). This proposal requires further study of a larger number of viral isolates with low passage history.

The infectious clone of DEN-2 provided the means of assessing specific changes on virus replication in a uniform genetic background. We chose to analyze E390 because this is 1 of 3 regions in the DEN-2 genome identified as possible primary determinants of DHF (the others are the 3' and 5' untranslated regions), which may exert their effects via replicative ability in monocyte or macrophages. The effect on

### Table 5

<table>
<thead>
<tr>
<th>MON601 (1.73 × 10^5)*</th>
<th>601D (2.79 × 10^5)*</th>
<th>601S (1.94 × 10^5)*</th>
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<tr>
<td>601D</td>
<td>0.025 &gt; P &gt; 0.01</td>
<td>P &gt; 0.25</td>
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<tr>
<td>601S</td>
<td>0.05 &gt; P &gt; 0.025</td>
<td>0.25 &gt; P &gt; 0.05</td>
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<tr>
<td>601A (4.05 × 10^4)*</td>
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</tbody>
</table>

* Mean titer in plaque-forming units per milliliter determined from experiments 5–7. Table 3. Titers for viruses from duplicate clones were combined.
viral tropism of single amino acid changes in the envelope glycoprotein is well documented for a number of viruses, including DEN-2. Furthermore, E390 is located in domain III of the flavivirus E glycoprotein, a domain linked to cell attachment, tropism, and virulence. The recombinant viruses derived from MON601 and containing Asp, Ser, or Ala in place of Asn at E390 all replicated relatively well, but titers of the parental virus (Asn) were consistently highest. A reproducible and significant reduction in titer compared with MON601 was observed for 601D and 601S. None of the recombinant viral yields were as low as PR152. We were particularly interested in the reduction in titer shown by 601D because Asp is the residue at E390 in strains of the Americas topotype. The consistent reduction in titer observed in 4 experiments that used 2 different preparations of MDMs and independently derived viruses from 2 different clones is strong evidence that Asp at this position does in fact reduce replication in MDMs, although it is not possible to assess the consequences of this reduction for the pathogenesis of dengue disease.

In summary, we have established a well-defined cell culture and infection system for DEN-2 that leads to reliably high viral growth in relevant human primary cells in the absence of enhancing antibody. By use of an infectious clone of DEN-2, the amino acid at E390 was identified as a determinant of replicative ability in MDMs. Further experiments will target other genetic differences between naturally occurring strains—for example, in the 3' and 5' untranslated regions—by the production of recombinant viruses with defined nucleotide changes.

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Authors’ addresses: Melinda J. Pryor, Andrew D. Davidson, and Peter J. Wright, Department of Microbiology, PO. Box 53, Monash University, Victoria 3800, Australia. Jillian M. Carr, Helen Hocking, and Peng Li, Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Box 14 Rundle Mall PO., Adelaide, South Australia 5000, Australia.

Reprint requests: Peter J. Wright, Department of Microbiology, PO. Box 53, Monash University, Victoria 3800, Australia, Telephone: 61-3-9905-4828, Fax: 61-3-9905-4811 (e-mail: Peter.Wright@med.monash.edu.au).

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