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

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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. 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 response. 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 and nucleotide sequencing. This variation may be reflected in the ability of a strain to replicate in leukocytes and monocyte or macrophages, either in the absence or presence 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, recombination 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. 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 were calculated as $2\sqrt{n/m}$, 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 topotypes and subtypes 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) ofuffy 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. Nonadherent cells were further depleted of monocytes by a second round of adherence. Adherent cells were vigorously washed with warm Hank’s 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 Hank’s 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
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 $^{130}$SpH1 and $^{242}$NheI 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 $^{130}$C6/36 cells were described previously. The culture medium from BHK-21 cells that had been electroporated with transcribed RNA and maintained for 5–7 days 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 previously. There are 22 nucleotide differences between the pDVWS501 sequence and the first published New Guinea C sequence. 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 numbering). 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, $^{130}$IpuI and $^{130}$MluI sites were introduced at nucleotide 7404 (T→G) and nucleotide 9732 (C→HpaI and 2427 SphI sites were introduced at nucleotide 7404 (T→G) and nucleotide 9732 (C→A), respectively, by overlap PCR that used appropriate subfragments of genomic cDNA (Butcher R, unpublished data).

These sites were not used in the experiments reported here.

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### Production of virus from genomic-length cDNA

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### 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. Antibody-dependent enhancement of infectivity may be an important factor, but viruses may also differ intrinsically in their ability to grow in these cells. Initial evidence suggesting such differences for DEN-2 replication in leukocytes both with and without enhancing antibody was obtained previously. 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 1980 and one Carib 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 data (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 $^{130}$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,
FIGURE 1. Replication curves of natural dengue virus type 2 (DEN-2) isolates and recombinant viruses in macrophages. (A) Yields of released (medium) and cell-associated (lysate) virus from infection of monocyte-derived macrophages (MDMs) with MON601. (B) Yields of released virus from infection with DEN-2 natural isolates. (C) Yields of released virus from infection with recombinant viruses derived from MON601. Titers shown with 95% confidence limits. Data at 48 hr after infection are also presented in Table 3, in experiments 1 (PUO-312 and D80-100), 5 (PR152), and 6A (601D#14, 601S#8, and 601A#1).

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 log_10 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 (log_{10}) were subtracted from the MON601 titer (log_{10}) 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

in subsequent experiments, only the culture medium was harvested. Samples were collected immediately after the wash after infection (time 0) and at least 3 times up to 72 hr. In all cases, virus titers peaked at 48 hr. Similar replication profiles were demonstrated for isolates and recombinant viruses (examples are shown in Figure 1B, C). The virus titers in samples collected after 72 hr continued to drop, and no virus was detectable by plaque assay at 9 days after infection (data not shown). Infection of MDMs by dengue virus was noncytopathic with no visual cytopathic effects detected.
epidemic in Thailand, and it is therefore possible that PUO312 and D80-100 may have caused either DF or DHF in patients, whereas strains of American origin such as PR152 have not been linked with DHF/DSS.

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, 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). 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 virus 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 determining growth in macrophages from the preceding experiments because of multiple genetic differences between the strains.

![Reduction in viral titer (log_{10}) compared with MON601](image)

**Figure 2.** Reduction in viral titer compared with MON601 (log_{10} MON601 titer − log_{10} 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.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MON601</td>
</tr>
<tr>
<td>1</td>
<td>(7.2 ± 1.4)</td>
</tr>
<tr>
<td>2</td>
<td>(1.5 ± 0.6)</td>
</tr>
<tr>
<td>3</td>
<td>(6.4 ± 1.4)</td>
</tr>
<tr>
<td>4</td>
<td>(1.5 ± 0.6)</td>
</tr>
<tr>
<td>5</td>
<td>(2.9 ± 0.8)</td>
</tr>
<tr>
<td>6A</td>
<td>(9.6 ± 5.0)</td>
</tr>
<tr>
<td>6B</td>
<td>(9.3 ± 4.8)</td>
</tr>
<tr>
<td>7</td>
<td>(2.1 ± 0.8)</td>
</tr>
</tbody>
</table>

* 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.

† Virus titer at 48 hr after infection in plaque-forming units per milliliter.
‡ Reduction in titer compared with MON601 in the same experiment expressed as log_{10} (log_{10} MON601 titer minus log_{10} virus titer).

**Table 3**

Replication of dengue virus type 2 strains and recombinant viruses in macrophages

<table>
<thead>
<tr>
<th>Virus</th>
</tr>
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<tbody>
<tr>
<td>MON601</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1.00</td>
</tr>
<tr>
<td>0.78</td>
</tr>
<tr>
<td>0.55</td>
</tr>
<tr>
<td>0.05</td>
</tr>
<tr>
<td>0.03</td>
</tr>
<tr>
<td>0.01</td>
</tr>
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</table>

Values of P attained in pairwise comparisons between virus isolates

<table>
<thead>
<tr>
<th>Virus</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUO-312 (1.87)*</td>
<td>D80-100 (2.23)*</td>
</tr>
<tr>
<td>D80-100</td>
<td>0.50 &gt; P &gt; 0.10</td>
</tr>
<tr>
<td>PR152 (2.75)*</td>
<td>0.05 &gt; P &gt; 0.02</td>
</tr>
<tr>
<td>0.10 &gt; P &gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Mean reduction in viral titer (log_{10}) from Figure 2.
considered a nondisruptive substitution.\textsuperscript{38} 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\textsubscript{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\textsubscript{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\textsubscript{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 at the same time as previous studies that used DEN-2 and peripheral blood leukocytes\textsuperscript{27} or monocytes\textsuperscript{26} suggested a correlation between disease severity and virus growth. Viruses retained their relative growth properties in the presence or absence of enhancing antibodies.\textsuperscript{27} 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\textsuperscript{22}. Each strain may well have been associated with cases of DHF and DF during the 1980 epidemic. However, our results are consistent with the proposal that strains from the Americas (genetic subtype V), which are not associated with severe dengue disease\textsuperscript{27,24,28} (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.\textsuperscript{24}

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),\textsuperscript{24} which may exert their effects via replicative ability in monocyte or macrophages. The effect on

### Table 5

Values of \(P\) attained in pairwise comparisons between recombinant viruses and MON601

<table>
<thead>
<tr>
<th></th>
<th>MON601 (1.73 × 10\textsuperscript{9})*</th>
<th>601D (2.79 × 10\textsuperscript{9})*</th>
<th>601S (1.94 × 10\textsuperscript{9})*</th>
</tr>
</thead>
<tbody>
<tr>
<td>601D</td>
<td>0.025 &gt; (P &gt; 0.01)</td>
<td>(P &gt; 0.25)</td>
<td>(P &gt; 0.05)</td>
</tr>
<tr>
<td>601S</td>
<td>0.05 &gt; (P &gt; 0.025)</td>
<td>(P &gt; 0.25)</td>
<td>(P &gt; 0.05)</td>
</tr>
<tr>
<td>601A (4.05 × 10\textsuperscript{9})*</td>
<td>0.25 &gt; (P &gt; 0.05)</td>
<td>0.25 &gt; (P &gt; 0.05)</td>
<td>0.25 &gt; (P &gt; 0.05)</td>
</tr>
</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,13,14 including DEN-2. Furthermore, E390 is located in domain III of the flavivirus E glycoprotein, a domain linked to cell attachment, tropism, and virulence.15,16 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.22,24 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 indeed 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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