Isolation of a Temperature-Sensitive Dengue-2 Virus Under Conditions Suitable for Vaccine Development

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Received for publication 21 May 1976

Dengue virus, type 2, in viremic human sera and after passage in cell cultures produces mixtures of small and large plaques when assayed in LLC-MK₂ cells. Clones of dengue virus type 2 obtained by plaque selection in primary green monkey kidney cell cultures were tested for temperature sensitivity in vitro and for virulence by intracerebral inoculation of suckling mice. Sublines of a small-plaque clone were found to have lower nonpermissive temperatures than the parent virus by both plaque formation and release of infectious virus into the culture media. Small-plaque sublines were significantly less virulent in suckling mice than was the parent virus. Sublines from a large-plaque clone were not temperature sensitive and closely resembled parent virus mixed-plaque morphology. When small-plaque sublines were serially passaged using undiluted inocula, reversion occurred as evidenced by the appearance of large plaques and return of mouse virulence. Small-plaque virus could be maintained through several serial passages without reversion by using low-input inocula. Desirable passage history as well as temperature-sensitive and attenuation characteristics of the S-1 small-plaque subline make it appear suitable as a vaccine candidate virus.

Passage in cell culture has resulted in reduced in vivo virulence for numerous flaviviruses. These include yellow fever (11), West Nile, and Russian spring-summer encephalitis (8), Central European tick-borne encephalitis (4), Japanese encephalitis (3), and Kyasanur forest (7) viruses. Plaque selection has been used as a final step in the purification of many of these viruses; however, plaque size and virulence have been related only rarely. Mayer (4) described a small-plaque variant of Central European tick-borne encephalitis virus obtained from a persistently infected human amnion cell culture. The small-plaque variant had reduced virulence for mice by the peripheral route when it was compared with the parent virus. Paul (7) obtained a spontaneous variant of Kyasanur forest virus by repeated passage in monkey kidney epithelial cell cultures. In addition to small-plaque and lesser in vivo virulence markers, replication of the variant was restricted at 40°C, a permissive temperature for the parent virus.

Mixed populations of large and small plaques have been frequently found in wild dengue virus type 2 (DEN-2) isolates from humans and mosquitoes in both Asia and the Caribbean region. Previous studies with DEN-2 demonstrated that large- and small-plaque clones could be readily derived from isolates in first cell culture passage; the clones were identical by neutralization tests, but the small-plaque clone had a markedly reduced virulence for suckling mice (P. K. Russell and P. Sukhavachana, unpublished data).

Many naturally occurring and induced temperature-sensitive mutants of other types of viruses have been associated with lower animal virulence (2). Indeed, a lower permissive temperature of replication than that of the parent virus has been associated with some viruses currently in use (1) and which have been proposed for use (6, 12, 13) as live-virus vaccines. This paper describes the isolation and purification of small- and large-plaque clones from a human serum isolate of DEN-2 virus. Plaque size is correlated with temperature sensitivity and with suckling mouse virulence for these clones.

MATERIALS AND METHODS

Virus. DEN-2 virus, PR-159 strain, was recovered from serum obtained in Puerto Rico on 19 August 1969 from a 31-year-old male patient with uncomplicated dengue fever. The serum was free of bacteria on culture, and radioimmunoassay for hepatitis B
surface antigen was negative. The virus was identified as DEN-2 by plaque reduction neutralization tests using reference antisera and was found to be antigenically identical to the PR-109 strain from the same epidemic (9).

Cells. Primary green monkey kidney (PGMK) cells were obtained from Lederle Laboratories, Pearl River, N.Y., as frozen suspensions. Lots of cells were tested by the supplier for the presence of simian adenovirus agents by inoculation of growth fluids in PGMK, Lederle 130 human diploid cells, and BSC-1 cells. Hemadsorption tests using guinea pig erythrocytes were also performed on monolayers of the pretested PGMK cells prior to their certification. Certified PGMK cells were grown in Eagle minimal essential medium containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, Md.), 2 mM L-glutamine, and 100 μg of streptomycin sulfate and 50 μg of neomycin sulfate per ml. All sera used in cell culture media were thoroughly screened for adventitious agents and bacteriophage prior to use. Upon formation of a monolayer, the PGMK cells were used immediately, or a 1:2 subpassage was made and secondary cell monolayers were used for virus passage and plaqueing. Growth medium with 2 or 5% fetal bovine serum was used to maintain confluent cell monolayers for virus passage.

A continuous line of rhesus monkey kidney (LLC-MK<sub>2</sub>) cells was grown in medium 199 containing 20% fetal bovine serum. The LLC-MK<sub>2</sub> cell line was subpassaged weekly and used for plaque assay of DEN-2 virus.

Plaque assays. Monolayers of LLC-MK<sub>2</sub> cells in 25-cm<sup>2</sup> plastic flasks were used for plaquing and characterizing DEN-2 virus clones. After virus inoculation and adsorption for 1 to 2 h, flasks received 7 ml of medium 199 containing 10% fetal bovine serum, 36 mM NaHCO<sub>3</sub>, 0.02% diethylaminoethyl-dextran, 0.5% 100× concentrated Eagle basal medium vitamins and amino acids, 1% purified or Noble agar (Difco), and antibiotics.

After incubation at a designated temperature for 6 days, flasks received 4 ml of a second overlay containing 1% Noble agar and 1:6,000 neutral red in Hanks balanced salt solution. Plaque flasks were incubated at 35°C for 4 h and overnight at room temperature before plaques were counted.

For plaquing in PGMK cell monolayers, the first overlay medium contained Eagle minimal essential medium supplemented with 10% fetal bovine serum, 36 mM NaHCO<sub>3</sub>, 0.02% diethylaminoethyl-dextran, 1% Noble agar, and antibiotics. After virus inoculation and adsorption for 1 to 2 h, flasks received 7 ml of the first overlay. After 6 days of incubation at 35°C, a second overlay containing 1% Noble agar and 1:6,000 neutral red in Hanks balanced salt solution was added. Plaques were counted the following day.

Viral replication. Adsorption was carried out for 1.5 h at 35°C, using undiluted virus suspensions in 25-cm<sup>2</sup> plastic flasks (Costar, Cooke Engineering, Alexandria, Va.) containing 2 × 10<sup>6</sup> cells. The inoculum was removed by washing the monolayers three times with Hanks balanced salt solution, and incubation was started at the indicated temperatures 2 h after addition of the virus. Samples of cell culture fluids were removed during the experiments, diluted 1:10 in growth medium, and frozen at -70°C.

Animal inoculation. One- to two-day-old suckling mice were inoculated intracerebrally with 0.02-ml volumes of diluted virus. The mice were observed for sickness and death for 21 days.

### RESULTS

Plaque purification of DEN-2 virus. Six passages of the DEN-2 isolate were made in PGMK cells using undiluted cell culture fluid for the inoculum at each passage. A large stock of seed virus was prepared at the 6th passage. This seed virus is referred to below as the parent virus. The original human serum isolate and the parent seed virus contained similar mixed populations of large (3 to 5 mm)- and small (1 to 2 mm)-plaque-forming virus (Fig. 1). Virus titers gradually increased from 1.7 × 10<sup>4</sup> to 3.0 × 10<sup>6</sup> plaque-forming units/ml during the first six passages in PGMK cells.

Plaque assays in LLC-MK<sub>2</sub> cells were used throughout this study to characterize the plaque morphology of all DEN-2 isolates and clones. All clone purification was performed in certified PGMK cells, where plaque morphology was variable and not distinctive. Plaque picking in PGMK cells was therefore necessarily followed by characterization of the selected clones in the LLC-MK<sub>2</sub> plaquing system. Passage history of the clones is summarized in Table 1.

Initial plaque purification was performed by locating a single, well-isolated plaque after neutral red staining, removing a plug of agar over the plaque, and growing the isolate in PGMK cells under fluid culture media. After two of these cycles, clones designated 5 and 6 were selected for further purification. Sublines of these two clones at the 10th passage level were carried to the 14th passage level by three cycles of plaque purification, the progeny of one plaque being directly plaqued again under agar for each cycle. Table 1 lists the results of the S-1 subline, one of eight sublines of clone 6, and the L-1 subline, one of five sublines derived from clone 5. Although all of the small-plaque sublines retained their small-plaque morphology, this was not true for the progeny of the large-plaque clone. All five sublines of clone 5 after three cycles of plaque purification contained mixed viral populations similar to the parent, passage 6 virus. Whereas small-plaque populations could not be eliminated from these prepa-
TEMPERATURE-SENSITIVE DENGUE-2 VIRUS

Fig. 1. Original DEN-2 human isolate and passage 6, parent virus, plaqued in LLC-MK₂ cells.

In vivo virulence of the purified clonal sublines. Small-plaque sublines had reduced intracerebral virulence for suckling mice when they were compared with the parent virus. Virulence ratios listed in Table 2 measure the infectivity in cell culture compared to the infectivity in suckling mice for the small- and large-plaque sublines and parent virus. Although significant differences in virulence were not seen between small- and large-plaque sublines, the small-plaque sublines were clearly less virulent for suckling mice than the parent virus. Also, the mean survival time for mice inoculated with the small-plaque viruses was at least 2 days greater than for mice inoculated with the parent virus. A higher plaquing efficiency for the small-plaque sublines may account for the lower virulence ratios. However, a longer mean survival time in suckling mice would indicate a real difference in lethality for these viruses as compared with the parent virus.

Effects of temperature on plaque formation. The first four small-plaque sublines (S-1 to S-4) at the 14th passage level had plaque-forming titers in LLC-MK₂ cells similar to those of a large-plaque subline (L-1) and the parent virus (6th passage level) when assayed at 35°C; however, only the parent virus and the large-plaque subline formed plaques at 39°C (Table 3). The S-1 subline was selected for the remaining experiments. Plaque formation by this virus was compared with the parent virus at increasing temperatures from 35 to 39°C (Table 4). The nonpermissive temperature for the small-plaque subline was between 38 and 39°C in LLC-MK₂ cells. An effect of increasing temperature on plaque formation by the parent virus was to gradually decrease the size of both
the small and large plaques until (by assumption) only the large plaques were visible at 39°C. In a separate test, 40°C was found to be nonpermissive for plaque formation by the parent virus.

Effects of temperature on viral replication in cells in fluid culture. The S-1 subline was compared to the parent virus at 35 and 39°C in

TABLE 1. Plaque size and titer of DEN-2, PR-159 virus after passage and clone selection in PGMK cell culture

<table>
<thead>
<tr>
<th>Virus</th>
<th>Passage</th>
<th>Plaque size in LLC-MK₂ cells (mm)</th>
<th>Titer (PFU/ml) at 35°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>0</td>
<td>1-2, 3-5</td>
<td>1 x 10⁹</td>
</tr>
<tr>
<td>Parent, passage 6*</td>
<td>6</td>
<td>1-2, 3-5</td>
<td>3 x 10⁹</td>
</tr>
<tr>
<td>Clone 5c</td>
<td>10</td>
<td>1-2, 3-5</td>
<td>1.1 x 10⁹</td>
</tr>
<tr>
<td>Clone 6c</td>
<td>10</td>
<td>1-2</td>
<td>5 x 10⁹</td>
</tr>
<tr>
<td>L-1 (from clone 5c)</td>
<td>14</td>
<td>1-2, 3-5</td>
<td>9 x 10⁹</td>
</tr>
<tr>
<td>S-1 (from clone 6c)</td>
<td>14</td>
<td>1-2</td>
<td>9 x 10⁹</td>
</tr>
<tr>
<td>S-1</td>
<td>18</td>
<td>1-2</td>
<td>2 x 10⁹</td>
</tr>
<tr>
<td>S-1</td>
<td>19</td>
<td>1-2</td>
<td>1 x 10⁹</td>
</tr>
</tbody>
</table>

* PFU, Plaque-forming units.

Clones derived by plaque selection at passages 7 and 9.

Clonal sublines derived by plaque selection at passages 11, 12, and 13; five large-plaque sublines derived from clone 5 and eight small-plaque sublines derived from clone 6 in identical fashion.

S-1 subline further plaque purified at passages 15, 16, and 17.

LLC-MK₂ cells and at 37, 39, and 40°C in PGMK cells (Fig. 3A, B). The parent virus replicated well in the primary cells at 39°C, whereas in the continuous cells infected at the same time its replication was substantially depressed at the same temperature. Replication of the S-1 subline in primary cells was significantly depressed at 39°C, but in continuous cells replication was totally nonpermissive at 39°C. When infected primary cells were incubated at 40°C (Fig. 3B), replication of the parent virus was further depressed, whereas replication of the small-plaque subline was totally restricted at this temperature, 1°C higher than in the continuous cells. The effect of temperature on the S-1 subline at 1°C intervals was tested in LLC-MK₂ cells (Fig. 4). Whereas the nonpermissive temperature was again 39°C, there was also a substantial depression of replication from 37 to 38°C.

Stability of small-plaque sublines on passage. It was observed that genetic stability of small-plaque sublines on serial passage in PGMK cells was influenced by the multiplicity of infection (MOI). Parallel lines of S-1 and S-6 viruses were passed three times using either undiluted inocula (MOI, approximately 10⁻⁴) from passage 14 to 17 or undiluted inocula (MOI, approximately 10⁻¹) from passage 14 to 17 (Table 5). With both S-1 and S-6 sublines, serial passage with a relatively high MOI resulted in

FIG. 2. Parent DEN-2 virus and clonal sublines S-1 and L-1 plaqued in LLC-MK₂ cells.
reversion of the homogeneous small-plaque virus to a population containing large plaques. The appearance of large plaques was accompanied by loss of temperature sensitivity and increased virulence for suckling mice. The small-plaque characteristic as well as reduced mouse virulence and temperature sensitivity could be maintained by passage of diluted virus at a low MOI for at least six passages. Yields of infectious virus produced at 7 days were approximately the same for cells inoculated with either a high or low MOI.

In vivo stability of the S-1 subline was tested after one passage in suckling mice. Brain homogenates from mice dying after intracerebral inoculation of the S-1 and parent viruses were assayed for the presence of small and large plaques in LLC-MK₂ cells. Only virus capable of producing small plaques was recovered from mice inoculated with S-1, whereas the parent virus-inoculated mice yielded progeny virus that produced a typical plaque mixture.

Tests for adventitious agents in the S-1 virus pool. The S-1 virus pool prepared at the 19th passage level was tested for adventitious bacterial and viral agents. Tests for bacterial and mycoplasmal contaminants were negative after inoculation of the S-1 pool in Trypticase soy, thioglycolate, and mycoplasma broths. Tests for adventitious viral agents were done in adult and suckling mice and in four types of cell cultures. Young adult mice remained healthy after receiving a combination intracerebral and intraperitoneal inoculation of the S-1 subline. Suckling mice inoculated by the same route using neutralized S-1 virus also remained healthy over a period of 3 weeks. Tests after two passages of the S-1 subline at 14-day intervals in WI-38, primary rabbit kidney, primary rhesus monkey kidney, and PGMK cell cultures were also negative for cytopathic or morphological changes in the cell monolayer.

**DISCUSSION**

The plaque size variants observed in the unpassaged DEN-2, PR-159 isolate and in the passaged parent virus appear to be due to viral
subpopulations with differing virulence and temperature sensitivity characteristics. A small-plaque clone was derived from the parent virus and multiple sublines were isolated and purified. Homogeneity of the original clone was substantiated by the similar characteristics demonstrated by the sublines. The failure to isolate pure large-plaque clones in spite of repeated attempts strongly suggests a consistent, high-frequency mutation from large plaques to a mixture of small plaques and large plaques. There appears to be a much-lower-frequency mutation of small-plaque to large-plaque morphology.

There is a strong positive correlation between small-plaque morphology, temperature sensitivity, and reduced suckling mouse virulence. The reason for reduced in vivo virulence of the small-plaque clones is probably partial suppression of viral replication processes at the body temperature of the host. The degree of suppression at a given temperature is determined in part by the virus and in part by the host cell. We have shown in this study that viral replication is suppressed at different restrictive temperatures in continuous and primary monkey kidney cells. Virulence or attenuation might be a reflection of which cells in a certain host are effectively replicating the virus. Thus, measurement of temperature sensitivity in vitro cannot generally be used to predict attenuation for a given host, but it is a practical screening method for variants or variants in many virus-cell systems. In the case of a DEN-2 small-plaque clone, temperature sensitivity appears to be a very useful laboratory marker, which is associated thus far with attenuation.

The small-plaque clonal subline S-1, at the 19th passage level, which has been tested more extensively than other DEN-2 clones, has several characteristics that indicate its potential as a vaccine candidate. These include temperature sensitivity, reduced neurovirulence for suckling mice, and, in other studies to be reported (Harrison et al., in preparation), reduced virulence for primates and low neurovirulence for rhesus monkeys. In addition, the S-1 subline is free of detectable adventitious agents and has only been passaged in cell cultures acceptable for human vaccine development. Passage experiments indicate that the genetic purity of the S-1 subline can be maintained during serial passage by using a low MOI.

ACKNOWLEDGMENTS

We thank C. Hampton for technical assistance and J. Lowenthal, S. Berman, and D. Dubois for helpful discussions.
**TABLE 5. Characteristics of the S-1 and S-6 sublines passaged at relatively high and low MOI in PGMK cells**

<table>
<thead>
<tr>
<th>Virus, passage (p)</th>
<th>MOI</th>
<th>PFU/mla</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;/PFU</th>
<th>Plaque morphology at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35°C</td>
<td>39°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-1, p-14</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>9 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>S-1, p-17</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.008</td>
</tr>
<tr>
<td>S-1, p-19</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.040</td>
</tr>
<tr>
<td>S-1, p-17</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>3 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>S-6, p-14</td>
<td>2 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.0008</td>
</tr>
<tr>
<td>S-6, p-17</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>0.006</td>
</tr>
<tr>
<td>S-6, p-20</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>Small</td>
</tr>
<tr>
<td>S-6, p-17</td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> PFU, Plaque-forming units.

<sup>b</sup> Suckling mouse intracerebral 50% lethal doses (LD<sub>50</sub>).

<sup>c</sup> ND, Not determined.

**LITERATURE CITED**


