Comparison of Three Methods Used to Isolate Dengue Virus Type 2

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During the 1969 dengue epidemic in Puerto Rico, human sera and Aedes aegypti mosquitoes were collected for virus isolation and identification. Three methods of isolation were used and compared. In the first method, we inoculated newborn mice by the intracranial route, noted any signs of illness, and serially passed specimens in mice until virus was isolated. In the second method, we inoculated tube cultures of LLC-MK2 cells, noted any cytopathic effect (CPE), and assayed fluids for virus by plaque formation in LLC-MK2 cell monolayers. The third method was different from the second only in that the original specimens were first inoculated into fluid cultures of Singh's A. albopictus cells. No significant CPE was seen in LLC-MK2 cultures; however, distinct syncytial CPE was observed in A. albopictus cells. About the same number of virus isolates were made in each isolation system. Virus isolates from both sera and mosquitoes were identified as dengue type 2 by a plaque-reduction neutralization test in LLC-MK2 cells. The utility of the three methods, individually or in combination, is discussed and related to diagnostic and epidemic situations.

The isolation of dengue viruses in newborn mice usually is a slow and frustrating procedure. It is often necessary to make several serial passages before virus titer reaches a detectable level; a period of adaptation must take place before titers of hemagglutinin, complement-fixing antigen, or virus are sufficient to react in standard identification systems.

In recent years, several cell culture systems have been used to isolate dengue viruses. Both primary African green monkey kidney (GMK) cells and a continuous line of GMK cells (BS-C-1) were reported to be superior to mice for isolating dengue virus type 3 (2). To detect the presence of dengue virus, cell cultures were challenged with poliovirus type 2 as described by Halstead et al. (1). Both direct and delayed plaque assay procedures in LLC-MK2 cell cultures were reported by Yuiil et al. (9) to be better than mice for isolating all four dengue serotypes. Singh's Aedes albopictus cell line was also reported to be more sensitive than mice for isolating dengue virus (6).

Since both LLC-MK2 and A. albopictus cells were reported individually to be superior to mice for isolating dengue virus, we decided to compare the three systems during the dengue epidemic in Puerto Rico in 1969 (C.H. Calisher et al., Bacteriol. Proc., p. 118, 1970) to determine the best one for an epidemic situation.

MATERIALS AND METHODS

Cell cultures. A culture of Singh's A. albopictus cells was obtained from S.M. Buckley, Yale University. The cells were grown and maintained by the procedures described by Singh (5).

LLC-MK2 cells were obtained from P.K. Russell, Walter Reed Army Institute of Research. They were grown in medium 199 containing 20% heat-inactivated fetal calf serum; the methods described previously were used (9). For maintenance, the serum content of the medium was 5%.

Collection and processing of specimens for virus isolation. Sera were collected from individuals in the acute stage of illness who had some combination of fever, headache, and rash or muscle pains. Since other viruses mimicking dengue clinically were concurrently circulating in the population, it was not possible to predict which patients were dengue-viremic; many of the specimens undoubtedly did not contain virus. Blood specimens were collected and allowed to clot on wet ice. Within a period of 4 to 6 hr, the sera were separated by centrifugation and stored in screw-cap vials at -60 C. One to 5 days later, sera were transferred to dry ice and shipped to the Center for Disease Control (CDC), Atlanta, where they were stored again at -60 C until tested.
Mosquitoes. *A. aegypti* mosquitoes were caught by aspiration in houses in Puerto Rico by Leslie Beadle, CDC, and members of the Vector Control Section, Puerto Rico Department of Health, under the auspices of Juan R. Palmer. Specimens were stored at \(-60\) C until they were shipped on dry ice to CDC; there they were stored at \(-60\) C until processed for inoculation in pools of 1 to 32 mosquitoes by the methods described by Sudia and Chamberlain (7).

**Virus isolation.** Sera diluted 1:10 and suspensions of pooled mosquitoes were inoculated simultaneously into four tube cultures of *A. albopictus* cells and LLC-MK\(_2\) cells. The volume of inoculum for each tube was 0.1 ml. After cultures were inoculated, they were kept at 35 C for 30 min for virus adsorption. Maintenance medium (1.5 ml) was then added without rinsing cultures. All cultures were incubated at 35 C until harvested. The same sera, undiluted, and mosquito suspensions were inoculated into six 2- to 4-day-old mice by the intracranial (ic) route in 0.02 volumes. Cell cultures were observed daily for cytopathic effect (CPE). All cultures were harvested on day 7 and inoculated simultaneously into LLC-MK\(_2\) cells and mice.

Mice were observed daily for 21 days for signs of illness. Ten per cent brain suspensions of sick mice were inoculated into both LLC-MK\(_2\) cells and newborn mice. Cell cultures were observed for plaque formation. Mice were challenged with a lethal dose (approximately 100 median lethal doses for weaned mice by the ic route) of dengue virus type 2 (strain Tr 1751) on the 28th day after inoculation to determine whether protection had been established by the original specimen.

**Plaque assay.** Virus titrations were performed in LLC-MK\(_2\) cells cultured in 1-oz (32 ml) prescription bottles by the method of Sukhavachana et al. (8).

**Virus identification.** Virus isolates were passed once or twice in LLC-MK\(_2\) cells or *A. albopictus* cells to increase infectivity titers so that the virus could be identified. Isolates were identified by plaque-reduction neutralization in LLC-MK\(_2\) cell cultures as described previously (3, 4).

**RESULTS**

No significant CPE was observed in cultures of LLC-MK\(_2\) cells; however, syncytial CPE (Fig. 1) in *A. albopictus* cells, when present, was very distinct and appeared as early as 3 days after inoculation with either serum or mosquito suspension containing virus. No plaques were observed when brains from inoculated mice were tested in LLC-MK\(_2\) cells.

Of the 32 isolates obtained from human sera and the 5 from mosquitoes, 25 were isolated in newborn mice, 20 in LLC-MK\(_2\) cells, and 29 in *A. albopictus* cells (Table 1). From 116 sera, 32 virus isolations were made. Virus was isolated from 11 sera in all three assay systems, from 2 sera in both cell cultures, and from 1 serum in LLC-MK\(_2\) cells only. Virus was also isolated from seven sera in *A. albopictus* cells, three sera in both mice and LLC-MK\(_2\) cells, four sera in mice and *A. albopictus* cells, and four sera only in mice. Virus was isolated from 5 of 22 mosquito suspensions. Three of the five suspensions yielded virus in all three systems, one yielded virus in *A. albopictus* cells only, and one yielded virus in mice and *A. albopictus* cells only.

Eighty sera that were collected from patients

![Fig. 1. (A) Aedes albopictus cells showing syncytial CPE 7 days after inoculation with mosquito suspension containing dengue virus type 2. (B) Normal A. albopictus cells.](image)
Table 1. Results of three systems used for isolating dengue virus type 2 from human sera and mosquitoes

| Isolation system       | No. positive |
|------------------------|-------------|
| Newborn mice           | LLC-MK₂ cell cultures | A. albopictus cell cultures | Sera | Mosquito pools |
| +³                    | +           | +                       | 11   | 3           |
| 0³                    | +           | +                       | 2    | 0           |
| 0                     | 0           | 0                       | 1    | 0           |
| +                     | +           | 0                       | 3    | 0           |
| +                     | 0           | +                       | 4    | 1           |
| +                     | 0           | 0                       | 4    | 0           |

³ Total sera = 116 (32 virus isolates).
² Total mosquito pools = 22 (five virus isolates).
³ Symbols: +, virus isolations made; 0, no virus isolations.

Table 2. Influence of antibody and time of collection on the isolation of dengue virus type 2 from human sera

| Time (day) serum collected | Virus isolations |
|---------------------------|------------------|
|                           | Sera with HI antibody | Sera without HI antibody |
| 0                         | 0/2             | 4/8             |
| 1                         | 0/4             | 7/13            |
| 2                         | 0/4             | 2/3             |
| 3                         | 2/7             | 5/8             |
| 4                         | 0/6             | 2/5             |
| ≥5                        | 0/13            | 0/7             |
| Totals                    | 2/36            | 20/44           |

Number of virus isolations/number of sera tested.

Before the 6th day of illness were tested for the presence of dengue viruses. Forty-four of these were free from dengue type 2 hemagglutination-inhibition (HI) antibody (<1:20), and 36 sera had HI titers in excess of 1:20. Neutralization tests were not performed on these sera. The influence of dengue virus antibody and the time of collection on the possibility of isolating dengue virus from human serum is reflected in the results presented in Table 2. Twenty virus isolations were made from the sera without antibody, and two were made from those containing HI antibody.

Discussion

Although the number of specimens tested was small, the results obtained strongly indicate that the A. albopictus cell system was superior to newborn mice for isolating dengue virus type 2 active in Puerto Rico in 1969. The presence of dengue virus in human serum or in mosquito homogenate could be readily demonstrated in mice by the virus challenge method; however, actual recovery of titratable dengue virus from mice required several serial passages. Isolation and recovery of virus by either cell culture method were accomplished in the initial isolation attempt. In all positive specimens, enough virus was recovered from the original tube culture to produce plaques. One or two serial passages were sufficient to amplify virus titer for identification by plaque-reduction neutralization.

There are three differences between the two cell systems used for virus isolation; the most important is the development of syncytial CPE in A. albopictus but not in LLC-MK₂ cell cultures 4 to 6 days after inoculation. Preliminary titrations for neutralization tests showed that concentrations of virus recovered from A. albopictus cultures were generally higher than those from LLC-MK₂ cells; therefore, fewer serial passages were required before carrying out identification tests. A. albopictus cells also present fewer growth and maintenance problems than do LLC-MK₂ cells.

Thus, all three systems individually and in combinations have their advantages and disadvantages for isolation and identification of dengue virus type 2. Ideally, all three systems would be used concurrently to assure maximum sensitivity. However, in a routine operation or when dealing with large numbers of specimens, the use of three systems is impractical. Although further confirmatory studies are indicated, the results obtained suggest that isolation and identification of dengue type 2 can be most simply, inexpensively, rapidly, and successfully carried out by inoculation of A. albopictus cell cultures for isolation and by subinoculating in LLC-MK₂ cells for identification by the plaque-reduction neutralization technique.

Of the 80 sera collected from patients before the 6th day of illness, 45% had HI antibody and 55% did not have HI antibody for dengue type 2 virus. The fact that only 2 virus isolations were made from 36 sera with antibody and 20 isolations were made from 44 sera without antibody indicates that sera from dengue patients should be tested by HI before inoculated for virus isolation. If only HI-negative sera are tested for virus isolation, there may be a low percentage of isolates missed; however, the saving in time and expense will be significant.

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