Simplified Plaque Reduction Neutralization Assay for Dengue Viruses by Semimicro Methods in BHK-21 Cells: Comparison of the BHK Suspension Test with Standard Plaque Reduction Neutralization

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A newly modified semimicro plaque reduction neutralization test (PRNT) in BHK cells was compared with a standard PRNT in bottles with LLC-MK2 monolayers and with an LLC-MK2 PRNT adapted to semimicro methods. The BHK semimicro PRNT compared favorably in terms of sensitivity in detecting dengue antibody (96%), specificity at a screening dilution (95%), and ability to detect seroconversion to dengue viruses of three serotypes (93%). Disagreements between the BHK test and the LLC-MK2 tests were attributed to greater sensitivity of the BHK test in detecting dengue type 2 (DEN-2) antibody in acute-phase sera and to apparent low-level DEN-1/DEN-3 cross-reactions in some sera in all three tests. The BHK PRNT was easier, faster, and more economical than either of the LLC-MK2 tests. Many of the benefits of the BHK PRNT derive from the fact that cells are infected while still in suspension, at the time of cell splitting, hence the term "BHK suspension test."

Standard antibody assays such as hemagglutination inhibition, complement fixation, and enzyme-linked immunosorbent assays are of limited value in dengue diagnosis and seroepidemiologic study because they often cannot identify the infecting serotype. Assay of neutralizing antibody is more specific and of similar sensitivity for individuals immune to a single dengue type, but detection of neutralization by standard plaque reduction methods (8) in glass prescription bottles ("macro test") is time consuming and expensive and requires relatively large serum volumes, precluding the use of blood obtained by finger-stick and drawn into capillary tubes or adsorbed onto filter paper. Several micro or semimicro methods for detecting dengue antibodies have been described (1, 3, 5, 6, 9, 10), but none is widely accepted. In some cases these tests are difficult to perform or interpret or require reagents of limited availability. An ideal test system should retain the simplicity and small-scale attributes of the best micro-method tests along with the sensitivity, specificity, and reproducibility of the macro test. We recently reported a simplified plaque reduction neutralization test (PRNT) adapted to semimicro methods in LLC-MK2 cells that was comparable in sensitivity and specificity to the standard macro test in bottles and required only microvolumes of serum (15 to 25 μ l) to measure antibody titers to all four dengue serotypes at a screening dilution (D. M. Morens, S. B. Halstead, and L. K. Larsen, submitted for publication). Here we report an improved semimicro PRNT in BHK cells which is of comparable sensitivity and specificity and which has the additional advantages of being less time-consuming, less expensive, simpler to perform and interpret, and capable of providing final results in 5 to 7 days. The major improvements in this assay over previous assays described by us and by others derive from infecting BHK

cells as a suspension; hence, we refer to it as the "BHK suspension test."

MATERIALS AND METHODS

Viruses. The following Caribbean strains of dengue viruses obtained from the Walter Reed Army Institute for Research were used in all neutralization tests: dengue type 1 (DEN-1) (CV.1636/77), passed seven times in FRhL cells; DEN-2 (PR-159), passed six times in PGMK and twice in FRhL cells; and DEN-3 (PR-6), passed five times in suckling mouse brain. All three virus strains were passed an additional one to three times in LLC-MK2 cells and divided into aliquots as single virus stocks of known titer ($\geq 1.0 \times 10^5$ PFU/ml). To examine variability in plaque size and morphology, we looked at 13 wild DEN-2 strains isolated from Thai patients in 1980. These strains, designated with the prefix "AHF," were kindly supplied by Donald S. Burke, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. For the same purpose we also evaluated Southeast Asian DEN-2 strain 16681, DEN-4 strain H-241 and Caribbean DEN-4 strain 341750, kindly supplied by K. H. Eckels, Walter Reed Army Institute of Research.

Serum specimens. Paired ("first" and "second") serum specimens were selected from among those received from the Centers for Disease Control Dengue Laboratories, San Juan, Puerto Rico, after the 1977 dengue epidemic. The specimens were kindly provided by John P. Woodall. Information about the surveillance system that generated these specimens has been published (D. M. Morens, J. G. Rigau-Pérez, R. H. López-Correa, et al., *Am. J. Trop. Med. Hyg.*, in press). Of the 500 specimen pairs received, 420 had been found to have monotypic antibody in the second specimens by macro PRNT. Eighteen of these pairs that had second specimens reactive with either DEN-1, DEN-2, or DEN-3 were selected at random for comparison. Fifty first speci-

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mens which lacked antibody to DEN-1, DEN-2, or DEN-3 by macro PRNT and which had been tested in a previous study comparing the LLC-MK2 macro and semimicro PRNTs (Morens et al., submitted for publication) were also selected. Sera were not tested against DEN-4 since that virus had not been prevalent in the Caribbean before the sera were obtained.

PRNT, macro method in bottles. The PRNT test with LLC-MK2 cells in glass prescription bottles has been described elsewhere (4). Briefly, serial fourfold serum dilutions beginning with 1:10 or 1:40 were prepared in 0.5% gelatin in phosphate-buffered saline (pH 7.95) and added to equal volumes of virus diluted to yield 40 to 80 PFU/0.1 ml. Virus-serum dilution mixtures of 0.6-ml total volume (for each dengue type) were incubated for 60 min at 37°C. Three replicate 1-oz (ca. 29.6-ml) prescription bottles containing 7-day-old LLC-MK2 monolayers were inoculated with 0.2 ml each of virus-serum mixtures and incubated for 90 min at 37°C. The inoculum was then removed, and the monolayers were overlaid with 1% Noble agar (42 to 45°C) containing 10% heat-inactivated calf serum, Eagle basal medium with Hanks buffered salt solution without phenol red (GIBCO Laboratories, Grand Island, N.Y.), glutamine, bicarbonate, antibiotics, and neutral red. Bottles were incubated, first at 37°C in the dark for 7 days, then covered at room temperature for an additional 7 days, before final plaque counts were made. Calculations of 50% endpoint plaque reduction neutralization titers were made by the method of Russell et al. (8).

PRNT, semimicro method. In the semimicro test, dilutions of serum were made in wells of sterile 96-well flat-bottomed polystyrene microtiter plates (Costar, Cambridge, Mass.) placed upon a bed of crushed ice in a laminar-flow hood. A 100- μ l volume of each serum dilution in 0.5% gelatin-phosphate-buffered saline (pH 7.95) was incubated at 37°C for 1 h with 100 μ l of a working dilution of virus calculated to give 10 to 20 PFU/50 μ l of the final volume of virus-serum mixture. For virus controls the working dilution was mixed 1:1 with (i) 0.5% gelatin-phosphate-buffered saline (pH 7.95), (ii) fourfold serial dilutions (10, 40, 160, 640, 2,560) of at least three dengue-negative sera, and (iii) one dengue-positive serum in the same microtiter plate and then incubated along with the virus-serum mixtures to be tested. In each test at least one well each of a 10^{-1} and 10^{-2} dilution in gelatin-phosphate-buffered saline of the working virus dilution was made.

At the end of virus-serum incubation, the 96-well plates were again placed on ice in a laminar-flow hood. The test proper was performed in 24-well polystyrene tissue culture plates (Costar) of 2-cm² bottom area per well, whose lids and bottoms had each been labeled to identify test materials.

BHK-21 clone 15 cells (2, 7) were obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md., and maintained in 75-cm² polystyrene tissue culture flasks (Costar) in Earle minimum essential medium (Flow Laboratories, McLean, Va.) with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), glutamine, bicarbonate, and antibiotics. Cells were split every 5 to 7 days by addition of trypsin-EDTA, passed into new flasks at 3×10^6 cells per 75-cm² flask in 30 ml of medium, and incubated at 37°C under 5% CO₂ atmosphere. When a test was to be performed, unpassed cells were removed and maintained in suspension at 3.0×10^5 cells per ml in Earle minimum essential medium in an Ehrlemeyer flask by gentle mixing with a stirring bar or occasional swirling.

The test proper was performed in a laminar-flow hood by addition of 0.5 ml of BHK cell suspension (1.5×10^5 cells) to each well of the 24-well polystyrene plates, one plate at a time. Immediately thereafter, 50 μ l of previously incubated virus-serum mixtures was added to the cell suspensions in triplicate with the aid of a micropipettor and disposable sterile tips. Plates were covered and incubated for 4 h at 37°C under 5% CO₂ atmosphere, allowing the cells to settle and lightly adhere to the well bottoms. Then the wells were overlaid with a medium consisting of 50 ml of medium-viscosity carboxymethyl cellulose (Sigma, St. Louis, Mo.), 100 ml of 2 $\times$ minimum essential medium without phenol red (Microbiological Associates, Walkersville, Md.), and 7% fetal bovine serum, glutamine, and antibiotics delivered by a Cornwall syringe dispensing 0.5 ml per well. Plates were incubated for 5 to 7 days (empirically determined; see below) at 37°C under 5% CO₂ atmosphere. After removal from the incubator, the medium was dumped by inverting plates over a receptacle containing sodium or calcium hypochlorite, and plates were rinsed gently under tap water and fixed and stained with 0.5 ml, per well, of a solution of naphthol blue-black prepared in 1-liter stock amounts by the addition of 1.0 g of naphthol blue-black, 13.6 g of sodium acetate, 60 ml of glacial acetic acid, and 940 ml of distilled water. Plaques were counted immediately, or plates were stored at room temperature for later counting. The 50 first-serum specimens were screened at a dilution of 1:40 and compared qualitatively by 70% plaque reduction criteria. Eighteen serum pairs were tested at serial fourfold dilutions from 1:10 to 1:640, and titers were computed by the method of Russell et al. (8).

RESULTS

Comparative specificity at a screening dilution. Fifty first-serum specimens without antibody to DEN-1, DEN-2, or DEN-3 by either macro or semimicro PRNT in LLC-MK2 cells (Morens et al., submitted for publication) were screened in the BHK suspension PRNT. Overall concordance for seronegativity (specificity) was 95%. Eight serum specimens without dengue antibody in either of the LLC-MK2 assays caused greater than 70% plaque reduction at the screening dilution; seven specimens with antibody to DEN-2 and one with antibody to DEN-3 produced the same result. Each was confirmed on repeat BHK assay and subsequently titered (Table 1). In seven of the eight cases the antibody detected in the BHK assay was against the dengue serotype to which seroconversion was documented by macro PRNT in LLC-MK2 cells, an outcome unlikely to have occurred by chance alone ($P < 0.01$, Fisher's exact test).

Comparative sensitivity in detecting dengue seroconversion. Titers of 18 serum pairs tested against the same strains of DEN-1, DEN-2, and DEN-3 in the macro PRNT and BHK semimicro PRNT were computed (Table 2) by the method of Russell et al. (8). All 18 first specimens were without detectable LLC-MK2 PRNT antibody to any dengue virus at a 1:10 dilution, while only 2 (17987 and 18258) neutralized DEN-2 in the BHK test, a concordance rate similar to that of the first-serum specimens (96% versus 95%) tested only at a screening dilution (see above). The overall concordance rate for positivity (sensitivity) of the second specimens was 96% at a positive titer threshold of 1:40. Of 30 seroconversions detected by macro PRNT, 93% were also detected in the BHK PRNT. The two exceptions were apparent low-level DEN-2 cross-reactions in the macro PRNT not detected in the BHK PRNT (pairs 16731-18499) and 73106-22981; Table 2). For each serum pair tested against all three DEN types,

TABLE 1. Reciprocal antibody titers in eight acute-phase serum specimens without dengue antibody by LLC-MK2 macro PRNT but with detectable antibody by BHK semimicro PRNT

Serum pairs ^a	Titer in LLC-MK2 macro PRNT			Titer in BHK suspension test		
	DEN-1	DEN-2	DEN-3	DEN-1	DEN-2	DEN-3
14120 (3)	<10	<10	<10	<10	120	<10
14998 (46)	<10	280	<10			
14228 (11)	<10	<10	<10	<10	90	<10
14349 (24)	15	1,700	<10			
15576 (4)	<10	<10	<10	<10	275	<10
17012 (19)	15	740	<10			
16313 (?)	<10	<10	<10	<10	<10	20
19025 (?)	45	50	1,050			
16519 (2)	<10	<10	<10	<10	340	<10
17077 (16)	<10	450	<10			
16801 (5)	<10	<10	<10	<10	40	<10
70506 (20)	25	50	2,000			
17203 (3)	<10	<10	<10	<10	30	<10
70464 (16)	15	380	<10			
17553 (4)	<10	<10	<10	<10	1,400	<10
92159 (21)	30	550	15			

^a First and second serum specimens of a pair, respectively. Parentheses indicate the day after illness onset on which specimen was obtained.

seroconversion to the highest titer (ideally, in primary infections, an indicator of the infecting serotype) was concordant in most cases (Table 2). Exceptions (pairs 24010-24332, 17986-91918, and 73106-22981) were sera with a broad heterotypic pattern. Examination of Table 2 suggests that in these instances determination of infecting type could be confounded by a one-directional DEN-1/DEN-3 cross-reaction, that is, a cross detected in neutralization of sera selected for DEN-1 neutralizing activity, but not of sera selected for DEN-3 neutralizing activity, but the data do not indicate which of the two tests identifies the true infecting serotype.

Comparison of plaque size and morphology. Dengue virus plaques in BHK suspension-infected monolayers were detectable as early as day 5 and no later than day 7, depending upon the infecting serotype and strain. For the three laboratory-adapted serotype strains (Fig. 1), distinct and readily countable plaques were present by day 5 for DEN-2 and no later than day 7 for DEN-1 and DEN-3. Plaques produced by the wild strains of DEN-2 were sometimes indistinct on day 5, but were always countable by day 6 or 7. Determination of when to stain the plates was based upon experience with the particular strain and, in the case of "new" strains, by direct visualization of plaques in unstained plates by low-power light microscopy. Generally, plaque size increased rapidly over the first 2 days after appearance, as shown by comparing DEN-2 16681 on days 5 and 6 (Fig. 1). For most viruses tested, plaques were small (2 mm) and evenly distributed at first appearance. Often 100 or more distinct plaques could be counted per well (Fig. 1). However, plaque size quickly enlarged; if assays were harvested late, crowding and overlapping made plaque counting difficult. For example, DEN-2 strain 16681 caused small plaques on day 5 and large, fluffy plaques on day 6 (Fig. 1). Morphological plaque differences

TABLE 2. Comparison of reciprocal PRNT titers in 18 paired sera^a

Serum pairs ^b	Titer in standard macro test (glass prescription bottles)			Titer in BHK suspension semimicro test		
	DEN-1	DEN-2	DEN-3	DEN-1	DEN-2	DEN-3
16731 (2)	<10	<10	<10	<10	<10	<10
18499 (16)	≥640	50	90	≥640	10	140
21042 (2)	<10	<10	<10	<10	<10	<10
22497 (16)	550	20	25	390	<10	<10
24010 (2)	<10	<10	<10	<10	<10	<10
24332 (52)	≥640	50	60	190	250	500
23875 (1)	<10	<10	<10	<10	<10	<10
24461 (70)	375	70	50	210	90	140
17986 (2)	<10	<10	<10	<10	<10	<10
91918 (12)	120	115	120	120	160	≥640
73106 (3)	<10	<10	<10	<10	<10	<10
22981 (24)	≥640	40	250	160	10	300
17596 (8)	<10	<10	<10	<10	<10	<10
92736 (29)	<10	460	<10	<10	160	<10
17987 (2)	<10	<10	<10	<10	15	<10
92728 (16)	<10	≥640	<10	<10	≥640	<10
17996 (19)	<10	<10	<10	<10	<10	<10
19382 (35)	<10	≥640	<10	<10	≥640	<10
18058 (0)	<10	<10	<10	<10	<10	<10
19727 (16)	<10	360	<10	<10	160	<10
18258 (5)	<10	<10	<10	<10	100	<10
71292 (19)	<10	290	<10	<10	≥640	<10
19176 (1)	<10	<10	<10	<10	<10	<10
21706 (26)	<10	≥640	<10	<10	≥640	<10
15067 (0)	<10	<10	<10	<10	<10	<10
15071 (13)	<10	<10	155	<10	20	120
92274 (1)	<10	<10	<10	<10	<10	<10
19783 (7)	<10	<10	160	<10	<10	≥640
72735 (1)	<10	<10	<10	<10	<10	<10
22937 (32)	70	<10	290	160	<10	≥640
90339 (3)	<10	<10	<10	<10	<10	<10
19349 (25)	15	30	500	<10	<10	40
92792 (4)	<10	<10	<10	<10	<10	<10
20513 (18)	15	25	≥640	<10	30	≥640
14279 (1)	<10	<10	<10	<10	<10	<10
14898 (?)	15	50	≥640	<10	70	230

^a Boldface indicates highest reciprocal titer to any of the three types for each serum pair on each test.

^b Parentheses indicate day after illness onset on which serum was obtained.

between the dengue serotypes were not as characteristic as those described for plaque assays in LLC-MK2 cells (Morens et al., submitted for publication). Wide variations in plaque size and morphology were noted to be characteristic of and reproducible for 14 DEN-2 strains tested, and limited experience with strains of the other three serotypes suggests a similar spectrum of size and shape, e.g., between DEN-4

FIG. 1. Dengue virus plaque size and morphology in polystyrene wells of 2-cm² surface area containing monolayers of BHK cells infected in suspension. Top row: Caribbean strains of all four dengue serotypes and, for comparison, Southeast Asian DEN-4 strain H-241. Rows 2 through 4: Thirteen Southeast Asian DEN-2 strains exhibiting variations in plaque size and shape. All plaques were photographed at day 7 of incubation except for laboratory-adapted DEN-2 strains PR-159 (row 1, column 2, photographed on day 5) and 16681 (row 2, columns 1 and 2, photographed as labeled on days 5 and 6).

strains H-241 and 341750 (Fig. 1). Some wild strains caused mixed plaque size and morphology, but both large and small plaques were neutralized to the same degree by serotype-reactive monoclonal antibodies and convalescent sera (data not shown), suggesting that this variability could reflect either normal variation in the rate of plaque evolution or heterogeneous growth of single strains.

DISCUSSION

The BHK semimicro PRNT compares favorably with the standard macro PRNT in terms of specificity (95%), sensitiv-

ity (96%), and ability to detect seroconversion to dengue viruses of three serotypes (93%). Comparison of the two tests suggests that in some respects the BHK test may be superior; almost all of the discordant results in screening sera without DEN antibody in the macro and semimicro LLC-MK2 tests (Table 1 and serum pairs 17987-92728 and 18258-71292 in Table 2) reflected detection in the BHK test of antibody to the infecting serotype, as determined by standard macro assay in LLC-MK2 cells, an event unlikely to have occurred by chance alone. Since most of the first specimens were obtained shortly after illness onset, the BHK test may actually be more sensitive than the other tests in detection of early antibody to an infecting DEN-2 serotype.

In qualitative terms, the BHK test was also 96% sensitive in detecting dengue antibody previously detected in the macro LLC-MK2 test, the only exceptions being low-level heterotype cross-reactions found in the LLC-MK2 macro test, but not in the BHK test. Thus the BHK test may be more specific than the LLC-MK2 test, although too few sera were tested to establish this.

In at least two cases of seroconversion with substantial heterotypic antibody (pairs 24010-24332 and 73106-22981), there was disagreement between the two tests as to the highest serotype antibody level, the macro test identifying DEN-1 and the BHK test identifying DEN-3. Although it is conceivable that in both tests these instances reflect secondary infection with subthreshold antibody in the first specimen and comparatively low-level antibody in the second, it is more likely that they represent primary infections with heterotype cross-reaction, perhaps in one instance because the second specimen was obtained early in convalescence. Examination of Table 2 suggests that there was substantial DEN-1/DEN-3 crossing in both tests, particularly for those specimens originally identified as seroconverting to DEN-1 (versus DEN-3) in the macro PRNT. These data cannot resolve the uncertainties about infecting serotype, but they underscore the well-known complexities in the interrelationships of the dengue serotypes.

The BHK suspension PRNT satisfies the previously mentioned criteria for an acceptable alternative to neutralization in bottles: it is of equal specificity and sensitivity and, for detecting antibody to DEN-2 virus, apparently is more sensitive. At the same time, its simplicity and ease of performance meet or exceed those of other improved tests reported in the scientific literature (1, 3, 5, 6, 9, 10). Specifically, the BHK suspension test is preferable to the macro PRNT in the following respects: (i) it can be performed with small volumes (60 μ l, versus 0.24 ml in the bottle PRNT, for screening all four dengue serotypes at 1:10 dilution); (ii) it can be interpreted at 5 to 7 days versus 14 to 21 days and can be performed more quickly, resulting in a substantial saving in personnel time; (iii) it is cost saving, requiring only 25% as much cell culture medium; (iv) it is less cumbersome, requiring about 5% as much incubator and storage space; and (v) it can be interpreted at any time and stored indefinitely for later interpretation or comparison. In the following respects it is preferable to the LLC-MK2 semimicro assay recently reported (Morens et al., submitted for publication): (i) it can be interpreted at 5 to 7 versus 14 to 21 days; (ii) it is simpler to perform because cells are infected in suspension immediately after normal splitting, without the need to grow a preformed monolayer; (iii) the plaques are easier to identify by visual inspection; (iv) the plaques were evenly distributed across the cell sheet rather than concentrated along the edges of the well where the inoculum

meniscus gathers in the LLC-MK2 test; (v) a higher number of PFU can be added to each well, resulting in considerable benefit in test specificity and sensitivity; and (vi) the test can be interpreted at any time after staining or stored for prolonged periods. We have had encouraging preliminary results (data not shown) with early staining of plates (day 4 to 5) and use of BHK cells after short passage-incubation, suggesting that the BHK suspension test may be sufficiently flexible that in urgent diagnostic situations results could be provided in as little as 4 days after the specimen is obtained. Also, we have used the BHK PRNT extensively with Southeast Asian DEN-4 strain 4328-S and with laboratory strains of DEN-1, 2, and 3 (from Southeast Asia) and JE (Nakayama) and found morphology and time of plaque appearance to be equivalent to those of the Caribbean strains reported here.

Low cost, ease of performance, relatively short test time, and improved test sensitivity suggest that in laboratories with cell culture capability the BHK suspension PRNT will prove a sound alternative to such standard serologies as hemagglutination inhibition and complement fixation.

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