PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 472–475 (1975)

Dengue Virus Plaque Formation on Microplate Cultures and Its Application to Virus Neutralization (38564)

NOBUYA FUJITA, MASAKO TAMURA AND SUSUMU HOTTA

Department of Microbiology, Kobe University School of Medicine, and Central Clinical Laboratories, Kobe University Hospital, Ikuta-ku, Kobe, Japan 650

In view of the medical and epidemiological problems concerning dengue and related infections, reliable procedures for the detection of anti-dengue antibodies in human or animal sera are necessary. The neutralization (NT) test is one of the standard measures for determining the titer of the specific antibodies. The plaque reduction test is performed on bottle or petri dish cultures of a cell system such as that of monkey or hamster kidney and KB cells in which the plaques by dengue viruses can easily be detected. The technique is sufficiently sensitive and reproducible, however, it requires comparatively large amounts of media and sera, and moreover, is time-consuming. Simpler and faster micromethods are therefore inevitably needed. Although NT antibodies against certain arboviruses have been titrated by microtiter methods of varying modifications (1–3), similar procedures have not yet been successfully applied to dengue viruses. In this paper, a microculture method for dengue virus detection and its application to antibody titration will be described.

Materials and Methods. Virus. Dengue virus type 1 (DEN-1) Mochizuki strain (4), in the form of an infected mouse brain homogenate in Hanks' balanced salt solution, was employed. Appropriate dilutions were made using Eagle's minimum essential medium (MEM) supplemented with 0.2% bovine serum albumin as diluent.

Cell culture. BHK-21 cells, clone 13 (obtained from Dr. C. L. Wisseman, Jr., University of Maryland, through Dr. H. Aoki) have been propagated serially in our laboratory with Eagle's MEM supplemented with 10% heat-inactivated bovine serum and 60 γ/ml kanamycin.

Plaque formation in microplate culture. Following 3–4 days of incubation at 37°, the bottle cultures were trypsinized and the cells were suspended in the medium. One-tenth ml of the cell suspensions was added in each well of microculture plates (Falcon Microtest II, 124 × 82 × 14 mm (5)). Usually 1–2 days thereafter, full cell sheets were formed. Just prior to use, the cultures were washed with MEM, and 0.025 ml of the virus suspension was introduced with a pipette dropper. They were placed into a CO_2 incubator at 37° for 1 hr and subjected to shaking at an interval of 15 min. After removal of the fluid from each well, 0.1 ml of overlay medium consisting of 1% methylcellulose (MC) and 2% heat-inactivated calf serum in Eagle's MEM (6, 7) was introduced. The microplates, prepared in this way, were loosely covered with a plastic lid and placed back into the CO_2 incubator. After 5–6 days of incubation, the overlay medium was decanted and washed off with Hanks' salt solution. The cultures were then fixed and stained with 1% crystal violet solution in 20% ethanol. After being washed thoroughly with water, the plaques were counted.

Plaque reduction neutralization in microplate. A modification of the method reported by Catalano *et al.* (8) was applied. Twofold serial dilutions, 0.025 ml each, of test serum were made in the wells of a transfer-plate on a holder using a microdiluter (Cooke Engineering Co.). One-fortieth ml of the virus, adjusted as to contain about 400 PFU/ml, was put into each well. After being shaken in a micromixer, the serum-virus mixtures were held in a CO_2 incubator at 37° for 1 hr. Thereafter the transfer-plate was placed on the top of the cell-containing tissue culture microplate, whose growth medium in each well had been replaced with 0.05 ml diluent. The serum-virus mixtures in the transfer-plate were transferred into the tissue culture plate, immediately after the bottom of the former and the surface of the latter

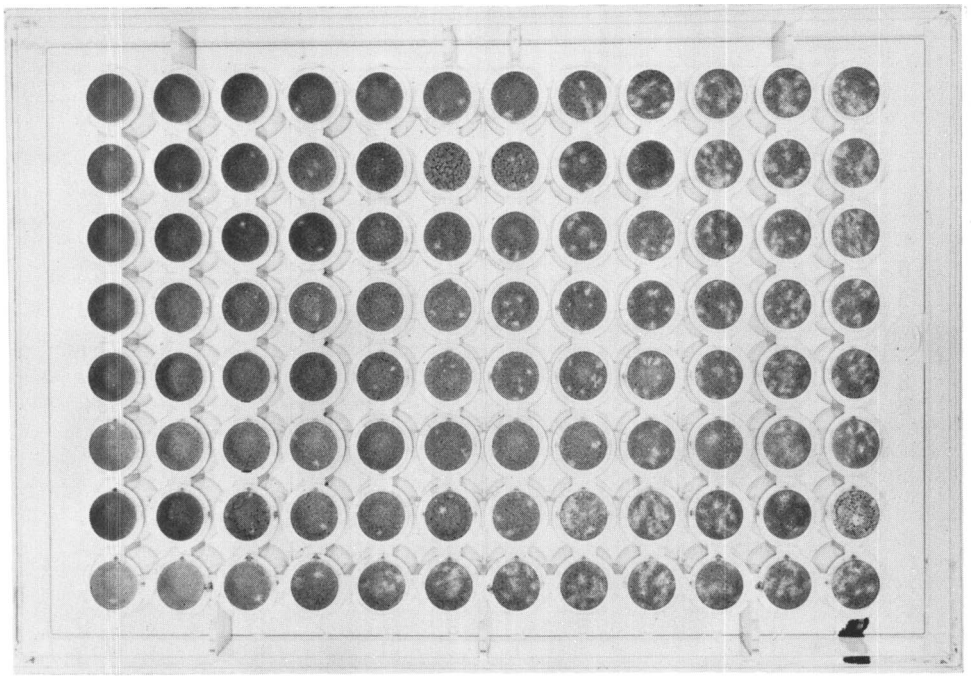

FIG. 1. Microplate cultures of BHK-21 cells, revealing plaques formed by DEN-1 virus. The wells on the right side reveal clearly visible plaques, whereas those on the left side lack plaques or show reduced number of plaques. Crystal violet stain. Size of plate, 124 × 82 mm.

came into contact. After being shaken in a micromixer, it was kept at 37° for an additional hr at 15 min shaking intervals. Then the fluids were removed from each well, and 0.1 ml of the MC overlay medium was introduced. After incubation in a CO_2 incubator at 37° for 5–6 days, the overlay medium was washed off with Hanks' solution, and the cultures were fixed and stained with the crystal violet solution. After rinsing the cultures with water, the plaques were counted.

Three lines of wells were used for one serum sample, and the total number of plaques from three wells for the same dilution was regarded as the PF titer. Control tests were performed by the same way replacing the serum with the diluent or control normal serum. Usually, 36 wells were used for one control test and the total plaque number was divided in twelve, which corresponded to the average number in three wells. The dilution grade of the test serum bringing about a half reduction of the plaque number of the control test, calculated by the Behrens–Kärber method, was regarded as a 50% plaque reduction neutralization titer.

Results. DEN-1 virus plaques were formed on the microplate cultures of BHK-21 cells under the MC overlay medium. The plaques, approximately 1 mm in diameter, were clearly visible 5 days after the beginning of the incubation at 37°. The plaque counting was easy, provided that the number of plaques in each well (having a diameter of 7 mm) did not exceed 15.

The microplate cultures combined with the transfer-plate were applied to neutralization tests of the dengue. Figure 1 is a picture of a microplate in which the virus-serum mixtures were inoculated. The wells on the left side which were inoculated with the virus mixed with higher concentrations of anti-DEN-1 immune rabbit serum, were lacking plaques or revealed fewer plaques than those on the right side wells which received the same virus mixed with the serum of lower concentrations.

An example of the protocols obtained is

TABLE I. DENGUE VIRUS NEUTRALIZATION TESTS BY USE OF BHK-21 CELL MICROCULTURE PLATES AND TRANSFER-PLATES.

Number of plaques in each well receiving virus[a] mixed with serum[b] diluted											
1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
1[c]	0	0	2	0	3	0	2	7	4	11	10
0	0	0	0	2	2	5	6	7	10	13	10
0	0	2	1	0	3	5	5	7	9	10	10
Average											
0.3	0	0.7	1	0.7	2.7	3.3	4.3	7	7.7	11.3	10
Control (Average of 36 wells) 8.5											
50% plaque reduction NT titer 1:147											

[a] Type 1 Mochizuki strain virus, in the form of an infected mouse brain homogenate.

[b] Obtained from a rabbit immunized with the Mochizuki strain virus, and inactivated by heating at 56° for 30 min.

[c] Three wells were used for one dilution of the serum.

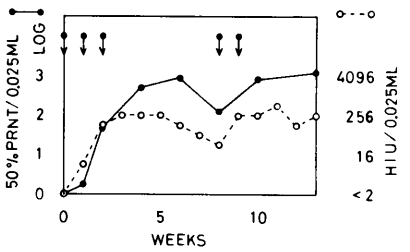

FIG. 2. Parallel titration of NT and HI titers of an anti-DEN-1 immune rabbit serum. The serum was obtained from a rabbit immunized with DEN-1 Mochizuki strain virus. Ordinate indicates titers of the serum; NT titers (black circles connected with a solid line) were determined by the micromethod described in the text, and HI titers (white circles connected with a dotted line) by the standard method (9) modified as adapted to microtiter technique. Abscissa indicates observation period in weeks, and arrows indicate the time of virus inoculation.

shown in Table I. When the plaque counts and serum dilutions were plotted on a section paper, a sigmoid curve was exhibited.

The compatibility of the NT titers obtained by this method with the hemagglutination-inhibition (HI) titers determined by the standard method by Clarke and Casals (9) (modified as adapted to the microtiter technique), was investigated using a serum sample from a rabbit inoculated with the dengue virus. The data thereof are illustrated in Fig. 2, in which it is shown that the NT titers are well parallel with the HI titers determined simultaneously.

Discussion and Summary. Dengue virus plaque formation on BHK-21 cell microplate cultures was described. The clear plaques were visible usually 5 days after incubation in a CO_2 incubator at 37°. The cells cultured in a 3-oz bottle were sufficient to prepare two microculture plates which were usually ready for use after 1–2 days of cultivation in the CO_2 incubator at 37°. The overall procedures were easy and of economic advantage.

It is to be stated in this connection that the affinity of dengue viruses to tissue culture cells is not necessarily high, so that the cell culture systems suitable for dengue virus plaquing have so far been limited; and even in such suitable systems the formation of clear plaques takes a much longer time of incubation than for other kinds of arboviruses in general. Some of the difficulties regarding this matter have been overcome by the techniques reported here.

By use of the microplate cultures, in combination with the transfer-plates reported by previous investigators (8), dengue neutralization tests were performed. Technical specifics, such as dilution of serum, transfer of virus-serum mixtures, etc., could be defined with good reproducibility. A sigmoid curve relationship was revealed between the decrease in virus titer and reciprocals of antiserum concentration. Within certain grades of the serum dilution, the same relationship was linear. This is in

general accordance with data obtained by other investigators (10) dealing with dengue virus plaques formed in bottle cultures. In our experiments in which the NT titers of particular serum samples measured by this method were compared with the HI titers of the same samples determined by the standard method (9), both values paralleled well with each other, indicating the compatibility of the former with the latter. In short, the dengue virus plaque techniques described here are comparatively rapid and easy to perform and can be applied to clinical diagnosis and epidemiological surveys of dengue and related virus infections.

1. Salim, A. R., Trans. Roy. Soc. Trop. Med. Hyg. **61,** 259 (1967).
2. De Madrid, A. T., and Porterfield, J. S., Bull. W. H. O. **40,** 113 (1969).
3. Hashimoto, N., Yamada, K., and Kanamitsu, M., Virus **21,** 55 (1971) (text in Japanese).
4. Hotta, S., J. Infect. Dis. **90,** 1 (1952).
5. Fuccillo, D. A., Catalano, L. W., Jr., Moder, F. L., Debus, D. A., and Sever, J. L., Appl. Microbiol. **17,** 619 (1969).
6. Schulze, I. T., and Schlesinger, R. W., Virology **19,** 40 (1963).
7. Yoshinaka, Y., and Hotta, S., Proc. Soc. Exp. Biol. Med. **137,** 1047 (1971).
8. Catalano, L. W., Jr., Fuccillo, D. A., and Sever, J. L., Appl. Microbiol. **18,** 1094 (1969).
9. Clarke, D. H., and Casals, J., Amer. J. Trop. Med. Hyg. **7,** 561 (1958).
10. Russell, P. K., Nisalak, A., Pairatana, S., and Vivona, S., J. Immunol. **99,** 285 (1967).

Received September 6, 1974. P.S.E.B.M. 1975, Vol. 148.