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SHORT COMMUNICATION

DEVELOPMENT OF A MICRO-NEUTRALIZATION TEST FOR CHIKUNGUNYA VIRUS

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A rapid, micro-scale focus reduction neutralization test for chikungunya virus was developed. In the test, cell monolayers are prepared in a 96-well tissue culture plate and the PAP (peroxidase-antiperoxidase) staining technique is used for detection of foci of chikungunya virus infected cells. This test is suitable for rapid diagnosis and epidemiological studies of the virus.

Chikungunya virus is widely distributed throughout Africa, south-east Asia and India (Chamberlain, 1980). It causes a disease characterized by high fever, severe joint pain and maculopapular rash. The disease occurs endemically as an unrecognized febrile illness or sometimes as large epidemics in many countries. In south-east Asia, the virus infection sometimes results in hemorrhagic manifestations, which were indistinguishable from those observed on dengue virus infection (Nimmannitya et al., 1969).

The disease has been diagnosed serologically by the hemagglutination-inhibiton (HI) and complement-fixation (CF) tests, and recently also by enzyme-linked immunosorbent assay (ELISA), which is more sensitive than the HI and CF tests (Nakitare et al., 1983). However, in these tests antibodies to chikungunya virus show cross-reactivity to other alphaviruses. Thus it seemed important to develop a neutralization (N) test that shows a type-specific antibody pattern for alphaviruses for clear diagnosis of the virus infection. Hahon and Hankins (1970) reported a rapid focus reduction N test for chikungunya virus in which infected foci were stained by immunofluorescence. The method, however, required laborious counting of foci under a fluorescence microscope. Recently, we have successfully employed a rapid, micro-scale N

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test for antibody titration of hemorrhagic fever with renal syndrome virus (Tanishita et al., 1984), mumps virus (Okuno et al., 1985) and Japanese encephalitis virus (Okuno et al., 1985). In the test, cells were cultured in 96-well tissue culture plates and foci of infected cells were stained by the PAP technique. This paper reports application of this method for an N test for chikungunya virus.

As a first step in developing the N test, we determined the optimum conditions for chikungunya virus infectivity assay. A chikungunya virus strain, African strain (Igarashi and Fukai, 1969), was propagated once in C6/36 cells (Igarashi, 1978) and the infected culture fluid was stored in liquid nitrogen as stock virus. Three cell lines, BHK-21, Vero and LLC-MK2 cells, were used in this study. They were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum.

Suspensions of the cells (about 5×10^4 cells/well for BHK-21 and LLC-MK2, and about 1×10^4 cells/well for Vero cells) were distributed in the wells of 96-well flat-bottom tissue culture plates (Falcon 3072, Becton Dickinson Lab., Calf. U.S.A.) and incubated in a CO_2 incubator at 37 C. The following day, volumes of 25 μl of serially diluted stock chikungunya virus were inoculated onto the cell monolayers in the wells. After adsorption for 2 h, the virus inocula were aspirated and the cells were covered with 0.1 ml of MEM containing 2% fetal calf serum. After appropriate incubation periods, the medium was removed and the cells were rinsed with phosphate buffered saline (PBS, pH 7.4) and fixed with absolute ethanol at room temperature for 10 min. Focus staining was done by successive treatments of the cells with rabbit anti-chikungunya serum (1:1,000), goat anti-rabbit IgG serum (1:100) and peroxidase-rabbit antiperoxidase complex (1:1,000) (Cappel Lab., Cochranville, PA., U.S.A.), for 40 min each with several washings with PBS between treatments. In the final step of the PAP technique, the peroxidase reaction was developed for about 5 min by the method of Graham and Karnovsky (1966) with 0.01% H_2O_2 and 0.3 mg/ml of 3–3′-diaminobenzidine tetrahydrochloride (Wako Chem. Indust., Osaka) in PBS. Then the wells were rinsed with tap-water and dried, and the stained foci were counted under a dissecting microscope. Numbers of foci are expressed as focus forming units (FFU).

First the optimum incubation periods for counting focuses in the three cell lines were determined. As shown in Fig. 1, foci were countable under a dissecting microscope 6 h after infection of Vero cells, 9 h after infection of BHK-21 cells and 12 h after infection of LLC-MK2 cells. Constant focus numbers were obtained between 9–18 h in Vero cells,

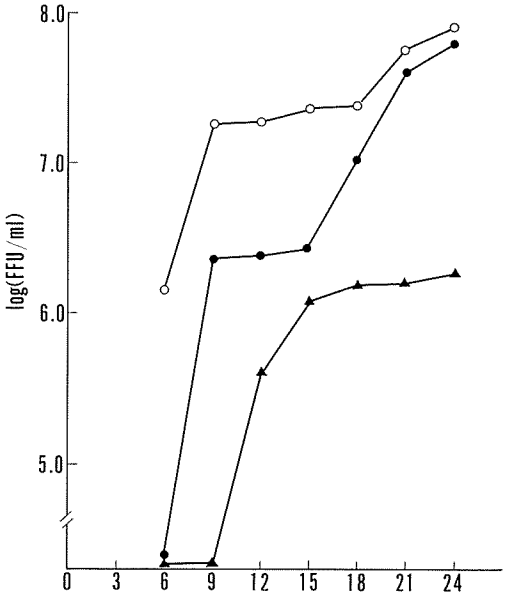

FIGURE 1. *Infectivity of chikungunya virus titrated by the micromethod at various times after infection. Stock virus was diluted serially and 25 μl volumes were inoculated onto monolayers in 96-well tissue culture plates. The cells in the plates were fixed from 6 to 24 hours after infection and stained by the PAP technique. Titers obtained with Vero (○–○), BHK-21 (●–●) and LLC-MK2 (▲–▲) cells are shown.*

9–15 h in BHK-21 cells and 15–24 h in LLC-MK2 cells. After these periods focus numbers increased because of the appearance of secondary foci. Thus the most appropriate incubation period was 12 h for Vero and BHK-21 cells and 18 h for LLC-MK2 cells. In these conditions at least 100 foci could be counted under a dissecting microscope.

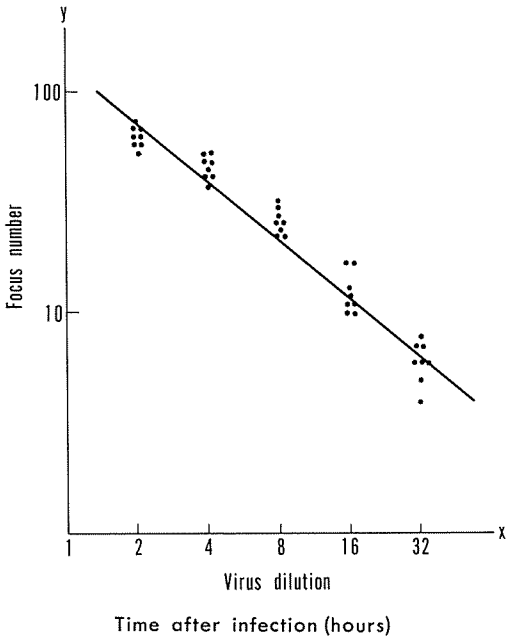

FIGURE 2. *Relation between chikungunya virus dilution and number of foci per well. The least squares regression line* ($Y=-0.256X+2.116$: $Y=\log_{10}y$, $X=\log_2 x$) *is shown.*

Figure 2 shows the linear relationship between the logarithms of the virus dilution and the number of foci per well measured with BHK-21 cells 12 h after infection. Similar results were obtained in experiments with Vero and LLC-MK2 cells. These results demonstrated that, irrespective of the cells used, this method is effective for infectivity assay of chikungunya virus. However, Vero cells seem to be the best of the three cell lines for infectivity assay of the virus because they showed the highest titer.

Next, the method was applied as an N test of chikungunya virus. Hyperimmune rabbit serum to chikungunya virus was serially diluted four-fold and each serum dilution or control diluent was combined with an equal volume of chikungunya virus adjusted to a final control count of about 100 FFU per well. The serum-virus mixtures were incubated for 2 h at 37 C and then 25 μl of each mixture was inoculated into the wells of 96-well tissue culture plates into which cells had been seeded the previous day. After adsorption for 2 h at 37 C, residual virus numbers were counted by the method described for infectivity assay. N antibody titers were expressed as the reciprocals of the highest dilutions that

TABLE 1. *Focus reduction and plaque reduction N tests for chikungunya virus using hyperimmune rabbit serum*

Serum dilution	Focus reduction N test			Plaque reduction N test
	Vero	BHK-21	LLC-MK2	BHK-21
640	0[a]	0.3	0	1[b]
2560	1.3	22.5	1.3	11
10240	31.8	40.8	40.8	23
40960	92.0	94.5	98.8	72
Control	103.0	114.8	120.0	110
N-titer	10240	10240	10240	10240

[a] Mean focus number per well for 8 replicate wells.
[b] Plaque number per plate.

reduced the number of foci to 50% of the control value or less.

Table 1 shows the results of the N test with Vero, BHK-21 and LLC-MK2 cells; values are mean focus numbers per well for 8 replicate wells. The hyperimmune serum showed the same N titer with the different cells. When the test serum was titrated by the plaque reduction method using the BHK-21 cell, the N titer was similar as that obtained by the micro N test (Table 1). These results indicate that any cells can be used for the micro N test and that the ordinary plaque reduction N test can be replaced by the micro N test.

The special advantage of this method is that the virus infectivity can be assessed within 18 h after inoculation of cell monolayers. Assay of chikungunya virus infectivity by plaque counting takes 3 to 4 days (Igarashi and Tuchinda, 1967). Moreover, as this is a micromethod, many specimens can be assayed at the same time. Thus the newly developed N test should be useful not only for rapid diagnosis but also for large scale epidemiological studies of chikungunya virus.

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