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RAPID TITRATION OF DENGUE VIRUS TYPE 4 INFECTIVITY BY COUNTING FLUORESCENT FOCI

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SUMMARY The infectivity of dengue virus type 4 was assayed by counting the number of fluorescent foci produced on infected BHK21 cell monolayers by the indirect fluorescent antibody technique. The foci, consisting of clumps of 10–20 cells with viral antigen in the cytoplasm, were clearly distinguished from the background of unstained cells. Basic conditions using this method for the rapid assay of the virus infectivity are described. The sensitivity of the method was almost the same as those of the conventional methods of plaque formation or mouse LD_{50} titration.

INTRODUCTION

Dengue hemorrhagic fever is an important viral infection in South East Asian Countries (Halstead et al., 1963; Halstead, 1966). Fundamental studies on the etiological agents, dengue viruses, are a prerequisite for the practical control of the disease. However, it is not always easy to assay the infectivity of viruses by plaque formation. Even in the well-studied case of dengue virus, type 2, it takes 6 days to form plaques (Schulze and Schlesinger, 1963; Russell et al., 1967). We tried to overcome this difficulty by counting the fluorescent foci obtained by the indirect fluorescent antibody technique.

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MATERIALS AND METHODS

1. *Viruses*

Dengue viruses, type 1, Hawaiian strain, (D1), at the 15th passage level, type 2, TR1751 strain, (D2), at the 10th passage level, type 3, H87, (D3), at the 26th passage level, and type 4, H241, (D4), at the 43rd passage level in suckling mouse brains (SMB) were kindly supplied by Dr. S. Ahandrik, Virus Research Institute, Bangkok, Thailand. The viruses were grown one to three times in SMB. Ten percent homogenates of infected SMB in 0.05 M borate buffer, 0.12 M NaCl, pH 9, were centrifuged at $1,500 \times g$ for 15 min. The supernatants were stored at -70 C as stock viruses.

2. *Antisera*

Hyperimmune mouse antisera were prepared in adult mice by 5 intraperitoneal injections of 0.1–0.2 ml of 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} dilutions and undiluted stock virus at one week intervals. Mice were bled one week after the last injection. Sera were

separated from the pooled blood of each type-specific antiserum. The titers of the antisera assayed by the hemagglutination-inhibition test (Clarke and Casals, 1958) were 1280, 1280, 640 and 2560 for anti-D1, anti-D2, anti-D3, and anti-D4 sera, respectively.

3. *Cells*

Established lines of baby-hamster kidney-cells, BHK21 clone 13, and African green monkey kidney cells (VERO) were grown with 10% calf serum in Eagle's minimum essential medium (MEM) (Eagle, 1959). The BHK21 cell line was further cloned by colony formation in petri dishes in a humidified CO_2 incubator. The continuous line of rhesus monkey kidney cells, LLC-MK_2, was originally obtained from Dr. R. N. Hull, Lilly Research Laboratories (Hull et al., 1962). The cells were cultivated with 10% calf serum in MEM.

4. *Plaque titration of dengue virus type 4*

The method described before (Igarashi et al., 1968) was slightly modified. The virus specimen was diluted serially 10-fold with 5% calf serum in MEM, and 0.2 ml of diluted specimen was inoculated onto monolayer cultures in 2 oz bottles. After adsorption for 2 hr at 37 C, spreading virus every 30 min, cell sheets were covered with the first overlay medium (5 ml/bottle) prepared by mixing equal volumes of 2% molten agar and double-strength MEM. Calf serum was added to the overlay medium at 2% final concentration. After incubation for 7 days at 37 C, 2 ml of overlay with 0.001% neutral red were added. Plaques were counted the next day and titers were recorded as plaque forming units (PFU) per ml.

5. *Mouse titration of dengue virus*

Volumes of 0.02 ml of serially 10-fold diluted virus specimens were inoculated into suckling mouse brain. The 50% lethal dose (LD_{50}) was calculated by Reed and Munch's method from the mortality in 3 weeks. All the mice used for mouse titration, viral growth and preparation of antisera were an inbred strain of C3H, kindly supplied by Dr. S. Tanabe, Department of Bacteriology, Medical School, Osaka University.

6. *Fluorescent antibody technique*

Cells were grown in Lab-Tek 8 chamber tissue culture slides (Miles, Illinois, U. S. A.) in a CO_2 incubator at 37 C. About 1×10^4 cells/well were inoculated with 0.1 ml of diluted virus specimen. After an appropriate incubation time at 37 C, spreading virus every 30 min, cells were rinsed with 0.5 ml of Hanks' balanced salt solution (HBSS) and then covered with 0.4 ml of 2% calf serum in MEM, and incubated at 37 C. After incubation for 2–4 days, fluids were removed and cells were washed with HBSS and fixed in acetone at 4 C for 10 min. Fixed cells were stained with the first serum (anti-D4 serum diluted 10-fold having a staining titer of 10^{-3}, unless otherwise specified) by incubation at 37 C for 30 min. The slides with cells were rinsed in HBSS and then stained with the second serum (fluorescent isothiocyanate-conjugated rabbit anti-mouse IgG serum, Pentex, Miles, Kentuckey, diluted 10-fold to a staining titer of 10^{-1}). After incubation at 37 C for 30 min, slides were thoroughly washed and then scanned with a Tiyoda ultraviolet microscope, FM-200, equipped with an Osrum 250 w high-pressure mercury arc lamp and UV-filter.

Staining titers of the sera were determined by applying sera serially diluted 3 fold to the infected cells. Titers were expressed as the highest dilution of the serum which showed definite positive fluorescence in the infected cells.

RESULTS

1. *Growth of dengue virus type 4 in 3 established lines of mammalian cells*

To assay viral infectivity by counting fluorescent foci, it seemed desirable to use a cell-virus system in which the virus or viral antigens were confined to infected cells, which could be stained brightly by the fluorescent antibody technique, and the fluid virus titer was much lower than the intracellular virus titer. In such a system cell-to-cell spread of the virus predominates over formation of secondary foci by release of progeny virus into the fluid medium.

Cultures of BHK21, VERO and LLC-MK_2 cells in 2 oz bottles were inoculated with 0.2 ml of 10-fold diluted stock of dengue virus type 4 at an input multiplicity of about 0.1 PFU/cell. After adsorption for 2 hr at 37 C, cells were washed with PBS and overlayed with 3 ml of 2% calf serum in MEM. Every day during

incubation at 37 C, duplicate cultures were taken. The fluid was pooled and the cell sheets were scraped into a volume of virus diluent equal to that of the fluid and homogenized in a Teflon homogenizer. Virus titers in the fluid medium and cell homogenate were assayed by plaque titration on BHK21 and VERO cells.

Fig. 1 shows that the virus titers in the infected fluids of VERO and MK_2 cells were almost the same as the titers associated with infected cells. However, in infected cultures of BHK21 cells, almost all the virus was associated with the cells, and less than 10% of the titer was released into the fluid for 5 days after infection. Thus, for the present purpose BHK21 cells were better than VERO or MK_2 cells although viral growth in the latter cell lines was as good as in BHK21 cells. The viral titer assayed by the conventional method of plaque titration was the same in BHK21 and VERO cells, though the plaque size in BHK21 (2 mm) was smaller than in VERO cells (4 mm). The PFU titer in MK_2 cells was a little lower than in the other two cell lines.

2. *Infectivity assay of dengue virus type 4 by counting fluorescent foci on BHK21 cells*

The appropriate adsorption time of the virus to the cells was determined first. BHK21 cells in 8 chamber slides were inoculated with D4 stock virus diluted serially 10-fold. After an appropriate time of adsorption at 37 C, cells at each dilution in duplicate wells were rinsed with HBSS and covered with fluid medium. On the second day after inoculation, cells were observed after staining with fluorescent antibody. Table 1 shows that the number of fluorescent foci increased in the first 2 hr of adsorption, but the number after adsorption for 4 hr was almost the same as that after 2 hr.

Next, the effect of the period of incubation after virus inoculation until fluorescent antibody staining was tested. BHK21 cells in 8 chamber slides were inoculated with 0.1 ml of serially 10-fold diluted virus specimen. After 2 hrs' adsorption at 37 C, cells were rinsed and

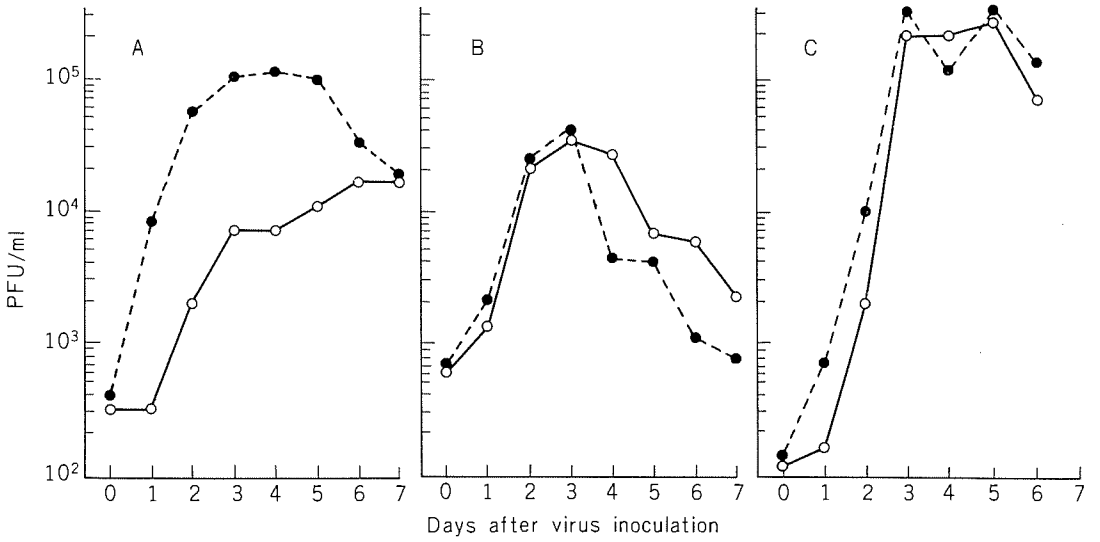

FIGURE 1. *Growth of dengue virus type 4 in BHK21, VERO, and LLC MK_2 cells. Cultures of BHK21 (A), VERO (B), or LLC MK_2 (C) cells in 2 oz bottles were inoculated with dengue virus type 4 at a multiplicity of infection of about 0.1 PFU/cell. Duplicate cultures for each cell series were sampled every day after inoculation and assay of virus in the fluid (○——○) and cells (●·····●) was done by plaque titration on BHK21 and VERO cells.*

covered with fluid medium. After 1–5 days' incubation at 37 C, slides were taken out, rinsed with HBSS, and kept at −20 C until all the specimens were ready for fluorescent antibody staining. On the first day after inoculation, fluorescent foci consisted of only 1–3 cells with viral antigen, so that virus infectivity was difficult to count. However, on the second day, each fluorescent focus consisted of clumps of 10–20 cells with brightly stained cytoplasm (Fig. 2). On the 3rd day, the number of foci was greater, but secondary foci appeared so that the number of foci could not be counted on the 4th and 5th days at low virus dilutions

TABLE 1. *Effect of Adsorption time of dengue virus type 4 on the formation of fluorescent foci on BHK 21 cells*[a]

Adsorption time (hr)	Number of foci per well	
	Virus dilution	
	10^{-2}	10^{-3}
0.5	42, 42	2, 4
1	49, 62	5, 6
2	89, 80	18, 18
4	85, 105	20, 13

[a] Duplicate wells in 8 chamber slides with BHK 21 cells were inoculated with diluted dengue virus type 4 and adsorbed for various lengths of time. Samples were taken on the 2nd day after inoculation for fluorescent focus counting.

FIGURE 2. *Fluorescent foci on BHK21 cells produced by dengue virus type 4 on the second day after inoculation.*

(Table 2). When anti-D4 antiserum was added to the fluid medium to 1% final concentration, the number of fluorescent foci on the second day was the same as in the control without antiserum, but development of secondary foci after the 3rd day was prevented.

From these experiments we adopted 2 hrs' adsorption of virus to BHK21 cells and 2 days' incubation at 37 C after virus inoculation until sampling as standard conditions.

TABLE 2. *Effect of the incubation time after inoculation of dengue virus type 4 on the formation of fluorescent foci on BHK 21 cells*

Days after inoculation	Fluorescent foci			
	Virus dilution			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	DC[a]			
2	160	29	4	0
3	176	30	2	0
4	confl[b]	confl	4	1
5	confl	confl	16	0

[a] Fluorescent foci contained only 1–3 cells so that they were difficult to count.

[b] Foci were confluent and so could not be counted.

3. *Validity and sensitivity of titration by counting fluorescent foci*

To see whether this method could be used for infectivity titration of D4 virus, we inoculated a serially 2-fold diluted virus specimen onto BHK21 cells in 8 chamber slides, using 6 replicate wells for each dilution, and counted the fluorescent foci on the second day after inoculation. Fig. 3 shows that there was a linear relationship between the relative virus concentration and the number of foci in each well with up to 200 foci/well. This confirms that each focus originated from a single infecting virus in the inoculum. The titers obtained by counting fluorescent foci were expressed as fluorescent focus forming units (FFU) per ml.

Three experiments with different lots of stock D4 virus were performed to compare the sensitivity of the FFU method with those of

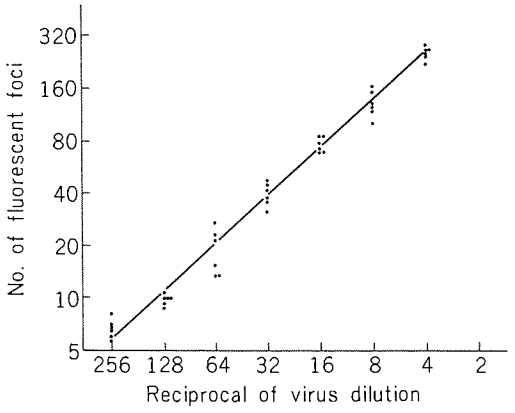

FIGURE 3. *Relationship between relative concentration of dengue virus type 4 and number of fluorescent foci on BHK21 cells. 0.1 ml of a serially 2-fold diluted specimen of dengue virus type 4 was inoculated onto BHK21 cell cultures in 8 chamber slides, using 6 wells for each dilution. Fluorescent foci produced on the 2nd day after inoculation were counted.*

TABLE 3. *Sensitivities of methods of titration of dengue virus type 4 by plaque formation, fluorescent focus formation, and mouse* LD_{50} *values*

Dengue virus type 4	Infectivity of dengue virus type 4 titrated by		
	FFU/ml	PFU/ml	LD_{50}/ml
Lot 1	3.00×10^6	5.30×10^6	NT[a]
Lot 2	1.28×10^7	1.20×10^7	1.3×10^7
Lot 3	4.70×10^5	4.75×10^5	NT[a]

[a] Not tested.

the well-established methods of PFU and mouse LD_{50} titration. Table 3 shows that the three methods have almost the same sensitivity in assay of infectivity of dengue virus type 4.

Formation of fluorescent foci on BHK21 cells by inoculation with D4 virus was inhibited by prior incubation of the virus with 10-fold diluted anti-D4 mouse serum. However in preliminary experiments the number of fluorescent foci produced by D4 virus was not reduced by anti D1 mouse serum. Thus, this may be a rapid method for titration of type-specific neutralizing antibody for dengue viruses.

The specificities of the type-specific antisera used as the first serum were tested. BHK21 cells in 8 chamber slides were inoculated with low dilutions of D1, D2, D3, and D4 viruses. After incubation at 37 C for 4 days, cells incubated with each type of dengue virus were allowed to react with the 4 kinds of type-specific anti-dengue sera diluted serially 9-fold. Then cells were stained with the common second serum (fluorescent dye-bound anti-mouse IgG rabbit serum) and observed under a fluorescent microscope. Table 4 shows the high cross-reactivities of these 4 kinds of antisera.

DISCUSSION

A rapid and quantitative method for assay of several arboviruses by counting fluorescent cells has been developed (Hahon, 1966; Hahon

TABLE 4. *Specificity of anti-dengue virus mouse sera used in fluorescent antibody staining of BHK 21 cells infected with dengue viruses*[a]

Dengue virus	type 4				type 3				type 2				type 1			
Dilution of antiserum	9	81	729	6561	9	81	729	6561	9	81	729	6561	9	81	729	6561
Anti-D 4	++	+	±	−	+	±	−	−	+	−	−	−	+	−	−	−
Anti-D 3	+	±	−	−	++	+	±	−	+	−	−	−	+	−	−	−
Anti-D 2	++	±	−	−	++	+	±	−	++	+	−	−	++	+	±	−
Anti-D 1	++	+	±	−	++	+	±	−	++	±	−	−	++	++	+	−

[a] Degree of fluorescence: ++, strongly positive; +, definitely positive; ±, slightly positive; −, negative.

and Cooke, 1967; Hahon and Haukins, 1970). Counting fluorescent foci has also been successfully used for cloning a certain strain of dengue virus type 2, which did not produce definite cytopathic effects (Lubinski et al., 1973). The primary foci formed with this strain consisted of groups of 30–100 stained cells surrounded by the unstained cells. We observed formation of similar fluorescent foci with dengue virus type 4 and BHK21 cells. The basic conditions for titration of virus infectivity with this system are reported in this paper. The merits of this method are that primary foci consisting of clumps of 10–20 cells are much easier to detect and count than single fluorescent cells and that results can be obtained after only 2 days of incubation, so that the method is much more rapid than the classical plaque titration method. Moreover, this methods is more economical than the latter method with respect to the number of cells and volume of medium and space for glassware required. The linear relation obtained between the relative virus concentration inoculated and the number of fluorescent foci in a chamber well indicated that a single focus originates from one infectious virus. Addition of antiserum to the medium after virus inoculation did not decrease the number of primary foci, but inhibited the appearance of secondary foci. Moreover the infective virus titer of the fluid medium was relatively low compared with that of the cells. Thus, in this system with dengue virus type 4 and BHK21 cells, cell-to-cell spread of virus seems to be predominant.

The sensitivity of this method is comparable to those of the conventional plaque titration and mouse LD_{50} titration methods. This together with the finding that focus formation is inhibited by prior incubation of the virus with antiserum, can be regarded as fundamental prerequisites for use of the method for infectivity titration of the virus.

The specificities of the anti-dengue sera used as the first sera were low because of the high degree of cross-reactivity between the 4 types of dengue viruses, as already indicated (Atchson et al., 1966). Thus before this method can be used for rapid identification and typint of dengue viruses type-specific antigen (Brandt et al., 1970; Trent and Qureshi, 1972) for each of the 4 types of dengue virus must be isolated, and more type-specific antisera must be prepared using these antigens. Dengue hemorrhagic fever is a very common and serious disease in South East Asian countries, so rapid and reproducible methods for titration of dengue viruses or type-specific antibody levels and for identification of types of viruses are urgently needed. We hope that the method reported here will be useful for these purposes, after overcoming various technical problems, including the isolation of type-specific antigen. The method is also valuable for studies on the fundamental virology of dengue viruses, because it is rapid and can be used to detect viruses that do not cause clear cytopathic effects but produce progeny virus and viral antigens.

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