Characterization of the Tacaribe Group of Arboviruses. 1. Propagation and Plaque Assay of Tacaribe Virus in a Line of African Green Monkey Kidney Cells (Vero). (32029)

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The propagation of Tacaribe virus in HeLa cells(1) and in hamster kidney tissue culture (HKTC)(2) has been described. However, marked and consistent cytopathic effect (CPE) following inoculation of Tacaribe virus has not been observed. Only incomplete CPE was noted in HeLa cells and a slight CPE in HKTC. Tacaribe virus has been shown to produce plaques under agar overlay without CPE in primary rhesus monkey kidney(MK)(3), continuous embryonic rhesus MK (MA-104)(4), and continuous newborn rabbit kidney (MA-111)(5) cell cultures.

This report describes CPE and plaque formation with Tacaribe virus in monolayer cultures of an African green MK *(Cercopithecus aethiops)* cell line (Vero). It was found that Vero cells not only support Tacaribe virus growth, but also develop cytopathic change with complete destruction of the cell sheet. The Vero cell cultures thus furnish a useful TC system for assay and propagation of Tacaribe virus.

Materials and methods. The virus used in this study was the TR 11573 strain of Tacaribe virus supplied by Dr. W. Downs. It had been through 21 passages in suckling mouse(SM) and one in suckling hamster (SH). Antiserum for Tacaribe, Junin and Machupo viruses was obtained from rabbits inoculated intravenously 3 or 4 times at 7-10 day intervals with a suspension of infected SM or SH brain diluted to 10^{-3} in Medium 199 (M-199)(6). Vero cells(7) were kindly furnished by Dr. Y. Yasumura, Chiba University, Chiba, Japan, in 1964. In this laboratory, the cells have been subcultured in M-199 containing 5% fetal calf serum, and maintained in M-199 with 2% fetal calf serum. They were dispersed with 0.25% trypsin and diluted in the growth medium. Cell suspension was adjusted to contain 1.5×10^5 cells per milliliter of medium. They were seeded into tubes (1 ml), 2 oz. bottles (5 ml), or 32 oz. bottles (40 ml), and were incubated for 3 to 4 days at 37°C. The medium was then changed, and the cultures were incubated for an additional 2 to 3 days and were ready for inoculation. Medium 199 without serum was used as virus and serum diluent. For the agar overlay, the medium was made by mixing equal parts of 3% agar in sterile distilled water and the following:

Lactalbumin hydrolysate (5%)	12.0 ml
Earle's balanced salt solution (without $NaHCO_3$) 10x	18.0 ml
$NaHCO_3$ (7.5%)	5.4 ml
Fetal calf serum (inactivated at 56° for 30 minutes)	3.6 ml
Neutral red (0.1%)	4.0 ml
Antibiotics (200 U penicillin, 200 γg streptomycin)	1.0 ml
Sterile distilled water	46.0 ml

Virus assays. Cell cultures in tubes were drained and inoculated with 0.2 ml of test material. Four tubes were usually used for each 10-fold serial dilution tested. After 1 hour of incubation at 37°C, 1 ml of medium was added. The tubes were incubated in stationary racks at 37°C, and examined for development of CPE. Final readings were made on the 12th day following inoculation and the 50% infectious endpoint was calculated by the Reed-Muench formula(8).

For *plaque assay,* monolayers in 2 oz. bottles were inoculated, in duplicate, with 0.2 ml of appropriate virus dilutions after removal of the medium and washing once with diluent.

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TABLE I. Serial Passage and Comparative Virus Titers of Tacaribe Virus (TR 11573 Strain) in Vero Cells and Suckling Mice.

Passage level	Cumulative $\log_{10}$ dilution of original inoculum*	Cumulative No. of days in tissue culture	Log_{10} virus titer		
			TCD_{50}	PFU	SM† LD_{50}
SM/21: SH/1			8.2	8.7	8.5
" Vero/1	7	8	7.8	—‡	—
" Vero/2	10	17	6.7	—	—
" Vero/4	16	31	5.9	—	—
" Vero/6	24	47	6.4	—	—
" Vero/8	32	60	7.5	—	—
" Vero/10	40	77	7.5	7.4	7.5

* Original inoculum was a suspension of infected hamster brain.
† Intracerebral inoculation of suckling mice.
‡ Not done.

The inoculated bottles were incubated for one hour at 37°C and washed twice. They were then covered with 5 ml of agar overlay, and were incubated in the dark at 37°C. Plaques were counted on the 10th to 12th day after inoculation, and results expressed as plaque-forming units (PFU).

Tacaribe virus was routinely assayed in 1-day old SM. Mice were inoculated by the intracerebral (IC) route with 0.02 ml of appropriate virus dilution and LD_{50} endpoints calculated with the Reed-Muench formula.

For cytopathology studies, cells were grown on coverglasses in Leighton tubes and stained by Giemsa's method.

Neutralization tests. Immune and pre-immune rabbit sera were heated at 56°C for 30 minutes. Neutralization (N) tests were performed by mixing 0.5 ml of a virus dilution, containing an estimated 100 PFU/0.2 ml, with 0.5 ml of 2-fold serial dilutions of serum. After incubation at room temperature for 1 hour, 0.2 ml of the mixture was inoculated per bottle and the overlay was added as described above. Titers were expressed as the highest serum dilution producing plaque reduction of at least 80% of the simultaneous control assay in a 1:8 dilution of pre-immune or normal rabbit serum. Neutralization tests in tubes, using 100 TCD_{50}, were also performed by the method described above.

Relationship of virus dilution to plaque number. Starting at 10^{-6} dilution of virus stock, 0.2 ml of serial 2-fold virus dilutions were inoculated into each bottle. After 1 hour, bottles were removed from the incubator, washed twice with diluent, and overlaid with agar medium described above.

Results and discussion. Cytopathic effects. Fig. 1 shows CPE produced by Tacaribe virus in Vero cells. Morphological changes can be noted as early as 5 days after inoculation. In general, in the case of tube cultures in a stationary position, the 10^{-5} dilution of the virus produced CPE by the 7th day. Examination of unstained cell cultures under low magnification (72×) revealed focal areas of shrunken and granulated cells surrounded by normal appearing cells (Fig. 1b). Later, the affected cells became detached from the tube wall with formation of macroscopically recognizable clear areas. There was extensive (Fig. 1c) or complete destruction of the cell sheet by the 10th day. The CPE in Vero cell cultures has been an all-or-none phenomenon. Thus, if even a few cells show evidence of morphological changes, the entire culture is eventually destroyed. Examination of infected cells of Giemsa stained cover-glass preparations revealed cytoplasmic changes described by Buckley(1). Polymorphic basophilic inclusions of varying size occurred either near the nuclear membrane or irregularly throughout the cytoplasm (Fig. 1d). The nucleus, however, appeared to be intact. Over a 2-month period 10 serial subcultures of Tacaribe virus were made in Vero cultures, resulting in cumulative final dilution of the original inoculum of 10^{-40} (Table I). Subcultures from infected to new cultures were carried out at 5 to 9 day intervals, and the nature of the CPE was not altered by these passages.

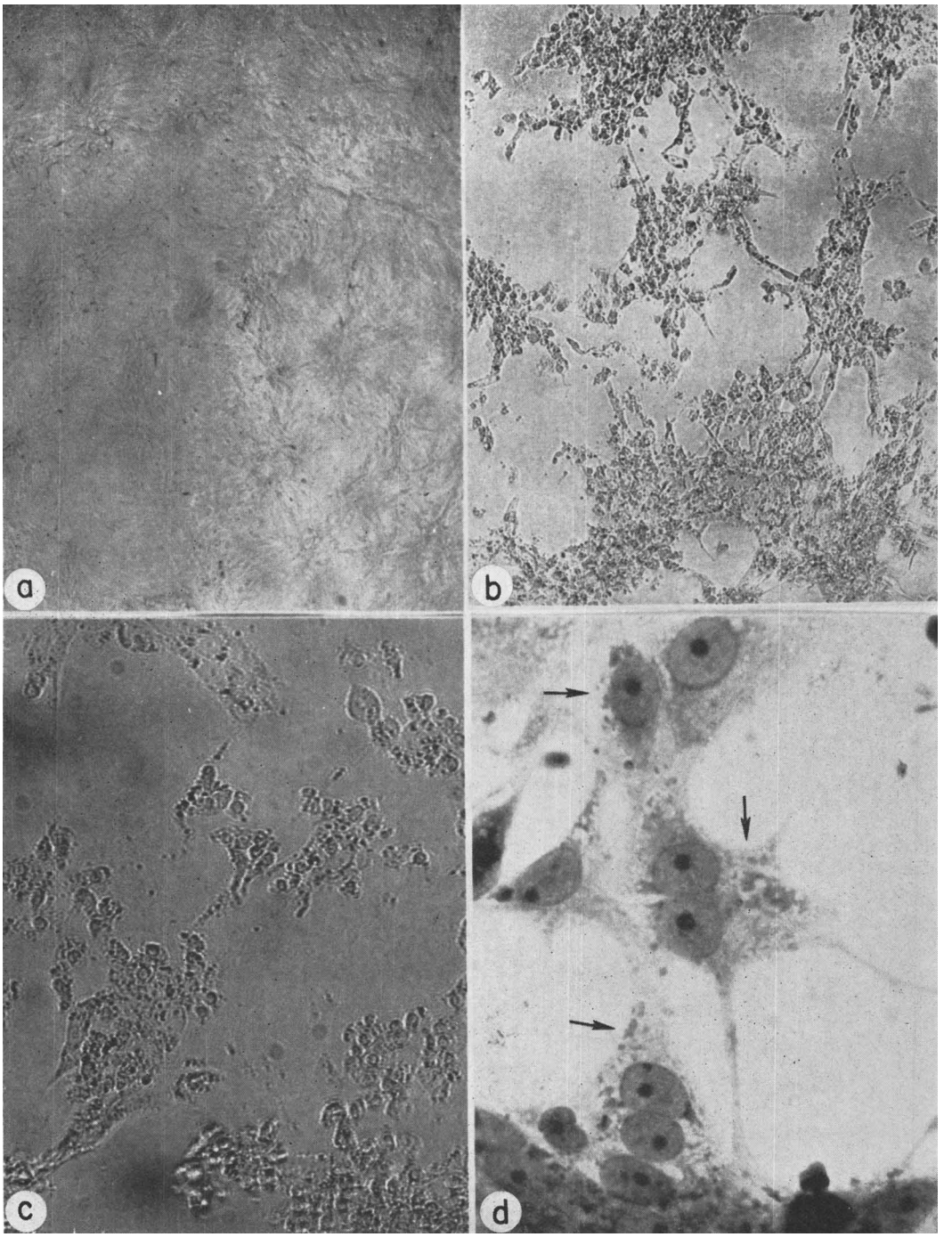

FIG. 1. Cytopathic effects (CPE) produced by Tacaribe virus (TR 11573 strain) in Vero cells. (a) Uninoculated Vero cells, unstained; 72X. (b) Early CPE of Tacaribe virus in Vero cells; day 7 unstained; 72X. (c) Tacaribe virus CPE in later stage in Vero cells; day 10; unstained; 120X. (d) The same as (c) but Giemsa stained; 270X. Note polymorphous basophilic inclusions (arrow) of varying size that occurred either near the nuclear membrane or irregularly throughout the cytoplasm.

TABLE II. Relationship Between Plaque Count and Virus Concentration.

Virus dilution (reciprocal)	Plaques/bottle No.	Avg	Log_{10} virus titer (PFU/0.2 ml)
1×10^6	86, 68, 81	78.3	7.8
2×10^6	40, 26, 36	35.3	7.8
4×10^6	17, 15	16	7.8
8×10^6	5, 7	6	7.7
16×10^6	2, 3	2.5	7.6

No CPE was observed in monolayer tube cultures of human embryonic lung (WI-26 and WI-38), continuous African green MK (BSC-1), MA-104 or MA-111 cells.

Plaque production. Small, round plaques were visible as early as the 7th and 8th day after inoculation. They increased in diameter to 0.5-1.5 mm by the 10th day; at that time, the plaques were clear, with sharp boundaries, and were easily counted when the bottle culture was held against a white background. There was little or no change after the 10th day. Tacaribe virus plaques were also obtained in MA-111 bottle cultures by using the overlay described above. However, the plaques were not as clear and the boundaries were not as distinct as those shown in Vero monolayers.

Table I gives results of comparative virus titrations by determination of TCD_{50}, PFU, and SM LD_{50}. Specimens of the 1st and 10th serial subcultures of Tacaribe virus, grown in Vero cells, were assayed, and the titers obtained by various methods were comparable.

The relationship between plaque count and virus concentration is shown in Table II. A linear relationship was observed between the number of plaques and the virus concentration, a result indicating that each plaque was produced by a single virus particle(9).

Neutralization tests. The development of either CPE or plaques by Tacaribe virus was inhibited by type-specific immune rabbit serum. As shown in Table III, when the virus seed pool of infected SH brain was used, a dose of 20 TCD_{50} was neutralized by a 1:150 dilution of Tacaribe virus immune serum, but not by 1:4 dilutions of Junin or Machupo virus antiserum. When virus from the 10th passage in Vero cells was used, a dose of 320 TCD_{50} was also neutralized by a 1:100 dilution of Tacaribe virus antiserum.

As shown in Table IV, when an average dose of 112 PFU of Tacaribe virus was used, 80% plaque reduction was obtained with a 1:256 dilution of Tacaribe virus antiserum. Plaque reduction was not obtained with a 1:8 dilution of normal (pre-immunization), Junin virus or Machupo virus antiserum. These results confirm earlier findings that Tacaribe virus is not related to Junin and Machupo viruses by the N test(3,4).

TABLE III. Serum Neutralization of Tacaribe Virus CPE in Vero Cells.

Source of virus, passage levels	Challenge dose TCD_{50}	PRS*	Rabbit immune serum†		
			Tacaribe	Junin	Machupo
SM/21: SH/1	20	<4	150	<4	<4
SM/21: SH/1: Vero/10	320	—	100	—	—

* PRS = Pre-immune rabbit serum.
† Neutralization titers shown as reciprocal of serum dilution inhibiting CPE.
SM = suckling mice; SH = suckling hamster. — = not done.

TABLE IV. Serum Neutralization of Tacaribe Virus Plaques in Vero Cells.

Antiserum used	Rabbit immune serum dilution 1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Titer*
PRS†	112‡	—	—	—	—	—	—	—	<8
Tacaribe	—	—	0	2	9	12	32	64	1:256
Junin	39	67	84	115	—	—	—	—	<8
Machupo	59	91	—	—	—	—	—	—	<8

* Titer shown as reciprocal of serum titer calculated for 80% reduction of plaque counts.
† PRS = Pre-immune rabbit serum.
‡ Average PFU/bottle. — = not done.

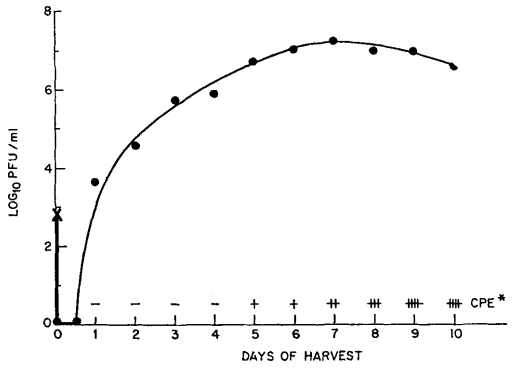

FIG. 2. Gowth curve of Tacaribe virus (TR 11573 strain) in Vero cells. *CPE – = no cellular change, + = 25%, ++ = 50%, +++ = 75%, ++++ = 100% cells affected. X: Virus dose = 9.1 x 10^2 PFU/4.1 X 10^6 cells = 1/4500.

Virus replication. Cell cultures in 2 oz. bottles were washed once and inoculated with 0.2 ml of a 10^{-5} dilution of the stock virus (9.1 $\times$ 10^2 PFU by assay). As there were 4.1 $\times$ 10^6 cells per bottle, this corresponds to an approximate dose of 1 PFU per 4500 cells (0.0002).

The virus inoculum was left in contact for 1 hour at 37°C: the cultures were then washed twice to remove unadsorbed virus and incubated at 37°C after covering with 5 ml of maintenance medium. At daily intervals, 2 bottles were frozen and stored at —70°C. The paired bottles were thawed, pooled and clarified by centrifugation at 2,000 rpm for 15 minutes, and their supernatant fluids were assayed for PFU. Results are shown in Fig. 2. The peak titer of 2.1 $\times$ 10^7 PFU/ml was obtained on day 7, at which time about 50% of the cells showed CPE. After day 7, the virus titer declined for 2 days, while CPE progressed to a complete destruction of the cell monolayer. It has been reported that the rate of Tacaribe virus multiplication in fluid cultures did not correspond to the rate of plaque development in cultures of primary Rhesus MK cells(3), as shown with other arbovirus TC systems. However, in this study, the rate of virus multiplication in fluid cultures did correspond to the rate of plaque development in Vero cells under agar overlay.

Summary. An African green monkey kidney cell line (Vero) has been found to support growth of Tacaribe virus. Cytopathic effects (CPE) were pronounced and appeared within 7 days after inoculation with 10^{-5} dilution of the virus. They were reproducible in 10 serial subcultures. Tacaribe virus also produced distinct plaques with sharp boundaries in monolayer cultures of Vero cells under agar overlay. A linear relationship was observed between plaque counts and virus concentration. The development of CPE and plaques was specifically inhibited by Tacaribe virus immune rabbit serum, but not by Junin or Machupo virus antiserum. Virus assays obtained by titration endpoints of CPE or by plaque counts in tissue culture were comparable to those obtained by titration in suckling mice. When the cultures were infected with <0.001 PFU/cell, virus yields reached their peaks within 7 days.

1. Buckley, S. M., Am. J. Trop. Med. Hyg., 1965, v14, 792.

2. Downs, W. G., Anderson, C. R., Spence, L., Aitken, T. H. G., Greenhall, A. H., ibid., 1963, v12, 639.

3. Henderson, J. R., Downs, W. G., ibid., 1965, v14, 796.

4. Tauraso, N. M., Wiebenga, N. H., Shelokov, A., Bact. Proc., 1964, p122.

5. Johnson, K. M., Wiebenga, N. H., Mackenzie, R. B., Kuns, M. L., Tauraso, N. M., Shelokov, A., Webb, P. A., Justines, G., and Beye, H. K., Proc. Soc. Exp. Biol. & Med., 1965, v118, 113.

6. Morgan, J. F., Morton, H. J., Parker, R. C., ibid., 1950, v73, 1.

7. Yasumura, Y., Kawakita, Y., Nippon Rinsho, 1963, v21, 1209.

8. Reed, L. J., Muench, H. A., Am. J. Hyg., 1938, v27, 493.

9. Dulbecco, R., Proc. Nat. Acad. Sci., U.S., 1952, v38, 747.

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