A Large and Small Plaque Variant of Sindbis Virus Obtained by Serial Passages in Tissue Culture of Chick Embryo Cells[1] (36564)

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(Introduced by W. W. Ackermann)
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It is well known that a stock culture of Sindbis virus may contain different populations of plaque-forming particles (1–3). Variants differing in the size of plaque they produce are obtained by cloning parent stock cultures (1–3). The present paper describes properties of large and small plaque variants of Sindbis virus obtained by serial passage in tissue cultures of chorioallantoic membrane and of chick embryo brain cells. The characteristics of these variants are contrasted with what has been reported previously by others (1, 4–6).

Materials and Methods. Virus. Sindbis virus 30698 (M-8) strain was kindly supplied by the Institute of Tropical Diseases Nagasaki University. The virus has been maintained by suckling mouse brain passages in our laboratory.

Plaque assay. Sindbis virus was assayed on monolayers of chick embryo fibroblast (CEF) cells, prepared by seeding prescription bottles with 4 ml of a cell suspension obtained by trypsinization of 10 or 12-day-old chick embryos. Cell suspensions were made in 0.5% lactalbumin hydrolysate, 10% inactivated bovine serum, and 0.002% phenol red in Hanks' balanced salt solution with antibiotics. After overnight incubation at 37°, cultures were ready for viral inoculation and agar overlay. The number of viral plaques produced was counted 2 days later. Viral titers were expressed as plaque-forming units (PFU)/0.2 ml of inoculum (7).

Isolation of a large plaque variant. To obtain a large plaque variant, the virus was serially passaged in chorioallantoic membrane (CAM). Two half portions of membrane were suspended in 5 ml of Hanks' balanced salt solution with 10% bovine serum and antibiotics in a 50-ml Erlenmyer flask. Cultures inoculated with 0.3 ml of undiluted virus material were incubated with shaking at 37°. Twenty-four hours after inoculation, the culture bottles were frozen in dry ice–acetone mixture and then thawed in water. After freezing and thawing three times, culture fluids with tissue cells were centrifuged at 3000 rpm for 10 min. Supernatant fluids were removed and stocked as virus materials to be used for transfer. For selecting a small plaque variant, one or two chick embryo brains (CEB) were used instead of CAM. In each transfer, viral yields and plaque sizes were measured using CEF monolayer cultures. Virus from an individual plaque, formed by inoculation of limiting dilutions of the virus after 50 serial transfers, was selected and cloned three times.

Pathogenicity for mice. Tenfold diluted materials (0.02 ml) were inoculated intracerebrally into adult and suckling mice. The lethal dose (LD_{50}) was calculated by the death rate according to the method of Reed and Muench (8).

Results. Infectivities of Sindbis virus during passages in tissue culture. In attempts to obtain a host-dependent variant having different properties from the parent virus serial passages of the virus in tissue culture cells were performed. Undiluted virus was inoculated into cultures containing CAM or CEB and incubated with shaking. Harvested tissue culture fluids obtained by the method described in Materials and Methods were serially transferred to freshly prepared tissue cul-

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TABLE I. Infectivities of Sindbis Virus to Chick Embryo Fibroblast Cell Monolayers During Passages in Chorioallantoic Membrane and Chick Embryo Brain.

Generation	Tissue culture cells[a]	
	CAM	CEB
1	7.0[b]	5.3
10	5.6	6.6
20	8.6	8.0
30	9.0	8.0
40	9.3	8.8
50-clone-2	9.0	7.7

[a] CAM = chorioallantoic membrane; CEB = chick embryo brain.

[b] Virus titer = $\log_{10}$ PFU/ml of mixture of culture fluids and cell extracts.

tures. Each time, the amount of virus propagated in tissue cultures was measured by plaque counting on CEF monolayers. The results obtained are shown in Table I. The Sindbis virus grew well in both tissue cells, CAM and CEB. In the 20th generation of tissue culture, a significant morphological difference was observed between plaques produced by viruses from CAM and CEB; that is, a plaque produced in CEF cells by the virus propagated in CEB was larger and clearer than that from CAM. However, this morphological finding gradually changed with passage; at the 26th generation, there was no distinct difference between plaques produced by virus from both chicken cells, but, at the 28th and 29th passages, plaques produced by virus from CAM culture promptly changed to be very large and clear. On the other hand, no change in the form and size of a plaque produced by virus from CEB was seen. These plaque characteristics of both viral lines were maintained until the 50th passage. Then from a typical plaque produced by each variant, viruses were removed and subjected to repeated clonal purification. The large and small plaque-forming viruses were referred to as lp or CAM-50 and sp or CEB-50, respectively.

Plaque sizes and infectivities of CAM-50 and CEB-50. The size of plaque formed by CAM-50 (lp) and CEB-50 (sp) on CEF cell monolayers from 10-day-old chicks were measured. At the same time, pathogenicities of both variants for adult and suckling mice were tested. Plaque diameters and biological properties of the clones of Sindbis CAM-50 and CEB-50 are shown in Table II and Fig. 1. There are distinct differences between plaque diameters of CAM and CEB passaged viruses; CAM-50 virus formed a large and clear plaque and CEB-50 virus produced a small but clear one. These plaque diameters depend upon the condition of cell monolayers, age of chick embryo used, and number of cells seeded, although no change in the relationship of plaque size between CAM-50 and CEB-50, was observed.

The relative biologic response of lp virus for CEF cells and suckling mice was 1 PFU/1 LD_{50} while that of sp virus was 1 PFU/3.3 LD_{50}. However in contrast to previous reports the lp virus obtained here had higher titers for CEF cells and for suckling mice than the sp virus (5). No adult mice inoculated by lp or sp virus died or developed signs of encephalitis. This lack of pathogenicity for adult mice is consistent with the known behavior of stock cultures of Sindbis virus.

Sensitivities to agar inhibitors. Others have reported that the mean diameter of lp variants was more than twice as large as sp variants under agar and that this difference disappeared under agarose, *i.e.*, plaques of the sp virus more than doubled in size (1,

TABLE II. Infectivities of Sindbis Virus Mutants in Chick Embryo Fibroblast Cell Monolayers and for Suckling Mouse Brains.

Sindbis virus (30698 strain)	Plaque diam (mm)	Log_{10} (PFU/ml)	
		CEF cells	Suckling mice
Parent in suckling mice	1.7 ± 0.8[a]	8.0[b]	8.7
CAM-50	4.9 ± 1.1	9.0	9.0
CEB-50	1.3 ± 0.1	7.7	8.2

[a] Mean diameter and standard deviation were calculated from 100 plaques.

[b] Comparative titration was done simultaneously in CEF cell monolayers (0.2 ml) and in suckling mice with intracerebral inoculation (0.02 ml).

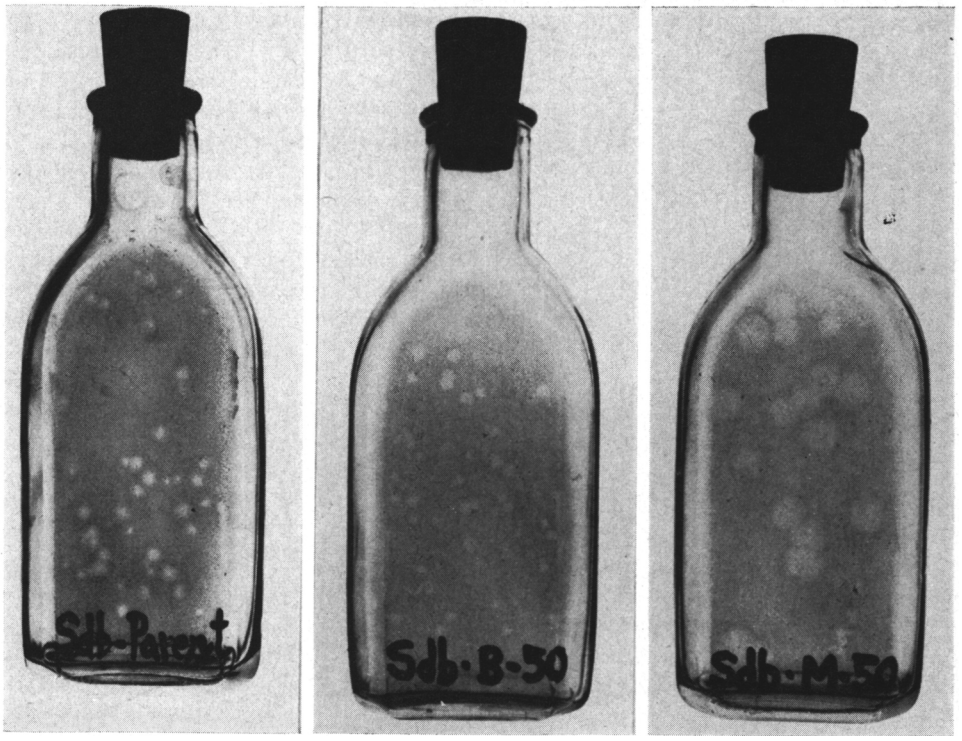

FIG. 1. Plaques produced with the parent strain of Sindbis virus in CEF monolayers (left); small plaques with Sindbis CEB-50 (middle); and large plaques with Sindbis CAM-50 (right).

5). Because of this, we studied the effect of viral inhibitors on the size of plaque produced in agar by our variants. To determine the effect, the plaque assays were done under 1.5% Bacto-agar (Difco), Bacto-agar containing protamine, and 1% agarose overlay medium. Protamine, which has been known to reverse the inhibitory effect of agar factor, was added to agar overlay medium at a final concentration of 400 μg/ml, then plaque formation of lp and sp virus was examined. In this experiment, CEF cell monolayers obtained from 12-day-old chick embryo were employed. The results were in opposition to data reported so far (1, 5), in that there were no effects on the plaque sizes of either lp or sp viruses when agarose or agar containing protamine were incorporated in the overlay medium. Moreover, protamine inhibited the plaque development of sp virus in both size and number.

Discussion. In order to obtain variants with altered virulence, it is not clear whether direct selection from plaques of the parent

TABLE III. Effects of Agarose and Protamine on the Diameters of Plaques Formed by CAM^{lp} and CEB^{sp} Variants.

Sindbis variants	Plaque diam (mm) and no.[a]		
	Bacto agar	Agarose	Protamine[b]
CAM^{lp}	4.0 ± 0.9 (25)	4.5 ± 0.8 (18)	4.2 ± 0.6 (22)
CEB^{sp}	2.3 ± 0.6 (46)	2.7 ± 0.8 (44)	1.6 ± 0.6 (39)

[a] The given diameters and based on 30 separate plaque measurements. Plaque counts are in parentheses and represent the means of duplicate counts.

[b] Protamine: 400 μg/ml.

virus, varying in size, is superior to a procedure in which an adaptation is employed before cloning. However, there are some differences between the properties of variants of Sindbis virus, arbovirus A group, obtained here and those which were selected by a direct cloning method, described by others (1–3). For instance, the large plaque mutant described here is highly pathogenic for mice, whereas, in the case of Western equine encephalitis virus, a large plaque mutant proved to be markedly avirulent for mice (6). On the other hand, the small plaque mutant, CEB^{sp}, obtained here is nonsensitive to agar inhibitors (4), but a small plaque mutant of Sindbis virus having an agar-inhitor sensitive character was selected by others (1, 5).

Clearly, these genetic markers are not obligately linked to those determining plaque characteristics. However, the properties of the large plaque variant are relatively stable. No change in properties was observed following repeated serial passage in chorioallantoic membrane, or monolayer cultures of chick embryo cells nor after one passage in chick embryo brain cells. Additional comparative experiments with these plaque variants (CAM^{lp} and CEB^{sp}) should be performed from the point of view of interferon sensitivity (1, 2, 5), effect of high concentration of NaCl on release of virus from infected cells (9), and temperature stability (10).

Summary. A large plaque variant of Sindbis virus was obtained by repeatedly cloning after serial passage 50 times in chorioallantoic membrane in tissue culture. This virus has a high infectious titer in CEF cells as well as suckling mice. On the other hand, a small plaque of Sindbis virus obtained by the same method above using chick embryo brains instead of chorioallantoic membrane, grows to somewhat lower titer than that of the large plaque variant, and is not sensitive to agar inhibitors.

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