Plaque Assay of Sendai Virus in Monolayers of a Clonal Line of Porcine Kidney Cells

HÔMU ITO

*Biological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd.,
Osaka (532), Japan*

Received for publication 29 August 1975

The MN strain of Sendai virus formed distinct plaques in monolayers of PS-Y15 cells, an established porcine kidney cell line. The plaque-forming ability was neutralized by specific antibody to the virus. A linear relationship was found between the concentration of virus and the number of plaques. The sensitivity of this assay was about equal to that of the in ovo titration. When applied to the serum neutralization test, the end points obtained were comparable to those of the hemagglutination-inhibition and complement-fixation tests.

Plaque formation by Sendai virus has been reported to occur in primary or secondary monolayer cultures of newly hatched chicken kidney (13), chicken embryo lung (1), chicken embryo skin-muscle (11, 12), monkey kidney (11, 12, 16) and calf kidney cells (6). The plaques formed in those cells were, however, faintly visible and difficult to count with accuracy. Furthermore, virus titers determined in terms of plaque-forming units (PFU) were lower than the egg-infectivity titers. Yoshii (17) found that a cell line established from porcine kidney, PS, was as sensitive to Sendai virus as were embryonated eggs. A clonal line of these cells, PS-Y15, was used in this study, which was aimed at improving the plaque assay for Sendai virus.

MATERIALS AND METHODS

Virus. Egg-passaged MN strain of Sendai virus, which was first isolated from a mouse lung by Fukumi et al. (2), was provided by K. Fujiwara, Institute of Medical Science, University of Tokyo. The virus was inoculated into the allantoic cavity of 10-day-old chicken embryos and incubated at 37 C for 2 days. After three consecutive passages, the allantoic fluid was harvested and centrifuged at $400 \times g$ for 10 min. The supernatant fluid was ampouled and stored at -70 C for seed virus.

Cell culture. A clonal line of porcine kidney cells, PS-Y15 (5) was donated by Y. K. Inoue, Institute for Virus Research, Kyoto University. The growth medium used in the present study was Eagle minimum essential medium prepared in Earles balanced salt solution; it was supplemented with 5% fetal calf serum, 10% tryptose phosphate broth (Difco), and 100 μ g of kanamycin sulfate (Takeda Chemical Industries, Osaka, Japan) per ml. The growth medium was sterilized by filtration through a membrane filter (Millipore Corp., 450-nm pore size). For stock cultures, the growth medium, containing 1×10^5

trypsinized PS cells/ml, was distributed in 50-ml amounts in Roux bottles (20 ounce [ca. 0.6 liter]) and incubated at 37 C for 3 days. The cells were dispersed by treating with a solution of 0.02% trypsin (Difco, 1:250) and 0.025% ethylenediaminetetraacetate made with Dulbecco divalent cation-free phosphate-buffered saline at 37 C for 10 min.

Preparation of immune sera. A stock preparation of Sendai virus was centrifuged at $92,000 \times g$ for 2 h, and the pellet was suspended in a 0.05 volume of cation-free phosphate-buffered saline. The virus in the suspension, usually titering 25,600 hemagglutinating units/0.025 ml, was inactivated by irradiation from a 15-W germicidal ultraviolet lamp (for 1 min at a distance of approximately 20 cm). Formalin was then added to a final concentration of 0.1%, and the mixture was allowed to stand in a cold room for 2 weeks. One milliliter of this inactivated-virus suspension was injected intraperitoneally into specific-pathogen-free male Donryu rats 4 weeks old. Sera were obtained 4, 7, 14, and 21 days after the inoculation.

Procedures for plaque formation. Growth medium (5 ml) containing 2×10^5 trypsinized PS cells/ml was added to each plaque bottle (2 ounce [ca. 0.06 liter]) and incubated at 37 C for 2 days. The growth medium was then removed and, without washing the cell sheet, serial dilutions of virus prepared in bicarbonate-free minimal essential medium supplemented with 10% tryptose phosphate buffer were inoculated in a volume of 0.2 ml per bottle. The bottles were allowed to stand at 37 C for 60 to 90 min with gentle agitation at 15-min intervals. At the end of the adsorption period, 5 ml of growth medium containing 0.8% agarose (Sigma) prewarmed at 40 C was added per bottle. After 7 days of incubation at 37 C, 3 ml of bicarbonate-free Earles balanced salt solution containing 0.8% agarose and 0.007% neutral red (Merck) warmed to 40 C was added to each bottle. After the bottles stood at room temperature in the dark for 1 to 2 days, the plaques were counted. Titers were expressed as PFU.

Infectivity titrations in eggs. For infectivity assays in eggs, serial 10-fold dilutions of a seed virus preparation were inoculated in 0.2-ml volumes into the allantoic cavities of 11-day-old chick embryos, using five embryos per dilution. After incubation at 37 C for 3 days, the allantoic fluids were harvested and assayed for viral hemagglutinin using chicken erythrocytes as described below. Titers were expressed as 50% egg infectious doses (EID₅₀), which were calculated by the Reed and Muench formula (8).

Serological procedures. Infected allantoic fluids were used as antigens in the hemagglutination inhibition (HI) and neutralization tests. In the complement fixation (CF) test, the fluid was used after heating at 56 C for 1 h. Pretreatment of sera to destroy nonspecific inhibitors for viral hemagglutinins was omitted, because no nonspecific inhibition was observed in the serum samples used in the present study. Sera were inactivated at 56 C for 30 min just before use.

Tests for CF and HI antibodies were carried out in microplates devised by Sever (10). In the CF test, reaction mixtures consisting of 2 full units of complement, 4 units of viral antigen, and test serum dilutions were kept at 4 C overnight for fixation; sensitized sheep red blood cells were then added. For the HI test, 4 hemagglutinating units of antigen and 1-day-old chicken erythrocytes were used, and sedimentation patterns were read after standing at 4 C for 90 to 120 min. In the neutralization test, 0.8 ml of a virus suspension titering approximately 400 PFU was mixed with an equal volume of each dilution, and both virus and serum were diluted in bicarbonate-free minimal essential medium supplemented with 10% tryptose phosphate broth. After standing at room temperature for 1 h, the mixtures were assayed for PFU as described above, using four culture bottles per mixture.

Antibody titers were expressed as reciprocals of the highest serum dilution which caused complete CF, complete HI, or more than 50% plaque reduction.

RESULTS

Plaque formation in PS cell monolayers. PS cell monolayers were inoculated with the virus and overlaid with agar medium, and neutral red staining was done on days 2 to 12. Plaque formation was first recognized on days 4 and 5, and the plaques increased in size and number until day 6. At this time the plaques were distinct, measuring 1 to 2 mm in diameter (Fig. 1). In subsequent experiments, the staining was carried out on day 7 after inoculation.

Next, the relationship between plaque numbers and the virus dose was investigated by assaying serial twofold dilutions of a stock virus preparation (Fig. 2). The number of plaques increased linearly with an increase in the inoculum size. This indicated that a single infectious Sendai virus particle formed each plaque.

Fig. 1. Plaques produced in a PS-Y15 monolayer culture by Sendai virus 6 days after inoculation.

As described below, the plaque formation was prevented by pretreatment of virus with specific immune sera.

The incubation time required for the maximum adsorption of Sendai virus was then examined (Table 1). It was found that adsorption was completed in 30 min. In subsequent experiments, adsorption of virus was allowed to take place for 60 to 90 min.

Parallel infectivity titrations. A seed virus preparation was assayed in parallel for PFU by the present assay and for EID₅₀. The titers obtained by the plaque assay were 9.2 and 9.7 log₁₀ PFU/ml. These values corresponded well with the egg infectivity titers of 9.3 and 9.5 log₁₀ EID₅₀/ml. These results indicated that the sensitivity of the two assay systems was almost equal.

Growth characteristics. Growth characteristics of the virus in PS-Y15 cells were then stud-

FIG. 2. Relation between the concentration of virus and the number of plaques produced in PS-Y15 cells. Each point represents mean $\pm$ standard deviation ($n = 6$). The relative virus concentration of 1 corresponds to a 10^6 -fold dilution of a stock virus.

TABLE 1. Adsorption of Sendai virus to PS-Y15 monolayer cells

Adsorption time (min)	No. of plaques/bottle ^a	% of maximum
10	62 $\pm$ 8	70
20	74 $\pm$ 4	84
30	88 $\pm$ 7	100
40	86 $\pm$ 7	98
60	82 $\pm$ 6	93
90	88 $\pm$ 7	100

^a Mean $\pm$ standard deviation ($n = 5$).

ied. Cell monolayers were inoculated with virus at a multiplicity of infection of about 1 PFU/cell. After incubation at 37 C for 1 h, unadsorbed virus was removed. The cell sheet was washed twice with phosphate-buffered saline and once with bicarbonate-free minimal essential medium containing 10% tryptose phosphate broth. To each culture bottle was added 5 ml of growth medium, and incubation was continued at 37 C. At appropriate intervals, the cultures were subjected to four cycles of freezing and thawing, and the supernatant fluid was assayed for HA and PFU titers (Fig. 3). After a lag of 4 h, PFU began to increase, attaining a peak of approximately 4.5×10^5 PFU/ml at 24 to 48 h and thereafter declined. On the other hand, the HA titer showed a lag of 6 h and reached a maximum titer of 512 in 24 h. In the case of virus harvested from embryonated eggs,

an HA titer of 512 usually corresponded to 10^8 to 10^9 PFU/ml. When compared with this empirical figure, the actual infectivity titer of the virus from PS cells was much lower. Nevertheless, the infectivity titers of PS-grown and egg-grown viruses were about equal in EID₅₀.

In the present experiments, cytopathic effect was also observed after infection of PS cells with Sendai virus. The effect was first noticed at 24 h, when virus multiplication reached a plateau. The entire cell sheet was extensively affected at 48 h and soon detached from the glass surface.

Application of plaque assay to the neutralization test. Neutralization of virus infectivity by antibody was examined by a plaque reduction test. Sera obtained from rats which had received a single injection of inactivated virus (Table 2) were tested for neutralizing, CF, and HI antibodies. Both neutralization and HI titers increased concurrently after the inoculation of inactivated virus. Titers determined on day 4 showed that the neutralization test had a higher sensitivity than the CF test. A serum sample from rat no. 26 (Table 2) was also assayed for neutralization using chicken embryos. The titer obtained in the in ovo test was 256, lower than the titer of 1,024 obtained by the plaque method. This finding suggested a higher sensitivity for the latter method.

FIG. 3. Growth curves of Sendai virus in PS-Y15 cells. Cytopathic effect was determined by microscopic observation and recorded as 0, 1+, 2+, 3+, or 4+.

TABLE 2. HI, CF, and neutralizing antibody titers of sera obtained from Donryu rats after a single injection of inactivated Sendai virus

Rat no.	Time post-inoculation ^a (days)	Antibody titer ^b		
		HI	CF	NT
1	0	0 (<4)	0 (<4)	0 (<16)
2		0	0	0
3		0	0	0
4	4	16	±4	16
5		16	±4	16
6		16	±4	16
7		16	0	16
8	7	64	32	64
9		64	32	64
10		128	32	64
16	14	512	256	ND ^c
17		64	32	64
18		256	128	ND
19		256	256	256
20		128	128	ND
25	21	512	256	512
26		512	256	1024
27		256	128	512
28		256	128	512
11	14	0	0	0
12		0	0	0
13		0	0	0
14		0	0	0
15		0	0	0
21	21	0	0	0
22		0	0	0
23		0	0	0
24		0	0	0

^a Rats no. 11 to 24 received no inoculation (cage-mate control).

^b Reciprocal of serum dilution. NT, Neutralizing antibody titer.

^c ND, Not determined.

DISCUSSION

Sendai virus is one of the most common pathogens in rats and mice. Detection of the virus by diagnostic tests and preventive measures for eradication have been laborious tasks in the routine care of experimental animals. Recently, however, the virus has attracted attention in modern fields of virology and cell biology. Analysis of its virion components in relation to the hemagglutinating activity (14) and induction of cell fusion (4, 7, 9) has provided new insights into somatic cell genetics. Furthermore infection in animals has provided a

model for human respiratory diseases (15). These studies have been greatly hampered, however, by the lack of a simple, sensitive, and reproducible virus assay method. To develop a plaque assay for Sendai virus, we tested various cell lines such as PS-Y15, Vero, L, RK 13, PK 15, and BHK-21. Of these, only the PS-Y15 cells gave satisfactory results, supporting the report of Yoshii (17) that the parental PS cells showed high sensitivity to Sendai virus and that virus growth was accompanied by a marked cytopathic and production of HA. The present plaque assay using PS-Y15 cells was as sensitive as the routine in ovo titration (3); also, it is a much simpler procedure and gives more reproducible results.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to K. Fujiwara, Institute of Medical Science, University of Tokyo, for the generous gift of the MN strain of Sendai virus and to Y. K. Inoue, Institute for Virus Research, Kyoto University, for the generous gift of PS-Y15 cells.

LITERATURE CITED

- Darlington, R. W., A. Portner, and D. W. Kingsbury. 1970. Sendai virus replication: an ultrastructural comparison of productive and abortive infections in avian cells. *J. Gen. Virol.* 9:169-177.
- Fukumi, H., F. Nishikawa, and T. Kitayama. 1954. A pneumotropic virus from mice causing hemagglutination. *Jpn. J. Med. Sci. Biol.* 7:345-363.
- Gresser, I., and J. F. Enders. 1961. The effect of trypsin on representative Myxo-viruses. *Virology* 13:420-422.
- Harris, H., and J. F. Watkins. 1965. Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. *Nature (London)* 205:640-646.
- Inoue, Y. K., and M. Yamada. 1964. Clonal line of porcine kidney stable cells for assay of Japanese encephalitis virus. *J. Bacteriol.* 87:1239-1240.
- Nagata, I., K. Maeno, S. Yoshii, and T. Matsumoto. 1965. Plaque formation by HVJ in calf kidney cells. *Arch. Gesamte Virusforsch.* 15:257-259.
- Okada, Y., and J. Tadokoro. 1963. The distribution of cell fusion capacity among several cell strains or cells caused by HVJ. *Exp. Cell Res.* 32:417-430.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493-497.
- Schneeberger, E. E., and H. Harris. 1966. An ultrastructural study of interspecific cell fusion induced by inactivated Sendai virus. *J. Cell Sci.* 1:401-406.
- Sever, J. L. 1962. Application of microtechnique to viral serological investigations. *J. Immunol.* 88:320-329.
- Shibuta, H. 1972. Effect of trypsin on the infectivity of Sendai virus grown in several host cells. *Jpn. J. Microbiol.* 16:193-198.
- Shibuta, H., M. Akami, and M. Matsumoto. 1971. Plaque formation by Sendai virus of parainfluenza virus group, type 1 on monkey, calf kidney and chick embryo cell monolayers. *Jpn. J. Microbiol.* 15:175-183.
- Shigeta, S. 1964. Plaque formation and growth characteristic of Sendai virus in chick kidney cell cultures. *Tohoku J. Exp. Med.* 83:114-120.

14. Shimizu, K., Y. K. Shimizu, T. Kohama, and N. Ishida. 1974. Isolation and characterization of two distinct types of HVJ (Sendai virus) spikes. *Virology* 62:90-101.
15. Van der Veen, J., Y. Poort, and D. J. Birchfield. 1970. Experimental transmission of Sendai virus in mice. *Arch. Gesamte Virusforsch.* 31:237-246.
16. Van Nunen, M. C. J., and J. Van der Veen. 1968. Experimental infection with Sendai virus in mice. *Arch. Gesamte Virusforsch.* 22:388-397.
17. Yoshii, S. 1964. Multiplication of myxovirus parainfluenza 1 (HVJ) and its persistent infection in PS cell. *Virus* 14:27-37.