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Isolation of a Sindbis Virus Variant by Passage on Mouse Plasmacytoma Cells

JANEY SYMINGTON* AND MILTON J. SCHLESINGER

Department of Microbiology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63110

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A variant of Sindbis virus has been isolated by growing a stock of virus, previously passaged on chicken embryo cells, in mouse plasmacytoma (MOPC 315) cells in suspension culture. An indirect immunofluorescence test and infective center assay showed that only a small fraction of cells could be infected by the stock wild-type virus, but that the population of virus accumulating after a few passages on the mouse cells had host-range properties distinct from the stock virus. The mouse-passaged virus retained its virulence for the original host and back-passaging of this virus on chicken cells did not change its newly acquired properties. Thus, this variant appears to be a genetically distinct form of Sindbis that adsorbs to and grows much better than the stock virus on several types of mouse cells including cultures of mouse macrophages.

The ability of viruses to adapt to new host organisms or tissue culture systems distinct from the normal, natural host of the virus is a phenomenon that has long been recognized (5). This property of viruses is of considerable importance, for the extent of viral adaptation has been found to profoundly influence both the pathogenicity of viral infections and the production of attenuated viruses useful as vaccines. With animal viruses, most investigations describing host-range properties and adaptation of viruses have used intact animals as the test organism, and the biological complexity of such hosts has by and large precluded extensive analysis of the adaptation phenomenon. Tissue culture and established cell lines derived from various animal tissues offer a simpler system for an analysis of a virus' host-range capacities and allow for easier and more exact quantitation of the biological activity of a virus.

One of these viruses that has proved very useful for studies carried out with cell culture systems is Sindbis virus, a group A togavirus that grows easily and rapidly in a variety of cells (11), including chicken embryo fibroblasts, BHK cells, mouse L cells, Chinese hamster ovary cells, mosquito cells (19), and HeLa cells (15). We have been studying some of the basic molecular events in the replication of Sindbis virus and have recently examined Sindbis infection of mouse plasmacytoma cells growing in suspension cultures. We were surprised to discover that these cells were initially a very poor host for our stocks of Sindbis that had been cloned and passaged on chicken embryo fibroblasts. Ultimately, we were able to alter the cell culture conditions so that a productive infection was achieved and titers of virus approached those recorded for growth of this stock of virus on BHK cells. The most important factors in increasing viral production were: (i) the state of the cells; they needed to be actively growing or capable of active, exponential growth; (ii) the concentration of cells in suspension (10^6 to 1.5×10^6 per ml); and (iii) the use of medium conditioned by prior growth with mouse plasmacytoma cells.

When we examined the kinetics of virus production under our best conditions of virus yield we discovered that very low amounts of virus appeared during the first 6 to 8 h even through virus production on chicken embryo cells is maximal at that time. The low titers that were detected at 6 to 8 h could result from an abnormal, slow production of virus by every cell or they could represent a normal infectious cycle carried out by only a small fraction of cells in the culture. To distinguish between these possibilities we scored the fraction of infected cells by an indirect immunofluorescence procedure. An immunofluorescence assay has been described for detecting Sindbis viral antigens in infected cell monolayers (22) and we adapted the technique for following infection of the plasmacytoma cells growing in suspension cultures. A sample of cells analyzed 6 h after

infection contained only a few cells forming Sindbis antigens in contrast to samples analyzed 24 h after infection. This result suggested that only a small fraction of cells were initially infected but these cells could sustain a normal 6 to 8 h cycle of Sindbis replication. The high yield of infected cells at 24 h was postulated to arise from a second and, possibly, a third round of infection by progeny virus of the first round of infection that was more efficient than the original sample in adsorbing to the plasmacytoma cells. This indeed appears to be the explanation, for we discovered that infection of mouse cells with an inoculum of virus previously passaged on mouse plasmacytoma cells led to rapid production of virus and a concomitant increase in the number of infected mouse cells (Fig. 1).

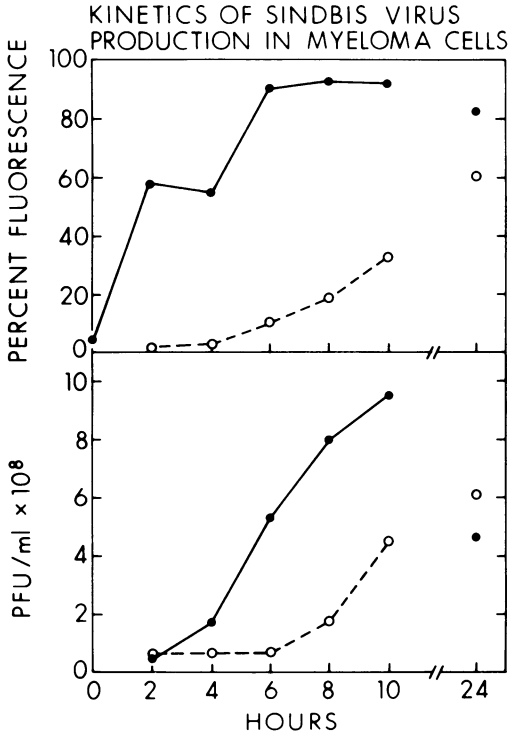

FIG. 1. *Kinetics of Sindbis virus formation in mouse plasmacytoma cells. Mouse plasmacytoma MOPC 315 was adapted for growth in tissue culture by P. Periman. Cells were grown in suspension at 37 C in a 5% CO_2 incubator in Leibowitz L-15 medium supplemented with 20% fetal calf serum. They were refed every 2 days (maintaining a cell concentration between 2 × 10^5 to 8 × 10^5 cells per ml). Twenty-four hours before infecting with virus, the cells were collected and suspended in fesh medium at a concentration of approximately 4 × 10^5 cells per ml. Immediately before infection, cells were centrifuged for 5 min at 85 × g and suspended at a concentration of 1.5 × 10^6 cells per ml in the same medium (1 day conditioned). Infection was initiated by addition of virus at a multiplicity of infection of 20. The inoculum was not removed from the cells. (Our standard preparation of Sindbis virus was a sample obtained from B. Burge [Massachusetts Institute of Technology] and was plaque purified on primary chicken embryo fibroblast cells.) Chick cell-passaged (- - -) or plasmacytoma cell-passaged (——) virus. Virus was plaqued on chicken embryo fibroblast cells (13). In scoring for fluorescent cells, a minimum of 400 cells were observed. For this immunofluorescent assay, samples of infected cells were centrifuged at 150 × g for 5 min, washed twice with 5 ml of L-15 medium containing 3% fetal calf serum, and suspended in a drop of cold 10 mM phosphate-buffered saline (pH 7.4) and a drop of fetal calf serum added. Samples of this cell suspension were spread on microscope slides, air dried, and fixed with acetone. A drop of rabbit anti-Sindbis serum, diluted 1 to 10, was added to the fixed cells and slides were incubated at 24 C for 30 min in a moist chamber. The serum (supplied by S. Schlesinger), at a 1:20 dilution, neutralized 90 to 95% of a Sindbis preparation containing 10^{10} PFU/ml. Serum was removed, the cells were washed twice with phosphate-buffered saline, and a 1:10 dilution of fluorescein-conjugated goat anti-rabbit gamma globulin (Miles Laboratories, Elkhart, Ind.) was added. Slides were incubated an additional 30 min at 24 C in the moist chamber and then washed twice with buffered saline. Fluorescent cells were scored on a Zeiss WL microscope fitted for fluorescence measurement.*

Figure 1 also shows the slower growth of chick-passaged virus on the same batch of mouse cells. A sample of virus previously passed on BHK cells showed the same course of infection on mouse cells as that depicted for chick-passaged virus.

A comparison of the ability of these various samples of viruses to grow on the homologous or heterologous host cells is presented in Table 1. Chick- and BHK-passaged virus grow equally well on BHK and chicken embryo fibroblast cells and equally poorly on the mouse plasmacytoma cells. As noted above, the mouse-passed virus grows much better on the homologous cell, yet it still is able to grow on the BHK and chicken embryo fibroblast cells at a level close to that of the BHK and chicken-passaged viruses. There appears to be little or no attenuation of virulence toward the original avian host cell in this system of passaging virus from avian cells to mouse cells.

Although the immunofluorescence assay provided some measure of the relative number of cells infected by mouse-passaged and chick-passaged virus samples, an infective center experiment was carried out to gain more precise

quantitation (Table 2). This infective center assay showed that the number of cells infected by chick-passaged virus was only 5% that measured for mouse-passaged virus and provided further evidence for a relatively poor ability of the chick-passaged virus to infect these mouse cells.

TABLE 1. *Ability of virus passaged on different hosts to grow on heterologous cells*

Host cells	Time of assay of virus (h post-infection)	Cells on which virus was previously passaged[a]		
		BHK	Chicken embryo	Mouse plasma-cytoma
BHK[b]	4	3	8	13
	6	9	36	73
	8	146	152	110
Chicken Embryo	4	10	29	39
	6	159	106	194
	8	556	505	325
Mouse Plasma-cytoma	4	3	8	18
	6	8	11	68
	8	17	31	157

[a] All plaquing was performed on chicken embryo fibroblast cells. Expressed as PFU/ml $\times 10^7$.

[b] BHK-21 were grown in minimal Eagle's medium, Earle's salts with two times glutamine and 6% fetal calf serum.

Enveloped viruses, such as Sindbis, are modified by their host so that the lipid and carbohydrates of the viral envelopes are host determined (4, 6). Thus, the differences between chick- and mouse-passaged virus might be simply a host modification and not a genetically stable property. We tested for this stability by back-passaging the virus produced in mouse plasmacytoma cells through chick cells. A sample of mouse-passaged virus that produced 1.1 $\times 10^9$ PFU/ml after an 8-h infection on plasmacytoma cells was passaged twice on chick cells and retested for its replication on plasmacytoma cells. This chick-passaged virus retained its ability to produce a high yield of virus (1.0×10^9 PFU/ml) on plasmacytoma cells after 8-h infection, in contrast to the stock parental virus which produced only 5×10^7 PFU/ml under the same conditions of infection on the mouse cells. We repeated this experiment on a separate sample of mouse-passaged virus and again observed that the variant retained its enhanced efficiency for replicating on mouse cells even after three back-passes on chick cells. The titer of virus produced after 8-h infection of plasmacytoma cells was 1.4×10^9 PFU/ml before passaging, and 0.9×10^9 PFU/ml after the third passage on chick cells. We conclude from these results that the virus isolated after passage through the mouse cells is a genetically distinct variant of Sindbis.

TABLE 2. *Assay for infective centers of mouse plasmacytoma cells*[a]

Host cells for production of inoculum	Tube no.	PFU/ml $\times 10^4$		Infected cells per ml $\times 10^4$ (A-B)	Cells infected[b] (%)
		(A) Final titer from a 60-min adsorption	(B) Residual titer in final wash		
Chicken embryo	1	2.3			
	2	2.8			
		(2.5)[c]	0.01	2.5	1.2
Mouse plasma-cytoma	1	56.6			
	2	44.8			
		(50.7)[c]	0.6	50.1	22

[a] Samples of virus were added to 2.5 ml of plasmacytoma cells, and after 1 h unadsorbed virus was separated from cells by 5-min centrifugation at $85 \times g$. Cells were washed once with an equal sample of growth medium and incubated with 2.5 ml of growth medium containing 0.25 ml of rabbit anti-Sindbis serum. After 20 min at 37 C, cells were harvested, washed twice, and suspended in 2.5 ml of growth medium. Dilutions of the cell suspension were made and plaqued on primary chicken embryo fibroblast cells. A sample of the undiluted suspension was centrifuged at $85 \times g$ for 5 min and this supernatant solution was also titered.

[b] Uncorrected for efficiency of plating. From the immunofluorescent assay, it has been repeatedly determined that 80 to 90% of the cells were infected by mouse plasmacytoma-passaged virus at the initial round (see Fig. 1). Based on this observation and assuming that the fluorescing cells are producing virus, we have calculated that the efficiency of plating in the infective center assay with these cells under our conditions is 26%.

[c] Numbers in parentheses show average.

We were interested to determine if the variant that was selected for growth on one kind of mouse cell would also have increased virulence for other kinds of mouse cells when compared with chick-passaged virus. Our variant is indeed more virulent for mouse L cells and mouse macrophages than the original virus (Table 3). There was a 2- to 13-fold increase in virus formation on monolayers of mouse L929 cells when using the variant. Interestingly, some macrophage preparations were virtually nonresponsive to chick-passage viruses while yielding significant amounts of virus with the variant as infectious agent.

On the basis of results from both the infective center and immunofluorescence assays, we propose that the variant differs from the stock virus in an ability to adsorb to mouse cells. Support for this hypothesis has come from studies comparing the amount of virus remaining free in the medium after virus was added to monolayers of mouse L929 cells. In three experiments, 17, 35, and 45% of mouse-passaged virus remained unadsorbed after 1 h, whereas the comparable values for chick-passaged virus were 87, 63, and 97% unadsorbed. Additional, preliminary experiments using preparations of radioactive virus and measuring the amounts of labeled virus adsorbed to plasmacytoma cells also support our proposal. Almost none of the labeled chick-passaged virus was adsorbed under conditions in which 30% of the labeled mouse-passaged virus became bound.

TABLE 3. *Infection of mouse L cells and mouse macrophages by chicken- or mouse plasmacytoma-passaged Sindbis virus*[a]

Host cells	Expt no.	No. of cells $\times 10^6$	Virus formed (PFU/cell)[b]			
			Chick-passaged virus		Mouse-passaged virus	
			8 h	24 h	8 h	24 h
L929	1	15.0	66	454	534	861
	2	11.7	4	141	51	877
	3	7.6	96	349	237	3090
Macrophage	1	1.8		0		5
	2	2.8		0		40
	3	1.6		2		123

[a] Mouse L929 cells were grown in the same medium with 10% fetal calf serum. Mouse peritoneal macrophages stimulated by injection of thioglycollate were from strains HA/ICR, BALB/c AnN, and C3H/Bi mice and were kindly supplied by C. Stewart (Washington University School of Medicine). These cells were infected after 2 weeks of growth. Details describing growth of these cells have been published (9, 20). They were counted at the time of infection by the modified pronase-cetrimide technique (21).

[b] Each figure is the average of two plates plaqued on chicken cells. L cell MOI = 20, macrophage MOI = 10.

It thus appears that infection by Sindbis virus of a culture of mouse plasmacytoma cells leads to the accumulation of a variant form of Sindbis which differs from the original stock virus by virtue of an enhanced ability to adsorb and infect mouse cells. Several other kinds of Sindbis variants have been described elsewhere; some of these showed a difference in plaque morphology when grown on chicken embryo cells (2, 3, 10), as well as differences in biochemical and antigenic properties (12). One attempt to locate a change in the primary structure of a virion protein in a variant by examining tryptic peptide fingerprints was unsuccessful (1). The variant described in our work was not isolated on the basis of plaque morphology and it has been difficult to observe reproducible differences between plaques made by the variant and the wild-type stock virus. Experiments have begun in an attempt to detect a change in the structure of a virion protein of the variant and to compare the antigenic properties of the variant with those of the wild type. In the presence of a large excess of antibodies raised against wild type Sindbis, the variant is neutralized to the same extent as the wild-type virus but subtle differences would only be detected in experiments by using limiting levels of these antibodies.

Often, variants isolated by passaging virus on a different, unrelated host become less able to grow on their original host. The variant described here retains its virulence for the original host cells and its growth cycle in chicken embryo cells is very much like that of stock virus. This latter result enabled us to carry out several critical genetic experiments including back-passaging of the virus through the original host, the isolation of clones of the variant, and a measure of infective centers.

The ability to enrich a virus stock for an existing variant will depend on the selection procedures. For host range variants, both differences in adsorption efficiency and intracellular replication in the new host will determine how many serial passages are required before the variant dominates the virus population. One Sindbis variant previously described that affected mouse cells required many passages on mouse L cells before it became the major type of virus in the stock (22). In contrast, the variant isolated in our work appeared very quickly, after only three or four serial passages. A

variant of mouse hepatitis virus was also isolated after a few passages on mouse macrophages (8). Conditions for cell culture also influences the appearance of variants (8), and we noted that rather carefully controlled conditions were required in culturing the plasmacytoma cells before selection of the variant was possible.

Our Sindbis variant grows much better than the stock virus on two other types of mouse cells. Of particular interest is the ability of this variant to infect cultured macrophages which appear quite resistant to the standard virus. Our variant grows better on mouse L cells as well. Inglot et al. (7) isolated Sindbis variants that also could grow much better on mouse cells and have shown that the variant would destroy a culture of L cells, whereas infection of the L cells by stock chicken-cell passage virus produced a persistent, cyclical type of infection in the tissue culture system. We were unable to show a persistent infection in the mouse plasmacytoma cells with chick-passaged virus, probably because the variant appeared so quickly. Inglot et al. demonstrated that production of interferon and appearance of defective particles were important in controlling the virulence of the stock-virus grown on L cells (7).

Variants adapted to new hosts in tissue culture also lead to different types of infection in animals, in some cases being much more virulent than the original virus (3, 17, 18). Some differences were noted in the fate of Sindbis variants when injected into mice (14, 16). We are also interested in determining if the variant we isolated will show an effect different than stock virus when given to mice and this study has recently begun. Possibly the most important aspect of these studies will be to utilize this kind of variant in seeking the biochemical basis for those specific interations between viruses and host cells during the initial stages of infection.

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