Semliki Forest Virus Replication in Cultured Aedes albopictus Cells: studies on the Establishment of Persistence

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SUMMARY

Semliki Forest virus (SFV) established a persistent, non-cytopathic infection in cultured Aedes albopictus cells with no effect on cumulative cell number as compared with control cultures. All cells were initially infected by SFV as judged by infective centre and immunofluorescence assay and released approx. 50 to 70 p.f.u./cell in the initial 24 h after infection. At 12 h after infection there was a 'shut-down' of virus-specific RNA synthesis followed by a sharp decline in the percentage of cells infected, stabilizing at about 2% by 48 h. Significant amounts of virus were released from about 2% of cells in persistently infected cultures. At 8 to 10 days a small-plaque, attenuated variant of SFV appeared in infected cultures; it replaced 'wild-type' virus completely by day 16.

The establishment of persistence could not be related to (i) the release of interferon-like activity from infected cells, (ii) the presence of a directly antiviral agent in infected cells or medium, or (iii) changes in the total levels or subcellular distribution of acid phosphatase.

Subculturing persistently infected cells stimulated cell division and promoted a (reduced) burst of virus production; simultaneous superinfection with SFV gave no increase in virus yields.

INTRODUCTION

Togaviruses are maintained in nature as a result of transmission between vertebrate hosts by haemophagous arthropods; they multiply in both their vertebrate and invertebrate hosts (Dalgarno & Davey, 1973). Infection of cultured vertebrate cells by alphaviruses is generally cytopathic, whereas infection of cultured mosquito cells generally results in a persistent infection with little evidence of c.p.e. (Rehacek, 1968; Buckley, 1971; Singh, 1971; Yunker, 1971). Only a small percentage of persistently-infected cells appears to be involved in virus production (Peleg, 1969, 1972; Yunker, 1971) and, although no gross c.p.e. is evident, a fall in the percentage of virus-producing cells concomitant with a drop in virus titre suggests a destruction of infected cells. In comparison with virus produced in mammalian cells, virus produced by persistently infected mosquito cells may show a markedly reduced virulence to mice and give rise to small plaques when assayed on mammalian cells (Singh, 1971; Sinarachatanant & Olson, 1973).

The mechanism by which persistence is established in mosquito cells is unknown. Homo-

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Heterologous interference has been observed in persistently infected cultures, but Peleg (1969, 1972) and Stollar & Shenk (1973) found no evidence for heterologous interference or for the production of interferon-like substances in alphavirus-infected cultured mosquito cells. In contrast, Enzmann (1973) concluded that the maintenance of the cell-virus equilibrium in Sindbis-infected Aedes albopictus cells was related to the presence of interferon-like substances. Ultrastructural studies show that during SFV and Ross River virus (RRV) infection of cultured A. albopictus cells, virus maturation may occur both at the cell membrane and within dense, membrane-bound, cytoplasmic inclusions (Raghow, Davey & Dalgarno, 1973a; Raghow et al. 1973b). This is noteworthy since a characteristic feature of the mumps virus carrier state in mammalian cells is the localization of virus-specific antigens in a small number of discrete, cytoplasmic masses (Walker & Hinze, 1962). At the time when persistent RRV infection of A. albopictus cells is being established, the contents of the inclusions are lost, resulting in their transformation into multivesicular vacuoles. It was proposed (Raghow et al. 1973b) that this may result from the fusion of inclusions with lysosomal microvesicles.

In this report we examine a number of aspects of the establishment and maintenance of persistence in the SFV-Aedes albopictus cell system.

METHODS

Cells and virus. Aedes albopictus cells (Singh, 1967) were grown in M-M medium according to Buckley (1969). Cells were counted in a Neubauer haemocytometer; viability was determined by staining with 0.4 % trypan blue in M-M medium (1:4, v/v). Virus stocks used in all growth experiments were prepared by intracerebral inoculation of suckling mice and assayed on Vero cells as previously described (Davey, Dennett & Dalgarno, 1973).

Virus growth experiments. Cells were concentrated and infected by replacing growth medium with a mixture of stock virus in growth medium (dilution 1:100 or 1:1000). After adsorption, cells were washed in growth medium to remove excess virus (Davey et al. 1973) diluted appropriately and incubated (see figures for details).

Infective centre assay. Infected Aedes albopictus cells were washed in growth medium (×3), resuspended at the appropriate dilution in growth medium and plated in duplicate on Vero cell monolayers (Davey et al. 1973).

Fluorescent antibody assay. Infected Aedes albopictus cells on coverslips were washed with PBS and fixed in ice-cold acetone. Immune ascitic fluid (Davey et al. 1973) was added; the coverslips were then washed in PBS and air dried. Fluorescein-conjugated, rabbit anti-mouse globulin containing 10 % rhodamine-conjugated bovine albumin (Microbiological Associates, Bethesda, Maryland) was added for 30 min, followed by washing in PBS. Fluorescence was observed with a Zeiss microscope with an Osram HB200 high pressure mercury vapour lamp. Uninfected cells or infected cells treated with non-immune ascitic fluid showed no fluorescence.

Acid phosphatase. (i) Total cellular activity: cell pellets were resuspended in water (approx. 10⁶ cells/ml) containing 0.2 % Triton X-100 and assayed as described by Linhardt & Walter (1965), using p-nitrophenylphosphate as substrate. (ii) Subcellular distribution: Aedes albopictus cells were fixed on slides in a solution containing 0.4 % formaldehyde, 0.1 % anhydrous CaCl₂ and 3 % sucrose and stored in 0.88 M sucrose. Slides were washed and incubated (60 min, 37°C) in a solution containing Na-β-glycerophosphate (0.01 M), Pb(NO₃)₂ (0.004 M) in sodium acetate buffer (0.05 M, pH 5.0). Slides were developed in dilute yellow ammonium sulphide (1 to 2 min) and counterstained with Harris’s haematoxylin blue and dehydrated.
SFV persistence in *A. albopictus* cells

with ethanol. Acid phosphatase was seen as black granules in blue cells; controls incubated with 0.01 M-NaF show no granules.

*Extraction of RNA* and *infectious RNA* assay. RNA was extracted directly from infected cell monolayers with a mixture of phenol/cresol and sodium aminosalicylate (Shine & Dalgarno, 1973). RNA was washed in ethanol and dissolved in phosphate-buffered saline (PBS) containing DEAE-dextran (1 mg/ml). For infectious RNA assay Vero cell monolayers were washed sequentially with PBS, PBS plus 0.4 M-NaCl and PBS plus 0.75 M-NaCl. Adsorption of RNA was for 20 min at 26 °C. Cells were washed with PBS plus 0.4 M-NaCl and finally with PBS before overlaying. The recovery of infectivity from stock virus was 0.1%.

*Rates of RNA synthesis.* Rates were determined by transferring cells to a defined nutrient medium (GM, Grace, 1962) which supports the growth of *Aedes albopictus* cells; SFV shows similar short-term growth kinetics in GM and in M-M medium. Duplicate infected monolayers (2.5 × 10^6 cells in 5 ml) were washed in GM and incubated for 3 h at 26 °C in GM containing [3H]-uridine (20 μCi/ml) and actinomycin (1 μg/ml). Cell monolayers were washed and precipitated at 0 °C in 5% trichloroacetic acid (TCA) containing 5 mM-uracil. After sedimentation the pellets were washed (× 2) in 5% TCA containing 5 mM-uracil and finally resuspended in 5% TCA (1 ml) and incubated at 90 °C for 30 min. After cooling and centrifuging, supernatant fluids were counted in 10 ml of dioxane-based scintillation fluid.

**RESULTS**

*SFV growth and the establishment of persistence*

The growth of SFV in cultured *Aedes albopictus* cells was assayed over 16 days at 26 °C (Fig. 1a). There was a latent period of 3 to 6 h and a maximum cell-associated virus (CAV) titre of 2.5 × 10^7 p.f.u./10^6 cells at 12 h. From 24 h there was a decline in titre which was variable in rate and in the final level reached (Fig. 1a, 2a, 3). Changes in extracellular virus (EV) titres followed those of CAV but EV titres generally stabilized at a higher level.

At 8 to 10 days after infection there was a 1 log unit increase in CAV and EV titres (Fig. 1a); this is due to the appearance of a virus which forms small plaques on Vero cells (Shenk, Kosshelnyk & Stollar, 1974). The small-plaque virus appeared at this time whether or not cells were previously subcultured. The titre of 'wild-type' virus was similar on days 8 and 10 (3 × 10^6 p.f.u./10^6 cells); on day 16 no 'wild-type' virus was detected. The small-plaque virus had reduced pathogenicity for mice; intracerebral inoculation of 10^5 p.f.u. had no apparent effect on adult mice whereas an equivalent dose of 'wild-type' SFV was lethal. The decreased virulence of Semliki Forest virus grown in mosquito cells has previously been correlated with a reduction in plaque size (Peleg, 1971).

*Cell division*

There was no significant difference in the cumulative cell number of SFV-infected or mock-infected *Aedes albopictus* cells over a period of 6 days (Fig. 1b). No difference in cell viability between the two cultures could be detected by trypan blue staining.

*Infectious centre and immunofluorescence assays*

At zero time, 36% of *Aedes albopictus* cells were infected as judged by infective centre assay; by 12 h, 81% of cells were infected (Fig. 1c). From 18 to 24 h, the number of infected cells decreased sharply; at 30 and 48 h respectively, 7 and 2% assayed positive. Since this decrease was not accompanied by any significant change in cell number (Fig. 1b) it was not due to generalized c.p.e
Fig. 1. For legend see opposite.
SFV persistence in A. albopictus cells

Similar conclusions were reached from immunofluorescence assays. At 12 h all cells demonstrated cytoplasmic fluorescence (Fig. 1c). There was a marked decrease in fluorescence intensity between 24 and 30 h. At later times, most cells did not fluoresce. The small proportion classed as positive fluoresced far less strongly than did 12 to 24 h samples.

Levels and distribution of acid phosphatase in infected cells

Acid phosphatase was used as a marker for lysozomal enzymes in infected and control cells. There was no significant difference in the total levels of acid phosphatase in infected and control Aedes albopictus cells over the first 3 days of infection (Fig. 1d); a slight increase in activity was observed in both cultures.

Microscopic examination of acid phosphatase distribution showed that the enzyme was localized in discrete, cytoplasmic granules, presumably lysosomes. No difference in the number or distribution of these granules could be detected between infected and control cultures.

Interferon-like activity in medium from infected cells

The release of interferon-like substances from infected Aedes albopictus cells was assayed. Medium from 1-, 2- and 3-day infected cultures (Fig. 1a) was centrifuged to remove most of the virus; approx. 5 x 10^2 p.f.u./ml remained after sedimentation. Uninfected cells were added to 2 x 10^6 cells/ml (final m.o.i. = 0.001), and the culture incubated at 28 °C. As controls, uninfected cells and SFV at the same final multiplicity were added to medium from uninfected cells and also to fresh growth medium. There was no significant difference in SFV growth kinetics under any of these conditions (Fig. 1e); maximum titres were not reached until 60 h after infection due to the low multiplicity.

In single step growth experiments, actinomycin at concentrations sufficient to inhibit up to 98% of host RNA synthesis was added to infected cells at zero time. At no concentration used was the observed cessation of virus production (Fig. 1a, 2a, 3) prevented. This result is also consistent with the establishment of persistence being independent of interferon-like agents.

Fig. 1. Growth of SFV in Aedes albopictus cells. A. albopictus cells (2.5 x 10^8) were adsorbed with SFV (added multiplicity, ~ 15), in 12 ml of M-M medium for 1 h at 26 °C. Cells were washed (x 3), diluted to 2.3 x 10^6 cells/ml and incubated at 26 °C. Control cultures were treated in parallel using gelatin–saline–tris (Davey et al. 1973) to replace the virus inoculum; the final cell concentration was 2.8 x 10^6/ml. (a) Virus growth. ●----●, CAV; ○-----○, EV; ■-----■, SFV titre in M-M medium without cells. (b) Cell growth. ●-----●, infected cells; ○-----○, uninfected cells. (c) Percent cells infected. Immunofluorescence assay; ●-----●, strongly fluorescent cells; ○-----○, weakly fluorescent cells; ■-----■, infectious centre assay. (d) Acid phosphatase assay. ●-----●, infected cells; ○-----○, uninfected cells. (e) Assay for interferon-like substances. Medium from 1-, 2- and 3-day infected cells was centrifuged at 120,000 g for 1 h in a Beckman L2 ultracentrifuge; 5 x 10^3 p.f.u./ml of virus remained in the supernatant fluid. Uninfected cells were added to each supernatant fluid to give 2 x 10^6 cells/ml and samples incubated at 26 °C. Virus growth did not differ significantly in 1-, 2- and 3-day medium and total virus titres from these three experiments are plotted together (●-----●). Uninfected cells were also added to medium removed from uninfected cells and to fresh medium, and virus added to give 5 x 10^3 p.f.u./ml; total virus in medium from uninfected cells (○-----○); in fresh medium (■-----■). (f) Assay for antiviral activity in growth medium. SFV (finally 10^3 p.f.u./ml) was added to the five different media described in (e) and incubated at 26 °C. Virus titres in 1-, 2- and 3-day infected media were not significantly different and are plotted as one (●-----●); titre in medium from uninfected cells (○-----○); titre in fresh growth medium (■-----■).
Fig. 2. Virus-specific RNA synthesis in SFV-infected *Aedes albopictus* cells. *A. albopictus* cells (10⁶) were adsorbed with SFV (added multiplicity, ~ l) in 120 ml of M-M medium for 1 h at 26 °C. Cells were diluted to 3 x 10⁵ cells/ml in M-M medium and incubated at 26 °C. (a) Virus growth and infectious RNA levels. ●●●●●●●, CAV; ○○○○○, EV; ○○○○○, infectious RNA. Release of EV was assayed by removing medium and replacing with 5 ml of fresh medium at various times; release was determined after 3 h (∓). (b) Cellular and virus-specific RNA synthesis: incorporation of [³H]-uridine was assayed in infected cells with actinomycin (●●●●●), in uninfected cells with actinomycin (○○○○○) and in uninfected cells without actinomycin (○○○○○).

Antiviral activity in medium and cells

There was no significant difference in the rates of inactivation of SFV over 5 days in the five different media used above (Fig. 1f). The decline in EV titre from 2 to 4 days (Fig. 1a) is therefore not due to any directly antiviral agent released from cells.

The antiviral activity of cell sonicates was tested. Uninfected cells and 1-, 2- and 3-day infected cells were sonicated in fresh growth medium and incubated with SFV for 12 h at 28 °C. There was no significant difference in the final SFV titres under any of these conditions. Thus the (variable) decrease in CAV titre between 1 and 3 days (Fig. 1a, 2a, 3) was not correlated with any detectable increase in intracellular, antiviral activity.

'Shut-down' of virus-specific RNA synthesis

Infectious RNA levels and the incorporation of [³H]-uridine into actinomycin-resistant RNA were assayed in SFV-infected *Aedes albopictus* cells (Fig. 2a, b). Infectious RNA levels were maximal at 9 h after infection; after this time the infectivity titre remained constant representing 0.1% of the CAV titre (Fig. 2a). Release of infectious virus decreased from about 12 to 18 h after infection (Fig. 2a).

Virus-specific RNA synthesis was also maximal at 9 to 12 h and represented ≥ 12% of total cellular RNA synthetic capacity (Fig. 2b). The rate of incorporation declined sharply from
**SFV persistence in A. albopictus cells**

Table 1. Distribution of SFV yields from individual Aedes albopictus cells during primary and persistent infection

<table>
<thead>
<tr>
<th></th>
<th>Primary infection</th>
<th>Persistent infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of samples</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>Expected number without cells*</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Observed number without virus†</td>
<td>40</td>
<td>54</td>
</tr>
<tr>
<td>Expected number with infected cells‡</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>Observed number with virus</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Plaque distribution (duplicates)</td>
<td>10, 14; 11, 17; 7, 8; 11, 13; 9, 10; 8, 10; 15, 16; 14, 18; 9, 11; 7, 11; 20, 25; 21, 23; 21, 21; 19, 20; 25, 27; 33, 34</td>
<td>32, 24; 30, 27</td>
</tr>
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(a) Aedes albopictus cells (2 x 10⁷) were adsorbed with SFV (added multiplicity, ~40) for 1 h at 26 °C in 2 ml of M-M medium. After washing (× 3), samples were (i) diluted and assayed for virus growth at 26 °C; EV titres increased from 10⁴ to 7.6 x 10⁵ p.f.u./10⁶ cells at 24 h; (ii) assayed for infective centres (36 %, see text); (iii) diluted in fresh growth medium to 1.5 cells/ml and 0.5 ml samples incubated for 24 h at 26 °C; 0.1 ml was plated directly (without sonication) on Vero cells. For 56 samples the Poisson distribution predicts that 1, 2 and 3 cells (infected or uninfected) will be present in 21, 8 and 1 samples respectively; the finding that about one-third of the samples contain approximately twice as many p.f.u. as the remainder is consistent with this prediction. Yields per cell (see text) are calculated by taking the average of the yields from the 10 lowest yielding samples (7 to 18 p.f.u./0.1 ml). (b) Five-day infected Aedes albopictus cells (Fig. 2) were diluted in fresh M-M medium to 1.5 cells/ml; 0.5 ml samples were incubated for 24 h at 26 °C and 0.1 ml samples assayed on Vero cells.

* Calculated from the Poisson distribution.
† Representing less than 1 p.f.u. in sample (0.1 ml).
‡ Assuming 36 % infected in primary infection; infective centres not determined in persistent infection (Fig. 2).

12 to 18 h (Fig. 2b). By 48 h the rates of actinomycin-resistant RNA synthesis in infected and control cultures were similar; there was a progressive increase in incorporation in both cultures at later times due to the increase in cell numbers (Fig. 2b).

**SFV release from individual cells**

Aedes albopictus cells were infected with SFV and assayed for the percentage of cells infected and for virus growth. A third sample was diluted in fresh medium and virus release from single cells examined (Table 1). From the infectious centre assay, 36 % of cells were infected at zero time; the growth experiment showed an average EV production of 76 p.f.u./cell by 24 h. After 24 h incubation of diluted samples, 16 out of 56 contained measurable levels of virus (Table 1); it was concluded that in those cells producing measurable levels of virus (Table 1, legend), approx. 50 p.f.u./cell were released from 0 to 24 h (Table 1).

Persistently infected cells were similarly assayed for virus production. After incubation for 24 h, 2 samples out of 56 (i.e. 6 %) produced significant levels of virus; plaque counts obtained represented the formation of 140 p.f.u./cell (Table 1).

**Effect of subculturing and superinfecting persistently infected cells**

Subculturing 5-day infected Aedes albopictus cells into fresh growth medium stimulated cell division (Fig. 3) and SFV production (Fig. 4a) although the maximum titre reached on subculturing was substantially less than during primary infection (Fig. 3, 4a). As in the primary infection, CAV titres decline at about 24 h after subculture (Fig. 4a). Dilution of 5-day infected cells into medium from 5-day infected cultures also stimulated cell division (M. W. Davey, unpublished results) and virus production (Fig. 4b). Thus the increased
Fig. 3. Effect of subculturing persistently-infected cells on cell division rate. *Aedes albopictus* cells ($2 \times 10^6$) were adsorbed with SFV (added multiplicity, 1) in 30 ml of M-M medium for 1 h at 26 °C. Cells were washed, resuspended at $3.7 \times 10^6$ cells/ml and incubated for 12 days at 26 °C; ●—●, CAV; ○—○, EV titre; ■—■, cell number. On day 5 a portion of the cells was diluted to $4 \times 10^6$ cells/ml in fresh growth medium; cell number (□—□).

Fig. 4. SFV growth after subculture or superinfection of persistently infected *Aedes albopictus* cells. (a) 5-day infected cells (Fig. 3) were diluted in fresh medium to $4 \times 10^6$ cells/ml and incubated. (b) 5-day infected cells were diluted to $4 \times 10^6$ cells/ml in medium from 5-day infected cells; EV titres are relatively high initially due to virus in 5-day infected medium. (c) 5-day infected cells were concentrated, adsorbed with SFV (added multiplicity, ~20) for 1 h at 26 °C and diluted in fresh medium. (d) equal vol. of uninfected and 5-day infected cells, both diluted to $4 \times 10^6$/ml in fresh growth medium, were mixed. Incubation was at 26 °C. ●—●, CAV titre; ○—○, EV titre.
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virus production on subculture into fresh medium is not due to the dilution of interfering substances present in 5-day medium, but probably results from increased cell growth. The stimulus is not due to cell detachment at the time of subculturing, since detachment alone does not yield increased virus titres.

Superinfection of 5-day infected cells with SFV at the time of subculture (added multiplicity, \(\approx 20\)) gave growth kinetics which were similar (Fig. 4c) to those for cells which were subcultured without superinfection (Fig. 4a).

Equal volumes of diluted (i.e. subcultured) 5-day infected cells, and diluted, uninfected cells were mixed. Virus growth kinetics reflected a primary infection cycle in the previously uninfected cells (Fig. 4d).

DISCUSSION

Infection of cultured Aedes albopictus cells with SFV results in the infection of all cells. By about 48 h a persistent infection has been established with a small percentage of cells producing infectious virus and a stabilization of CAV and EV titres. During the establishment of persistence there is no change in the cumulative cell number as compared with control cultures, nor any evidence of c.p.e., consistent with the observations of Peleg (1969, 1972) with other togavirus-cell systems. The decrease in virus production during the establishment of persistence is not accompanied by the release of agents which have either an interferon-like or a directly antiviral activity; nor is this decrease prevented by actinomycin. Our results therefore support the view (Peleg, 1969) that persistence is not established or maintained by interfering substances released into the medium. Ultrastructural changes previously observed in RRV- and SFV-infected Aedes albopictus cells (Raghow et al. 1973a, b) suggested an involvement of lysosomal enzymes in the destruction of cell-associated virus during the establishment of persistence (Raghow et al. 1973b). However, there is no significant alteration in the level or distribution of acid phosphatase in persistent cultures as compared with control cultures.

SFV-infected Aedes albopictus cells show a striking reduction in the rate of virus-specific RNA synthesis at 12 to 15 h; by 40 h the rates of incorporation in control and infected cultures are similar. The ‘shut-down’ of incorporation of \(^{3}H\)-uridine into virus-specific RNA is accompanied by a cessation of net infectious RNA synthesis; it is followed by a reduction in virus production, a decrease in virus release and a sharp fall in the percentage of infected cells. It seems likely that these latter events result from a primary inhibition of virus-specific RNA synthesis.

The inhibition of RNA synthesis is not total; there is a limited (approx. 10 % of maximum levels) incorporation of \(^{3}H\)-uridine into 45S virus RNA in 5-day infected cultures (M. W. Davey, unpublished results). Consistent with this, the single cell release experiments show virus production in a small percentage of cells in 5-day infected cultures. Since the extent of virus production in these cells was apparently normal, the establishment of persistence appears to be regulated at the level of the individual cell (Walker, 1964).

The effect of subculturing 5-day infected cells is to increase cell division rate and to stimulate production of infectious virus; this is followed by a stabilization of titre as is found during the primary infection cycle. The stimulation of virus production on subculturing presumably reflects a dependence of virus replication on some cellular activity which is promoted by subculturing. Although only a small percentage of cells in persistent cultures assay as infective centres, superinfection (at the time of subculturing) gives rise to no extra virus production. It therefore appears probable that all persistently-infected cells harbour the virus genome in some form which is reflected in homologous interference although it
cannot be expressed as infectious virus in the infective centre assay. The fact that at 18 to 24 h after subculturing 5-day infected cultures, all cells assay as infective centres (M. W. Davey, unpublished results), suggests that the latent form may represent the complete genome.

At 8 to 10 days after primary infection, a small-plaque virus of reduced virulence replaces wild-type SFV in infected cultures; this occurs at 8 to 10 days whether or not cells are subcultured into fresh medium at earlier times. Shenk et al. (1974) have shown that *Aedes albopictus* cells chronically (2 to 3 months) infected with Sindbis virus produce a small-plaque, temperature-sensitive mutant which is inactivated by antiserum prepared against wild-type Sindbis virus. By analogy, the small plaque virus found in SFV-infected cells is therefore probably SFV rather than a contaminating virus. Its appearance may represent a response to long-term infection at reduced temperature which is similar to that found in Sindbis-infected cells (Shenk et al. 1974).

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REFERENCES


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