Properties of Sindbis Virus Variants from Infected Culex tarsalis Mosquitoes

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SUMMARY

Plaque-purified Sindbis virus was passed three times in Culex tarsalis mosquitoes and progeny viruses were isolated by plaque purification on a cloned line of Aedes albopictus cells. Nine of ten clones examined differed from wild-type (wt) virus with respect to their plaque morphology characteristics in chick embryo fibroblast (CEF) and/or A. albopictus cells. Seven clones were temperature-sensitive and failed to replicate or synthesize viral RNA in CEF cells at 41 °C. At 35 °C in CEF cells the majority of isolates synthesized less viral RNA than wt virus. In contrast, all cloned isolates synthesized viral RNA more rapidly than wild-type virus in A. albopictus cells.

INTRODUCTION

Sindbis virus is a member of the alphavirus genus in the family Togaviridae. Like other alphaviruses, Sindbis is transmitted in nature by mosquito vectors which become infected when they take a blood meal from a viraemic vertebrate host. The virus replicates in the epithelial cells of the mosquito gut, spreads throughout the invertebrate's tissues and generates a focal infection in the salivary glands. The glands remain persistently infected for the insect's lifespan, thus permitting life-long ability to transmit virus to vertebrate hosts during the acquisition of blood meals (McLintock, 1978).

Alphaviruses such as Sindbis, Semliki Forest virus (SFV) and Western equine encephalitis (WEE) virus replicate efficiently in a variety of vertebrate and invertebrate cell lines in culture. Virus infection of vertebrate cells such as BHK-21 or chicken embryo fibroblast (CEF) cells leads characteristically to the death of the infected cells. In contrast, infection of mosquito cells such as those derived from Aedes albopictus or Aedes aegypti (Singh, 1967), generally does not lead to cell death but to the routine establishment of a persistent virus infection (Davey & Dalgarno, 1974; Igarashi et al., 1977).

Alphavirus persistence in A. albopictus cells in culture leads to the generation of small plaque temperature-sensitive (SPTS) variants which produce plaques smaller than those of wild-type (wt) virus in CEF cells at 35 °C and fail to replicate efficiently in CEF cells at 41 °C. SPTS variants from A. albopictus cells persistently infected with Sindbis virus or WEE virus may be RNA⁺ or RNA⁻ (Shenk et al., 1974; Maeda et al., 1979) and may contain alterations in either virus glycoprotein E2 or its precursor (Eaton, 1982; Simizu et al., 1983). Little is known of the effects on the virus of replication and persistence in mosquitoes. Stollar et al. (1978) artificially infected Culex triseriatus mosquitoes with plaque-purified Sindbis virus and found that 8% of the cloned viruses isolated 12 weeks post-infection were temperature-sensitive in CEF cells at 41 °C.

The experiments described here were done in order to characterize variants generated during Sindbis virus replication and persistence in the mosquito Culex tarsalis. The results show that following three 35-day replication cycles in C. tarsalis mosquitoes, nine out of ten cloned isolates of Sindbis virus differed from wt virus in their plaque morphology in both CEF and mosquito

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cells and seven clones were temperature-sensitive and RNA- at 41 °C in CEF cells. The majority of clones synthesized less viral RNA than wt virus in CEF cells at 35 °C but made RNA more rapidly than wt virus in A. albopictus mosquito cells.

METHODS

Virus and cells. Sindbis virus (strain AR339) was plaque-purified three times in CEF cells and stock virus (titre 8 x 10⁸ to 3 x 10¹⁰ p.f.u./ml) was obtained by infection of CEF cells at an m.o.i. of 0:01 p.f.u./cell. Primary CEF cells, prepared from 10-day-old embryonated eggs, were grown in Eagle's minimum essential medium (MEM) and 10% newborn calf serum. A. albopictus cell clone AAC29, isolated by Dr M. Kobayashi, was grown in plastic tissue culture flasks in A1 medium as described by Eaton (1979). AAC29 cells gave a marked cytopathic effect after Sindbis virus infection and were used in plaque assays for both wt Sindbis virus and variants from infected C. tarsalis mosquitoes.

Penetration of CEF and AAC29 cells by virus. Virus penetration was determined by the method of Baric et al. (1981). Cell monolayers were infected with either wt or Sin-C4 viruses and at different times post-infection antiserum to wt virus was added to neutralize virus which had not penetrated the cells. After 10 min at 37 °C, antiserum was removed and the cell monolayer was washed and agarose overlay was added. The number of plaques obtained after a 45 min adsorption in the absence of antiserum was taken as 100%. Rabbit antiserum to wt virus neutralized wt and Sin-C4 viruses with equal efficiency (50% neutralization endpoint 1:2048 for both viruses).

Analysis of intracellular viral RNA. Monolayers of CEF or AAC29 cells were infected with Sindbis virus at an m.o.i. of approximately 25 p.f.u./cell as estimated in CEF and AAC29 cells respectively. At different times post-infection, cells were labelled with [3H]uridine (25 μCi/ml) in the presence of 4 μg/ml actinomycin D and 4 μg/ml ethidium bromide.

Intracellular viral RNA was assayed by precipitation with 5% (w/v) trichloroacetic acid (TCA) on ice for 60 min. Samples were filtered on GF/A glass fibre filters (Whatman), washed four times with cold 5% TCA, four times with cold 95% ethanol and once with ether. Filters were dried and the radioactivity determined using a toluene-based scintillation fluid.
RNA was also extracted with phenol as described previously (Eaton, 1979) and analysed by zonal centrifugation in 15 to 30% (w/w) sucrose gradients in TNES (10 mM-Tris-HCl, 100 mM-NaCl, 1 mM-EDTA, 0.1% SDS) at 24000 rev/min for 14 h in a SW28.1 rotor.

**RESULTS**

**Plaque characteristics of virus isolated from mosquitoes**

Four days after emergence from the pupal stage, adult *C. tarsalis* mosquitoes were allowed to feed on rabbit blood containing $10^8$ p.f.u./ml of wt Sindbis virus. The normal life span of *C. tarsalis* mosquitoes was approximately 40 days after emergence. Consequently, mosquitoes were homogenized 35 days after infection. The titre of such mosquito-derived virus preparations was too low to guarantee infection of more newly emerged mosquitoes by either feeding or intrathoracic injection. Virus titre was therefore amplified by a single passage in BHK-21 cells. BHK-21-derived virus was mixed with blood and fed to newly emerged mosquitoes. Following a further 35-day period, infected *C. tarsalis* mosquitoes were homogenized and 43 clones of Sindbis virus were located by plaque purification on BHK-21 cells. Cloned virus isolates were compared to the original wt virus with respect to plaque size both in CEF cells at 35 °C and 41 °C and in AAC29 cells at 28 °C. The results indicated that a majority of the virus clones isolated (55.8%) were indistinguishable by these criteria from wt virus. The remaining 44.2% of the isolates could be differentiated from wt virus on the basis of only one or two of the plaque morphology characteristics examined. Only 4.7% of the isolates were temperature-sensitive and failed to replicate in CEF cells at 41 °C.

The number and properties of virus variants isolated after two 35-day passes in *C. tarsalis* mosquitoes indicated that variants appear much more slowly *in vivo* than in *A. albopictus* cells in culture. Seventy days after Sindbis virus infection of *A. albopictus* cells in culture, the majority of the cloned virus isolates differ from wt virus in a number of ways. They generate small plaques in CEF cells at 35 °C, are temperature-sensitive for replication at 41 °C in CEF cells and produce larger plaques than wt virus in mosquito cells at 28 °C (Igarashi *et al.*, 1977; Eaton, 1982). In an attempt to increase the proportion of variants in mosquito-derived virus, Sindbis virus isolated after two passages in *C. tarsalis* mosquitoes was passed once in BHK-21 cells and passed for a third time in mosquitoes. After 35 days, virus was extracted and 10 viruses were cloned by plaque purification in AAC29 mosquito cells. Cloned viruses were compared to wild-type virus with respect to their plaque morphology and ability to replicate in CEF cells at 35 °C and 41 °C and in AAC29 cells at 28 °C and 35 °C. The results in Table 1 show that only one of the cloned virus isolates (Sin-C6) had plaque morphology characteristics in CEF and AAC29 cells identical to those of wt virus and the remainder differed from wt virus with respect to at least one of the characteristics examined. Seven of the 10 clones were found to be temperature-sensitive in CEF cells at 41 °C (e.o.p. ranging from $10^{-3}$ to $<10^{-7}$). Five of the temperature-sensitive isolates also generated large plaques in AAC29 cells at 28 °C. One of the latter clones was Sin-C4 and in the next experiment we examined the ability of Sin-C4 to replicate and synthesize viral RNA in CEF cells at 28, 35 and 41 °C.

**Temperature sensitivity of Sin-C4 in CEF cells**

The titres of wt and Sin-C4 viruses in CEF cells at 28, 35, 37 and 41 °C are shown in Fig. 1 and confirm the temperature sensitivity of Sin-C4. Viral RNA synthesized in infected CEF cells was labelled with $[^3]H$uridine and analysed by zonal centrifugation in sucrose gradients. The results in Fig. 2 show that at 41 °C Sin-C4 is an RNA$^-$ temperature-sensitive mutant. Interestingly, Sin-C4 synthesized only 35% and 25% of the viral RNA synthesized by wt virus at 28 °C and 35 °C respectively.

**Growth characteristics of wt and Sin-C4 viruses in AAC29 and CEF cells**

Wild-type and Sin-C4 viruses were titrated in AAC29 cells and monolayers of AAC29 cells were infected at an m.o.i. of 25 p.f.u./cell. The results in Fig. 3(a) show that infected mosquito cells released approximately the same amount of infectious wt and Sin-C4 viruses. Infection of
Fig. 1. Analysis of the temperature sensitivity of Sin-C4 by reduction of plaque titre. ▲, wt virus; ●, Sin-C4.

Table 1. Characterization of Sindbis virus clones isolated after three 35-day cycles of replication in Culex tarsalis mosquitoes

<table>
<thead>
<tr>
<th>Virus</th>
<th>35 °C</th>
<th>41 °C</th>
<th>E.o.p.*</th>
<th>35 °C</th>
<th>41 °C</th>
<th>Plaque size</th>
<th>% wt RNA†</th>
<th>28 °C</th>
<th>35 °C</th>
<th>E.o.p.*</th>
<th>28 °C</th>
<th>35 °C</th>
<th>% wt RNA†</th>
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<tr>
<td>wt</td>
<td>L†</td>
<td>L</td>
<td>8·3 × 10⁻¹</td>
<td>100</td>
<td>100</td>
<td>S‡</td>
<td>S</td>
<td>2·1 × 10⁻¹</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
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<td>C1</td>
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<td>S</td>
<td>6·4 × 10⁻²</td>
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<td>L</td>
<td>L</td>
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<td>164</td>
<td>312</td>
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<td>S</td>
<td>ND‡</td>
<td>&lt;3·0 × 10⁻⁷</td>
<td>53</td>
<td>0</td>
<td>S</td>
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<td>1·5 × 10⁻¹</td>
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<td>137</td>
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<td>S</td>
<td>7·0 × 10⁻²</td>
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<td>256</td>
<td>L</td>
<td>L</td>
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<td>160</td>
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<td>L</td>
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<td>L</td>
<td>8·1 × 10⁻¹</td>
<td>196</td>
<td>160</td>
<td></td>
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</table>

* E.o.p. is titre at 41 °C/titre at 35 °C in CEF cells or titre at 35 °C/titre at 28 °C in AAC29 cells.
† % wt RNA is the ct/min of [³H]uridine incorporated into acid-precipitable intracellular viral RNA expressed as a percentage of that incorporated into wt virus RNA from 3 to 6 h post-infection and 18 to 24 h post-infection in CEF and AAC29 cells respectively.
‡ L, Large plaque (see Methods); S, small plaque (see Methods); ND, not detectable.

AAC29 cells with Sin-C4 led, however, to the development of a c.p.e. at 12 h post-infection, 10 h before the appearance of a c.p.e. in wt virus-infected cells. Monolayers of Sin-C4-infected cells started to recover at approximately 48 h post-infection, a time when wt virus-induced c.p.e. was maximal. The severity of the c.p.e. generated by both viruses was similar and was characterized by cell fusion and lattice formation (Sarver & Stollar, 1977).

The data in Fig. 3(b) show the growth of wt and Sin-C4 viruses following infection of CEF cells at 35 °C at an m.o.i. of 25 p.f.u./cell (calculated on the basis of virus titrations done in CEF
cells). Although the kinetics of virus production were similar for the two viruses, the amount of infectious virus released from Sin-C4-infected cells was approximately $2 \log_{10}$ units less than that produced by wt virus-infected cells. Infection of CEF cells with wt virus lead to complete destruction of the cell monolayer by 24 h post-infection. In contrast, Sin-C4-infected CEF cells showed a mild c.p.e. and remained as a monolayer up to 38 h post-infection.

In the next experiment we examined the rate of viral RNA synthesis following wt and Sin-C4 virus infection of AAC29 cells at 28 °C and CEF cells at 35 °C. The kinetics of incorporation of [3H]uridine into TCA-precipitable material in mock-infected and virus-infected AAC29 cells in the presence of actinomycin D and ethidium bromide at times from 0 to 48 h post-infection are shown in Fig. 4(a). The results indicate that there is a rapid onset of viral RNA synthesis in Sin-C4-infected cells beginning at 8 h post-infection and reaching a maximum at 22 h post-infection. Wild-type viral RNA synthesis, in contrast, was not detected by this procedure in significant amounts until after 16 h post-infection and although it reached approximately the same level as that in Sin-C4-infected cells, the peak of viral RNA synthesis occurred at 32 h post-infection, 10 h later than that observed in Sin-C4-infected cells. It is interesting that the more rapid production of viral RNA in Sin-C4-infected cells was not accompanied by a similar rapid release of infectious virus (Fig. 3a).

Fig. 2. Sucrose gradient analysis of viral RNA synthesized in CEF cells by wild-type (a to c) and Sin-C4 (d to f) viruses at various temperatures. Cells were infected and labelled with 50 μCi/ml [3H]uridine in the presence of actinomycin D from 3 to 6 h post-infection. RNA was extracted with phenol and analysed by zonal centrifugation in 15 to 30% sucrose gradients in TNES. The positions of 42S and 26S RNA are indicated in (c). (a, d) 28 °C; (b, e) 35 °C; (c, f) 41 °C.
Fig. 3. Kinetics of virus synthesis at 28 °C in AAC29 cells (a) and at 35 °C in CEF cells (b). Progeny virus was titrated in CEF cells at 35 °C. ▲, wt virus; ●, Sin-C4.

Fig. 4. Time course of RNA synthesis of Sin-C4 and wt viruses in AAC29 cells at 28 °C (a) and CEF cells at 35 °C (b). Cells were infected and at the times indicated they were pulsed for 1 h with 50 μCi/ml [3H]uridine. ▲, wt virus; ●, Sin-C4; ■, mock-infected control.

Analysis of [3H]uridine incorporation into viral RNA in CEF cells (Fig. 4b) showed that whereas wt virus RNA synthesis peaked at approximately 4 h post-infection, Sin-C4 RNA synthesis did not reach a peak until 6 h post-infection. Sin-C4 produced approximately 25% of the viral RNA produced by wt virus in CEF cells.

Experiments were done to determine whether the kinetics of synthesis of Sin-C4 viral RNA in AAC29 and CEF cells was due to a more rapid adsorption or penetration to mosquito cells and
an inefficient adsorption or penetration of the virus to vertebrate cells compared with wt virus. Results indicated that wt and Sin-C4 viruses adsorbed to CEF and AAC29 cells at the same rate. Approximately 60% and 75% of both viruses adsorbed to the two cell types within 10 min and 30 min respectively. In addition, the kinetics of penetration of wt and Sin-C4 viruses into CEF and AAC29 cells were very similar (data not shown).

Viral RNA synthesis by cloned isolates from infected mosquitoes

The data presented above indicate that Sin-C4 differed from wt virus, not only with respect to several plaque morphology criteria, but also in the efficiency with which it synthesized viral RNA in both vertebrate and mosquito cell lines. We next determined whether the other clonal isolates from infected mosquitoes showed an altered pattern of RNA synthesis in CEF and A. albopictus cells. The results in Table 1 show that nine of the isolates behaved like Sin-C4 and synthesized more viral RNA than wt virus in AAC29 cells at both 28 °C and 35 °C and less RNA than wt virus in CEF cells at 35 °C and 41 °C. Sin-C3 synthesized more RNA than did wt virus in both AAC29 and CEF cells.

DISCUSSION

The results presented here show that replication of Sindbis virus in mosquitoes results in the generation of virus variants. Passage of the virus three times in C. tarsalis mosquitoes led to the appearance of variants which differ from wt virus in a number of ways. The data in Table 1 show that nine of the ten cloned isolates examined produced plaques on CEF and AAC29 cells which were smaller and/or larger respectively than those generated by wt virus. Six of the isolates were temperature-sensitive in CEF cells at 41 °C. Two properties were common to the majority of cloned isolates, namely the ability to synthesize viral RNA rapidly in AAC29 cells compared to wt virus and the inability to make as much viral RNA as wt virus in CEF cells. At 41 °C in CEF cells the amount of viral RNA synthesized by the variants (except Sin-C3) ranged from undetectable up to 55 % of that generated by wt virus under the same conditions. Most temperature-sensitive variants were therefore identified as RNA-. Even at 35 °C in CEF cells, all but one cloned isolate generated less viral RNA than wt virus.

The efficiency with which Sin-C4 viral RNA is synthesized in A. albopictus cells and the low levels of virus RNA synthesized in CEF cells suggest that, compared with wt virus, Sin-C4 may have an alteration(s) in a component required for RNA synthesis. There is a growing body of evidence suggesting that a host cell factor is required for the synthesis of negative strands of viral RNA (Tooker & Kennedy, 1981; Baric et al., 1983a, b). Thus, one or more of the virus-specified components of Sin-C4 RNA polymerase may differ from their wt counterparts and may interact more efficiently with the host cell factor in mosquito cells and less efficiently with the CEF cell factor. Determination of the number of ct/min of [3H]uridine incorporated into viral single-stranded RNA and double-stranded replicative form (RF) RNA in wt and Sin-C4 virus-infected CEF cells indicated that although the total amount of virus-specific RNA in Sin-C4-infected cells was markedly reduced compared with wt virus-infected cells, the ratio of ct/min of [3H]uridine in single-stranded RNA to that in RF RNA was approximately the same for both viruses (data not shown). This suggests that the low level of Sin-C4 viral RNA synthesis in CEF cells may be due to inefficient synthesis of viral negative-stranded RNA.

In addition to postulated alteration(s) in the RNA polymerase of Sin-C4, analysis of structural proteins synthesized in CEF cells revealed small differences in the electrophoretic mobility of the envelope glycoproteins of the cloned variants compared with their wt virus counterparts. For example, PE2 synthesized in Sin-C4-infected CEF cells appeared as a very diffuse band on polyacrylamide gel electrophoresis. In contrast, the unglycosylated form of PE2 (synthesized in the presence of tunicamycin) appeared in polyacrylamide gels as a sharp band which migrated more slowly than its wt counterpart. This suggests that changes may have occurred in the amino acid content and glycosylation profile of Sin-C4 PE2. Such an alteration was, however, not characteristic of all cloned isolates examined.

‘Host range’ or ‘host-adapted’ variants of alphaviruses have recently been recognized. Kowal & Stollar (1981) chemically mutagenized Sindbis virus and isolated two variants which were
temperature-sensitive for growth in CEF cells at 41 °C, replicated efficiently in CEF cells at 35 °C but were inefficient in replication in A. albopictus cells at 35 °C. These viruses fell into complementation group F and examination of a non-temperature-sensitive revertant showed that the temperature-sensitive lesion was responsible for the host restriction. Simizu & Maeda (1981) isolated RNA− variants of WEE virus from persistently infected A. albopictus cell cultures and showed that these variants exhibited a marked improvement for growth in mosquito cells compared to the wt virus. In addition, they showed restricted growth in vertebrate cells. Thus, the variants of WEE virus isolated from persistently infected cells in culture show some similarities to the Sindbis virus variants isolated from C. tarsalis mosquitoes and described here.

The variants described in this paper were derived from passages in live mosquitoes and the data therefore suggest that mosquito-adapted variants may arise in the field. For this to occur, however, it would appear that mosquitoes would have to remain infected for long periods of time. McLintock & Iversen (1975) have suggested that WEE virus may be maintained (for periods of over 4 months) in overwintering C. tarsalis mosquitoes and some evidence for this was obtained by Reeves et al. (1958). An alternative method of long-term persistence is by transovarial transmission of the virus. This has already been shown to occur for the flavivirus yellow fever virus (Beaty et al., 1980). Such long-term infections could therefore give rise to mosquito-adapted viruses under field conditions. Igarashi et al. (1981) have reported field isolations of Japanese encephalitis and Getah viruses that could only be detected on A. albopictus C6/36 cloned cells and not in suckling mouse brains or vertebrate cells.

REFERENCES


Sindbis virus variants from mosquitoes


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