Growth Patterns of Temperature-sensitive Mutants of Western Equine Encephalitis Virus in Cultured *Aedes albopictus* (Mosquito) Cells

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SUMMARY

Several temperature-sensitive (ts) mutants of Western equine encephalitis virus (WEEV) have been isolated previously from persistently infected cultures of mosquito cells and divided into three groups: early passage RNA⁻ mutants, early passage RNA⁺ mutants and late passage RNA⁻ mutants (Maeda et al., 1979). The growth patterns of these groups, as well as of several ts mutants isolated after chemical mutagenesis and of wild-type (wt) WEEV, have been compared in BHK cells and in two strains of mosquito cells. The late passage ts mutants grew much better in mosquito cells than either the wt WEEV or the chemically induced mutants. When mosquito cells were co-infected with a late passage mutant (A125) and wt WEEV, infectious virions of both parental types as well as phenotypically mixed particles were produced. Infection of mosquito cells with WEEV resulted in a slight suppression of host DNA and protein synthesis during the acute stage of the infection (the first 1 or 2 days). Virus growth in a line of cloned mosquito cells in which WEEV produced a cytopathic infection (c.p.e.) was analysed with the result that the viruses could be divided into two groups: one in which wt WEEV, chemically induced ts mutants and early passage RNA⁺ mutants all induced maximal c.p.e., and another in which late passage RNA⁻ mutants and one early passage RNA⁻ mutant induced very little c.p.e., but released much more infectious virus into the culture fluid. Electron microscopy showed that in these cloned mosquito cells infected with a virus of the first group, large amounts of virus accumulated on or in the plasma membrane.

INTRODUCTION

Comparative studies of the growth of alphaviruses in cultured vertebrate and invertebrate cells have been undertaken by several laboratories (Schlesinger, 1975; Davey et al., 1973; Brown et al., 1976; Renz & Brown, 1976). The infection in vertebrate cells is strongly cytopathic and produces high virus yields within a few hours after infection. In addition, the infection results in rapid inhibition of host cellular DNA synthesis (Simizu et al., 1976; Koizumi et al., 1979a) and usually leads to total destruction of the host cells.

The infection of invertebrate cells is biphasic, consisting of an acute phase for the first 1 or 2 days in which the cells produce high virus yields without cytopathic changes, followed by a chronic infection during which the cells produce low levels of virus, and grow at a normal rate (Stevens, 1970; Raghow et al., 1973). The mechanism of the establishment of these persistent infections in mosquito cells has been studied extensively (Riedel & Brown, 1977, 1979).

Maeda et al. (1979) demonstrated that small-plaque mutants and/or ts mutants of Western equine encephalitis virus (WEEV) arose in cultures of one of Singh’s lines of *Aedes albopictus*.
cells (Singh, 1967) when persistently infected with WEEV. They found that single-step mutants (both RNA+ and RNA−) could be isolated during the early stages of infection, but that the virus which predominated after many passages in culture consisted of multiple mutants of the RNA− phenotype. These multiple mutants showed defects in at least two complementation groups: group III which affects the RNA phenotype, and group IV which affects the structural proteins. Since these mutants arise long after the establishment of the chronic infection it is likely that they have a significant selective advantage in these cells, even though they are not necessary for either the establishment or maintenance of persistent infection.

In order to determine whether late passage mutants have a selective advantage, we compared the growth patterns of these multiple mutants with the growth of single mutants and wt WEEV using BHK cells, uncloned mosquito cells and a cloned mosquito cell line (C6/36) in which WEEV produces cytopathic effect (c.p.e.). There were several differences in the growth of the late passage mutants in both vertebrate and invertebrate cells. Furthermore, the multiple mutants have almost completely lost the capacity to produce c.p.e. in C6/36 cells, although the single mutants isolated from early passage persistent infection retained this ability. One goal of our present study was to investigate the interaction of mutated gene expression of WEEV with invertebrate host cell functions involved in virus replication.

METHODS

Cells and virus. Preparation and culture conditions for primary chicken embryo fibroblasts (CEF) and for BHK-21 cells have been described previously (Simizu et al., 1973). Two lines of Singh's A. albopictus cells: the uncloned and MEM-adapted (Sarver & Stollar, 1977), and a cloned line C6/36 (Igarashi, 1978) were used. The cloned cell line culture was provided by Dr A. Igarashi, Nagasaki University, Japan. They were cultured and maintained as previously described (Maeda et al., 1979).

The preparation of wt WEEV and the isolation of ts mutants of WEEV have been described previously (Simizu & Takayama, 1972; Hashimoto & Simizu, 1978; Maeda et al., 1979). A list of the mutants which were used in this study and their properties are given in Table 1.

Plaque assay. Virus infectivity was assayed by plaque assay on confluent monolayers of CEF cells in 60 mm Petri dishes. Virus samples diluted in Eagle's minimum essential medium (MEM) were inoculated on to the monolayers and allowed to adsorb at 34 °C for 60 min. At this time the monolayers were overlaid with 8 ml MEM containing 1% calf serum and 1% special Noble agar (Difco) and 0.003% neutral red. Plaques were counted after incubation at 34 °C for 2 to 4 days.

Polyacrylamide gel electrophoresis (PAGE) of virus-specific polypeptides. BHK or mosquito cells were cultured in 35 mm Petri dishes. Cells were infected with WEEV at an m.o.i. of 10 and incubated at 34 °C (BHK) or 28 °C (mosquito). At different times post-infection the culture fluid was replaced with a labelling medium (Earle's salt solution containing essential amino acids at one-tenth the normal concentration and 2% dialysed calf serum) and incubated for 1 h more. Then, this medium was removed and fresh labelling medium containing 3 μCi/ml 14C-labelled amino acid mixture (The Radiochemical Centre, Amersham) was added. After the labelling period the monolayers were washed twice with cold phosphate-buffered saline (PBS) and dissolved in 0.4 ml SDS-containing buffer (50 mM-tris–HCl pH 6.8, 2% SDS and 10% glycerol). To 0.1 ml of each sample was added 5 μl 2-mercaptoethanol and the samples were heated to 70 °C for 30 min. The samples were applied to 12-cm slab gels containing 7.5% acrylamide and 0.2% Bis (Bio-Rad, Richmond, Ca, U.S.A.) using the discontinuous system of Laemmli (1970), modified according to Hashimoto & Simizu (1979). After electrophoresis the gels were stained with 0.05%
Growth of WEEV ts mutants in mosquito cells

Table 1. List of Western equine encephalitis virus ts mutants used

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Derivation</th>
<th>RNA phenotype</th>
<th>Complementation group</th>
</tr>
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<tbody>
<tr>
<td>A106</td>
<td>Early passage*</td>
<td>-</td>
<td>III</td>
</tr>
<tr>
<td>A108</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A114</td>
<td>Early passage</td>
<td>+</td>
<td>IV</td>
</tr>
<tr>
<td>A117</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A124</td>
<td>Late passage†</td>
<td>-</td>
<td>I/III/IV</td>
</tr>
<tr>
<td>A125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A143</td>
<td>Late passage</td>
<td>-</td>
<td>III/IV</td>
</tr>
<tr>
<td>ts2</td>
<td>Chemically induced‡</td>
<td>-</td>
<td>II</td>
</tr>
<tr>
<td>ts3</td>
<td>Chemically induced</td>
<td>-</td>
<td>III</td>
</tr>
<tr>
<td>ts39</td>
<td>Chemically induced</td>
<td>+</td>
<td>I</td>
</tr>
</tbody>
</table>

* Single mutants isolated from the early stage of persistently infected A. albopictus cultures (5 to 30 days after initial infection).
† Multiple mutants (I/III/IV, III/IV) isolated from the late stage of persistently infected A. albopictus cultures (80 to 170 days after initial infection).
‡ Mutants ts2 and ts3 were isolated by treatment with nitrous acid; ts39 was isolated by treatment with nitrosoguanidine.

Coomassie Brilliant Blue, dried in vacuo and autoradiographed for 10 days on a Kodak XR-5 film.

Electron microscopy. BHK-21 or C6/36 mosquito cells in 35 mm Petri dishes were infected with wt or ts mutants of WEEV and incubated at 34 °C or 28 °C respectively. At appropriate times after infection the culture fluid was removed and the monolayers fixed with 3% glutaraldehyde in 0.1 M-cacodylate buffer for 1 h at 4 °C. The cells were scraped off the plate with a rubber policeman and centrifuged at 1000 rev/min for 15 min to obtain a pellet. The pellet was post-fixed in 1% osmium tetroxide in cacodylate and embedded in Spurr’s epoxy resin (Spurr, 1969). Sections were cut with an ultramicrotome using a glass knife, stained with 2% uranyl acetate and lead citrate and examined with a Hitachi H-500 electron microscope at 75 kV.

RESULTS

Growth of ts mutants in mosquito, BHK and CEF cells

Late passage multiple ts mutants isolated from the late stage of persistent infection in mosquito cells may have some selective advantage over early passage ts mutants or wt WEEV for production of progeny virus. To test this possibility we compared the growth of one such mutant (A125) and wt WEEV in cultures of mosquito, BHK and CEF cells (Fig. 1). Aliquots of the culture fluids were taken at various times after infection and assayed for infectious virus by plaque assay on CEF monolayers at 34 °C. All the strains used grew equally well on CEF cells (Fig. 1 a, d) and formed plaques on CEF with equal efficiency. The A125 mutant grew faster in both the uncloned and the cloned C6/36 mosquito cell lines, but did not grow as well in BHK cells. Wild-type WEEV, on the other hand, produced infectious virus more slowly than A125 during the early stages of infection in mosquito cells (Fig. 1 a, b), although the yields are almost equivalent at 3 days after infection.

We also compared the growth of the early passage ts mutants and the chemically induced ts mutants in mosquito and BHK cells. In order to compare the relative growth of the various viruses in different cells, all of the titres were normalized to the relative yield of wt WEEV in the different hosts (Table 2). In all cases the monolayers were infected at 10 p.f.u./cell and the yield of virus was measured at 8 h post-infection as p.f.u. on CEF cells. As shown in Table 2, we found that the ts mutants isolated from persistent infection, especially the late passage
Fig. 1. Virus growth curves of A125 mutant and wild-type WEEV in mosquito, BHK and CEF cells. Cultured monolayers were washed once with PBS and then infected with each virus at an m.o.i. of 10. After adsorption for 90 min at 28 °C for mosquito cells or 45 min at 34 °C for BHK and CEF cells, the inocula were removed, and monolayers washed three times with PBS, and the culture media for each cell culture were added. Infected cultures were incubated at 28 °C for mosquito cells or 34 °C for BHK and CEF cells, and the samples for plaque assay were taken at the indicated times. Plaque counts were determined by CEF cultures at 34 °C. (a) Uncloned A. albopictus cells; (b) cloned C6/36 mosquito cells; (c) BHK cells; (d) CEF cells. ○, A125 mutant; □, wild-type WEEV; △, mixed infection of A125 (5 p.f.u./cell) and wild-type WEEV (5 p.f.u./cell).

Table 2. Growth of wild-type and ts mutants in mosquito (C6/36) and BHK cells

<table>
<thead>
<tr>
<th>Cultures</th>
<th>wt WEEV</th>
<th>ts3*</th>
<th>A106</th>
<th>A108</th>
<th>A114</th>
<th>A117</th>
<th>A124</th>
<th>A125</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosquito cells at 28 °C</td>
<td>2.7 × 10^6</td>
<td>1.2 × 10^6</td>
<td>4.0 × 10^6</td>
<td>5.4 × 10^6</td>
<td>1.6 × 10^6</td>
<td>1.3 × 10^6</td>
<td>2.8 × 10^7</td>
<td>2.4 × 10^7</td>
</tr>
<tr>
<td>BHK cells at 34 °C</td>
<td>3.0 × 10^8</td>
<td>2.4 × 10^8</td>
<td>3.4 × 10^7</td>
<td>1.7 × 10^7</td>
<td>1.1 × 10^8</td>
<td>2.1 × 10^7</td>
<td>3.7 × 10^6</td>
<td>2.1 × 10^7</td>
</tr>
<tr>
<td>Relative yield †</td>
<td>1</td>
<td>0.56</td>
<td>1.3</td>
<td>35</td>
<td>1.6</td>
<td>6.9</td>
<td>840</td>
<td>130</td>
</tr>
</tbody>
</table>

* The other chemically induced mutants (ts2 and ts39) showed similar growth.
† Yield of each mutant in mosquito cells at 28 °C / Yield of wt WEEV in mosquito cells at 28 °C

Multiple mutants A125 and A124, produced as much as 100 to 800 times as much infectious virus in 8 h in the cloned mosquito cell line compared with other mutants or wt WEEV.

Mixed infection of BHK and mosquito cells with a late passage mutant and wt WEEV

There have been several reports of homologous interference when a persistently infected mosquito cell culture is superinfected with alphaviruses (Stollar & Shenk, 1973; Renz &
Growth of WEEV ts mutants in mosquito cells

Table 3. Mixed infection with wild-type and A125 mutant in BHK or mosquito cells

<table>
<thead>
<tr>
<th>(a) Virus</th>
<th>Titre at 34 °C†</th>
<th>Titre at 41.5 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heat (−)‡</td>
<td>Heat (+)</td>
</tr>
<tr>
<td>A125</td>
<td>2.4 × 10⁷</td>
<td>6.1 × 10³</td>
</tr>
<tr>
<td>A125 + wt</td>
<td>9.5 × 10⁶</td>
<td>4.2 × 10⁵</td>
</tr>
<tr>
<td>wt</td>
<td>5.3 × 10⁶</td>
<td>2.0 × 10⁶</td>
</tr>
<tr>
<td>(b) A125</td>
<td>2.9 × 10⁶</td>
<td>8.8 × 10²</td>
</tr>
<tr>
<td>A125 + wt</td>
<td>3.1 × 10⁶</td>
<td>7.4 × 10³</td>
</tr>
<tr>
<td>wt</td>
<td>7.8 × 10⁵</td>
<td>3.0 × 10⁵</td>
</tr>
<tr>
<td>(c) A125</td>
<td>5.0 × 10⁷</td>
<td>2.3 × 10³</td>
</tr>
<tr>
<td>A125 + wt</td>
<td>3.5 × 10⁸</td>
<td>4.6 × 10⁷</td>
</tr>
<tr>
<td>wt</td>
<td>7.9 × 10⁷</td>
<td>1.2 × 10⁸</td>
</tr>
</tbody>
</table>

| (d) Sample (mix-infected) | wt protein | ts protein || || wt protein | ts protein || || wt genome | ts genome || || wt genome | ts genome |
|---------------------------|------------|-------------|| || --- | --- || --- | --- |
| Mosquito (24 h) cells     | 5.1 × 10⁵ | 4.8 × 10⁵ | 6.9 × 10⁴ | 7.8 × 10⁶ | 90% |
|                          | (5.3%) | (5%) | (7.3%) | (82%) |
| Mosquito (48 h) cells     | 2.5 × 10⁴ | 3.0 × 10⁴ | 1.7 × 10⁶ | 1.1 × 10⁶ | 91% |
|                          | (8.1%) | (1%) | (55%) | (36%) |
| BHK cells (8 h)           | 2.8 × 10⁸ | 9.0 × 10⁷ | 2.0 × 10⁷ | 0 |
|                          | (72%) | (23%) | (5%) | (0%) |

* Virus titres were determined at (a) 24 h, (b) 48 h after infection in mosquito cells, (c) 8 h after infection in BHK cells.
† Titration was done at 34 °C or 41.5 °C in CEF cells.
‡ Virus suspension harvested was either heated at 50 °C for 30 min or left unheated before titration.
§ Virus titre was calculated from (a), (b), (c). Data corrected for thermal lability of wild-type and reduced plaque efficiency at 41.5 °C. The various classes of particles have been calculated from the data in this table as follows: A, virus titre heated and titrated at 41.5 °C; B, virus titre unheated and titrated at 41.5 °C; C, virus titre heated and titrated at 34 °C; D, virus titre unheated and titrated at 34 °C. Column heading 1 = A, 2 = B − A, 3 = C − A, 4 = D − A − B − C.
|| A virion contains ts proteins at least as a part of its structural proteins.

Brown, 1976; Riedel & Brown, 1977). We have also found this effect when cultures persistently infected with WEEV are superinfected with wt WEEV (data not shown). However, when uninfected monolayers of BHK or mosquito cells were infected simultaneously with a late passage mutant and the wild-type virus, interference did not occur (Fig. 1a, e). In such a mixed infection we can determine which viral components are being produced and what type of infectious virions are being produced.

Mosquito or BHK cells were infected simultaneously with A125 and wt WEEV. As shown in Table 3, the culture fluids were collected and the infectivity was assayed by plaque titration in CEF cells. Mutant A125 did not form any plaques at 41.5 °C. Furthermore, the A125 virions are thermal-sensitive and heating an aliquot of the culture fluid to 50 °C for 30 min reduced the titre of A125 by a factor of 10⁴. On the other hand, wt WEEV made plaques at 34 °C and 41.5 °C with almost the same efficiency, and the titre of wt WEEV was only reduced by about a factor of 3 by the heat treatment (see Table 1 in Maeda et al., 1979). Using the ability to make plaques at 41.5 °C as a measure of ts or wt genomes and the sensitivity to heat as a measure of ts or wt proteins, we can separate the two possible classes of mixed particles as shown in the summary of Table 3 (d). (The figures have been corrected for the slight thermal sensitivity of wt WEEV and for the slight decrease of plaque efficiency at 41.5 °C of wt WEEV.)
Fig. 2. Proteins synthesized in C6/36 and BHK cells infected with WEEV. Monolayers in 35 mm dishes were infected with ts mutants and wt WEEV and labelled with 3 μCi 14C-labelled amino acid mixture per dish for 30 min at 4 h post-infection at 34 °C (BHK) and 48 h post-infection at 28 °C (C6/36). One-hundred μl of each sample containing 125 μg (C6/36) or 150 μg (BHK) protein was loaded on to the gel. PAGE was performed as described in Methods. (a) Mosquito cells; (b) BHK cells; M, mock-infected cells; 1, wild-type WEEV; 2, A124; 3, A125; 4, A108; 5, A106; 6, A142; 7, persistently infected cells. The persistently infected cells were a 1-year-old culture which was producing about 0.04 p.f.u./cell/day. The locations of the glycoproteins E1 and E2, and core (C) proteins were determined by running a purified virus control in parallel.

In mosquito cells, 90% of the particles contained the mutant (ts) genome at either 24 or 48 h after infection as expected since the mutant grew 10 times as well in a single infection. In fact the mixed infection appears to be equivalent to the sum of two single infections, i.e. there is no interference. In BHK cells the situation was somewhat different. Although A125 could be produced alone, only 5% of the released particles had ts genomes and these all appeared to have wt proteins associated with them. The ts genome encapsidated in ts proteins was apparently not produced in the mixed infection.

From these experiments we can conclude that in mosquito cells both the genomes and the proteins of the A125 mutant are produced 10 times as effectively as those of wt WEEV at both 24 and 48 h after infection, but that once produced these components appear to be part of the same pool as the wt components, and readily produce phenotypically mixed infectious particles which are released into the culture fluid.

Synthesis of virus and cellular proteins in BHK and mosquito cells infected with ts mutants of WEEV

We have examined the intracellular proteins produced after infection of BHK and mosquito cells by ts mutants and wt WEEV. For this series of experiments the C6/36 mosquito cell line was used, since it was easier to detect virus-specific polypeptides in these cells. Fig. 2 shows PAGE patterns for wt-infected and mutant-infected monolayers.

In BHK cells wt WEEV appeared to shut off host protein synthesis almost completely, while the two late mutants did not suppress protein synthesis effectively. In C6/36 cells, wt
Growth of WEEV ts mutants in mosquito cells

WEEV or A108 (an early passage RNA\(^+\) mutant) effectively suppressed host protein synthesis, while A106 (an early passage RNA\(^-\) mutant) and all of the late passage ts mutants did not suppress host protein production. Furthermore, the two glycoproteins E1 and E2 were always clearly seen in infected mosquito cell monolayers, while in BHK cells primarily PE2 and E1 were found, except after a pulse-chase experiment. This implies that fully processed E2 is present in the membranes of the mosquito cells. Either this E2 is present in the form of mature virions, or the final processing of E2 is not coincident with maturation of virions in these cells as it is in vertebrate infection.

**Cytopathic effect and host DNA synthesis in mosquito cells infected with various ts mutants and wt WEEV**

In our previous report (Maeda *et al.*, 1979) we showed that specific types of multiple mutants, in particular those of complementation groups III and IV, seemed to be
Table 4. **Host DNA synthesis in mosquito cells infected with ts mutants and wt Western equine encephalitis virus**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Labelling time after infection (h)</th>
<th>Mock-infected</th>
<th>wt</th>
<th>A106</th>
<th>A117</th>
<th>A125</th>
<th>A136</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncloned mosquito cells</td>
<td>4</td>
<td>100</td>
<td>99</td>
<td>85</td>
<td>100</td>
<td>92</td>
<td>85</td>
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<tr>
<td></td>
<td>6</td>
<td>100</td>
<td>81</td>
<td>77</td>
<td>78</td>
<td>75</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>100</td>
<td>107</td>
<td>107</td>
<td>124</td>
<td>102</td>
<td>104</td>
</tr>
<tr>
<td>Cloned mosquito cells</td>
<td>5</td>
<td>100</td>
<td>92</td>
<td>95</td>
<td>ND†</td>
<td>87</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>100</td>
<td>21</td>
<td>30</td>
<td>ND</td>
<td>15</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Multi-well (24) Linbro plates were seeded with mosquito cells and incubated at 28 °C. Three days later monolayers were infected with either the ts mutants or wt WEEV at an m.o.i. of 10. At the indicated times, individual cultures were pulsed with 5 μCi [3H]thymidine for 30 min. Pulses were terminated by chilling the monolayers on an ice-bath and washing them once with PBS. Monolayers were collected by trypsinization and acid-precipitable (with 5% TCA) counts were determined on triplicate well samples. The [3H] counts incorporated into mock-infected cultures were set as 100%.

† ND, Not done.

We have also examined the inhibition of host DNA synthesis in these two strains of mosquito cells after infection with wt WEEV and some of the ts mutants, and the results are shown in Table 4. Uncloned *A. albopictus* cells showed only a slight suppression of host DNA synthesis at 6 h post-infection and by 24 h post-infection the cultures appeared to have recovered completely. This parallels the results obtained by counting cell numbers after infection. In this case (data not shown) infected wild-type mosquito cells did not divide during the first 24 h, but thereafter appeared to recover and divide normally. Suppression of host cellular DNA synthesis was much more extensive in the case of the C6/36 cells (Table 4). However, in neither case was there a clear difference between the wt WEEV and the ts mutants. Thus, it appears that c.p.e. is not correlated with changes in DNA synthesis after infection in mosquito cells.
Growth of WEEV ts mutants in mosquito cells

Fig. 4. Electron micrographs of C6/36 cells infected with wt and A125 mutant of WEEV. (a) wt-infected cells; (b) enlargement of a portion of (a); (c) A125 mutant-infected cells (arrows indicate virions budded from the cytoplasmic membrane); (d) an A106 mutant-infected cell. All samples were fixed at 48 h post-infection at 28 °C. Bar marker represents 500 nm.
We studied the development of several ts mutants and wt WEEV in C6/36 mosquito cells at the ultrastructural level. At 8 h post-infection, both in cells infected with wt WEEV and with some mutants, a few electron-dense bodies appeared in the cytoplasm, but neither virus cores nor complete virions were seen. At 24 and 48 h post-infection (as shown in Fig. 4) there were some morphological changes, but these were not as extensive as those in infected BHK cells (Hashimoto et al., 1975). Some membrane-rich vesicular structures were found, similar to those described by Brown et al. (1976) (data not shown). Some cells contained many electron-dense vesicles such as have been seen in Sindbis-infected cells, and also inclusion bodies similar to those reported by Raghow et al. (1973) for mosquito cells infected with Ross River virus. We could see virus cores and enveloped virions inside the vesicles, as well as virus budding from the surface of the cytoplasmic membrane.

On the cytoplasmic membrane of C6/36 mosquito cells infected with wt WEEV or in the spaces between two cells can be seen large numbers of particles. These are probably virions which are not quite completed, or for some reason fail to be released from the cell surface. On the other hand, in cells infected with A106 or the late passage multiple mutants, only a few particles were scattered throughout the cytoplasm or seen budding from the cytoplasmic membrane (Fig. 4 c, d). Therefore, it appears that the late passage mutants may adapt to bud from mosquito cells much more efficiently.

**DISCUSSION**

In a culture of *A. albopictus* cells persistently infected with WEEV and cultured for many cell passages, the virus types which predominate in the late stages of the infection are multiple mutants with temperature-sensitive defects in at least two cistrons (Maeda et al., 1979). These mutants have at least one defect in RNA synthesis and belong to complementation group III; some mutants with a second defect(s) affecting the structural proteins (which makes the virions much more thermal-labile to heating at 50 °C) belong also to group IV. It should be pointed out that these mutants are temperature-sensitive for replication in chick embryo cells, with a permissive temperature of 34 °C and a non-permissive temperature of 41.5 °C. All of the experiments infecting mosquito cell cultures with these mutants were performed at the permissive temperature of 28 °C. Similar or identical mutations appear to arise repeatedly in persistently infected mosquito cells, which implies that these mutations convey a selective advantage for growth. These mutants probably produce altered virus-specific products which are slightly different from those of wt WEEV. However, it is not clear whether these mutant products are necessary for persistent infection. Persistent infections by alphaviruses in mosquito cells are readily established and during the maintenance of these infected cultures ts mutants (Shenk et al., 1974), antiviral factors (Riedel & Brown, 1979) and defective-interfering particles (Eaton, 1979; Logan, 1979) are reported; however, the relative importance of each of these factors in persistence has not been determined. From our previous results we suspect that although the mutations appear to belong to only two groups or cistrons, it is possible that many of these isolates have multiple lesions in each of these functions.

In order to examine the nature of these mutations, we have examined various aspects of their replication in CEF, BHK and mosquito cells, and compared them with parallel wt WEEV infections and with other ts mutants, both single mutations isolated after chemical mutagenesis, and with single mutants which arise in the early stages of persistent infection. The first result is that these late passage mutants grow faster in mosquito cells and release large amounts of infectious virus into the culture fluid as compared to the single mutants and to wt WEEV (Fig. 1 and Table 2). However, they do not grow better than wt WEEV in BHK
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cells. This difference is not due to the relative temperatures for the two infections (28 °C and 34 °C for mosquito and BHK respectively) because late passage mutants grown in BHK cells at 28 °C and mosquito cells at 34 °C produce more virus in mosquito cells, when the yields are normalized for wt WEEV production under these conditions (data not shown).

Stollar et al. (1974) reported that a mutant of Sindbis virus from persistently infected Aedes aegypti cells interferes with wt Sindbis virus replication in both vertebrate and invertebrate cells. However, our late passage mutants did not interfere with wt WEEV. In simultaneous infection of the same culture with late passage mutants and wt WEEV, the same phenomenon occurs. The infectious particles produced contained predominantly the ts genomes and the ts proteins in mosquito cells, and in BHK cells contained predominantly the wt genomes and the wt proteins. However, phenotypically mixed particles also occurred, showing that the products of the mutants are compatible with the wild-type components.

We have also compared the suppression of DNA synthesis after infection with the late passage mutants and with wt WEEV and the single mutants. DNA synthesis in uncloned mosquito cells was slightly suppressed during the acute phase of the infection (Table 4). DNA synthesis was more strongly suppressed in the C6/36 mosquito cell line. However, in this respect the late passage mutants did not differ in their effects from wt WEEV. These results support our concept that WEEV-infected vertebrate or invertebrate cells produce the nucleoside triphosphate phosphohydrolase which is probably a virus-coded non-structural polypeptide (Ishida et al., 1981). The enzyme has a primary role in virus replication and the inhibition of cell DNA synthesis is a secondary effect of its action (Ishida et al., 1981; Koizumi et al., 1979 a, b). However, when we examined intracellular proteins, it was clear that the late passage mutants did not suppress host protein synthesis nearly as much as wt WEEV or early mutants in either BHK or C6/36 cells. On this basis we can separate the late passage mutants and an early RNA− mutant (A106) in one class, and the other mutants and wt WEEV in the other class.

The difference in the replication of these two classes of WEEV was even more evident when we examined the replication of these viruses in C6/36 cells and uncloned mosquito cells. Both mosquito cell lines supported virus growth equally well and the lesions leading to cell damage did not seem to affect the ability of the cells to support virus replication as is the case with Sindbis virus (Sarver & Stollar, 1977). However, the late passage class of mutants showed a slight cytopathic infection in C6/36 cells, even when the cells were producing large amounts of virus. When these cells were examined in the electron microscope, it was seen that C6/36 cells infected with wt WEEV and not producing much virus into the culture fluid had relatively large amounts of virus (perhaps incomplete or defective virus) accumulating on the plasma membrane and in the spaces between cells. On the other hand, the cells from the asymptomatic infection with a late passage mutant showed virus budding from the surface, but no accumulation of particles at the membrane. From this we conclude that the mutations in the late passage mutants allow them to mature virus more efficiently and release it into the culture fluid. These observations are similar in the case of SV5 virus infections in MK and BHK cells SV5 virus multiplies to high titre in MK cells with minimal c.p.e., but in BHK cells produces little virus and extensive c.p.e. (Choppin, 1964; Compans et al., 1964). Virus release of alphaviruses from infected cells to culture fluids may depend on many factors, for instance ionic strength of culture fluids (Waite et al., 1972; Strauss et al., 1980). In all mosquito cell infections E2 as well as PE2 and E1 are seen in the monolayers. It is possible that unlike infection of vertebrate cells (Strauss & Strauss, 1977), the processing of PE2 is not the last step in maturation of infectious virions.

Another possibility is that the defect in RNA synthesis which is shared by A106 and the late passage mutants may contribute to their greater efficiency of replication with less c.p.e. in mosquito cells. The c.p.e. of the mosquito cells by alphavirus infections is different from that
of vertebrate cells. The former c.p.e. is not lethal and the aggregation of the infected cells seems to be an important step to overcome a crisis of the infected cell population. This aggregation property of togavirus-infected mosquito cells is reported in many instances (Igarashi, 1978; Sarver & Stollar, 1977; Suitor & Paul, 1969) including our case. Thus, decrease in c.p.e. may be a crucial factor in the establishment of persistent infection in vertebrate cells, and many viruses isolated from such persistently infected vertebrate cells were RNA^-ts mutants (Preble & Youngner, 1975; Youngner et al., 1976, 1978; Ju et al., 1980). In any event, it does not appear that a single mutation is responsible for the selective advantage in persistent infection, for all of the late passage mutants contain multiple lesions as is the case with vesicular stomatitis and measles viruses in vertebrate cells (Holland et al., 1979; Ju et al., 1980). Thus, both the establishment of persistent infection and the maintenance of the chronically infected state may depend on many factors.

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REFERENCES


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