A single amino acid change in the E2 glycoprotein of Venezuelan equine encephalitis virus affects replication and dissemination in *Aedes aegypti* mosquitoes

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Four monoclonal antibody-resistant variants (MARVs) of Venezuelan equine encephalitis (VEE) virus were used to study mosquito–virus interactions. *In vitro* experiments using an *Aedes albopictus* cell line, C6/36, demonstrated that an amino acid change in the glycoprotein E2a epitope (MARV 1A3B-7) decreased virus growth when compared with the wild-type, Trinidad donkey virus, and its vaccine derivative, TC-83. The MARVs replicated as efficiently as the parent virus when inoculated into *Aedes aegypti* mosquitoes, but MARV 1A3B-7 was restricted in its ability to infect and disseminate from the midgut following oral infection. These results demonstrate that a single amino acid change in the E2 glycoprotein can affect the ability of VEE virus to replicate and disseminate in *Ae. aegypti* mosquitoes.

**Introduction**

Venezuelan equine encephalitis (VEE) virus is an *Alphavirus* in the family Togaviridae. Alphaviruses contain single-stranded, positive-sense RNA surrounded by an icosahedral nucleocapsid. The nucleocapsid is enclosed in a host-derived lipid envelope containing heterodimers of glycoproteins E1 and E2 in the form of trimers (Strauss & Strauss, 1977). The E2 glycoprotein carries the major neutralization epitopes (Dalrymple *et al.*, 1976), and the E1 glycoprotein provides the fusion properties of the virus (Kielian *et al.*, 1990; Boggs *et al.*, 1989).

On the E2 glycoprotein, the E2c, E2f, E2g and E2h epitopes are located in the same spatial domain of the glycoprotein spike and are defined by VEE virus-neutralizing monoclonal antibodies (MAbs) (Roehrig *et al.*, 1982; Roehrig & Mathews, 1985). The E2a and E2b epitopes are the most conserved E2 epitopes. The E2c epitope of the E2 glycoprotein defines the major neutralization site of VEE virus (Roehrig *et al.*, 1982; Roehrig & Mathews, 1985). Antibodies against all four E2 glycoprotein epitopes can passively protect mice from lethal peripheral challenge with virulent VEE virus (Roehrig & Mathews, 1985).

To define these E2 epitopes further, we have prepared and sequenced MAb-resistant variants (MARVs) using the VEE virus vaccine, TC-83. All of the isolated MARVs have amino acid changes only in the E2 glycoprotein. MARV 3B4C-4, MARV 1A4D-1, MARV 1A3A-9 and MARV 1A3B-7 have changes at amino acid residues 182, 183, 199 and 207 which correspond to the E2c, E2f, E2g and E2h epitopes, respectively (Johnson *et al.*, 1990; Roehrig *et al.*, 1982; Roehrig & Mathews, 1985). VEE virus structural proteins expressed in vaccinia virus also identified amino acid residue 209 as being within the E2h epitope (Kinney *et al.*, 1988).

Events involved in productive arbovirus infection of vector species include infection of vector midgut cells, dissemination of virus into the haemocoel, infection and replication in secondary target organs and subsequent transmission to vertebrate hosts (Hardy, 1988; Grimstad, 1988; Beaty & Bishop, 1988). The time necessary for these events to occur is designated the extrinsic incubation period and can vary depending upon particular vector–virus pairs. For example, dissemination of the bunyavirus La Crosse (LAC) from *Aedes triseriatus* midgut cells may require 6 to 9 days (Beaty & Thompson, 1978), whereas eastern equine encephalomyelitis (EEE) virus can be detected in secondary target organs within hours of blood meal ingestion (Scott *et al.*, 1984). Rapid dissemination of EEE virus may be due to cytopathic effects in the midgut cells of vectors (Weaver *et al.*, 1988).

Multiple barriers to productive vector infection exist, including the midgut infection barrier, midgut dissemina-
tion barrier and salivary gland barrier (Hardy, 1988; Turell, 1988; Beaty & Bishop, 1988; Paulson et al., 1989). Molecular determinants of these barriers remain to be elucidated. There is an infection threshold involved in the midgut infection barrier to VEE virus in *Culex taeniopus* (Sherer et al., 1981); 1000 to 5000 p.f.u./ml of virus in the blood meal is required to infect vectors efficiently. Efficient vector infection appears to be associated with the fusion of viral glycoproteins with midgut villi (Sundin et al., 1987). Specific receptor events may not be involved in midgut infection (Beaty et al., 1982).

MARVs are useful tools for identifying viral determinants of infection. Sundin et al. (1987) exploited this approach to determine the role of the LAC virus G1 glycoprotein in vector infection. One MARV, which was reduced in fusion capability and was not neuroinvasive in mice, was markedly reduced in its ability to infect vector midgut cells. The MARV was as infectious as the parent strain when intrathoracically inoculated into mosquitoes.

We report here the use of the VEE virus MARVs to investigate alphavirus genetic determinants of productive vector infection. These viruses were also used to investigate viral replication in vertebrate and invertebrate cells.

**Methods**

**Cells and viruses.** Baby hamster kidney (BHK-21), African green monkey kidney (Vero) and *Ae. albopictus* (C6/36) cells were cultivated in MEM supplemented with 10% fetal calf serum (FCS). Wild-type VEE virus, Trinidad donkey (TRD) virus, and its vaccine strain, TC-83, were obtained from the reference collection of the Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, Co., U.S.A. Isolation and characterization of the E2 glycoprotein MARVs have been previously described (Johnson et al., 1990).

**MAbs.** Isolation and characterization of the MAbs have been previously described (Roehrig et al., 1980, 1982). All of the MAbs used in the experiments recognize epitopes on the E2 glycoprotein of TC-83 and/or TRD viruses.

**Plaque assays.** Stock virus and experimental samples were titrated and sampled at limiting dilutions by plaque assay (Monath et al., 1976). Briefly, 0.2 ml of sample per well was adsorbed onto Vero cells, overlaid with primary agarose in maintenance medium containing neutral red and incubated for 3 to 6 days. Titres were calculated and expressed as p.f.u./ml.

For virus growth experiments, viruses were adsorbed to cells at an m.o.i. of 0.1. Viruses on Vero cells were adsorbed for 1 h at 37 °C, and those on C6/36 cells were adsorbed for 2 h at 28 °C. Unattached virus was removed by rinsing cells with PBS pH 7.4. Growth medium containing 5% FCS was then added. Samples were taken at 12 h intervals beginning at 0 h unless otherwise stated.

**Mosquitoes and mosquito infection.** The *Ae. aegypti* mosquito colony was obtained from Rexville, Puerto Rico, and was in the fourth generation. Mosquitoes were reared at 27 °C with 80% relative humidity and a photoperiod of 16 h of light, 8 h of dark. Mosquitoes were fed on a 5% sucrose solution.

For intrathoracic inoculation of mosquitoes, stock viruses were diluted in BA-1 [M199-H medium (Earle's salts, L-glutamine and 25 mM-HEPES buffer), 0.05 M-Tris-HCl pH 7.6, 1% BSA, 0.35 g/l sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml amphotericin B] to equal titres. Sixty female mosquitoes were injected with approximately 10 to 12 p.f.u. of each virus. Ten virus-infected mosquitoes were taken at intervals of 0, 12, 24, 36, 72 and 96 h post-inoculation. Each mosquito from each interval was then homogenized in 1 ml of BA-1, centrifuged in a microfuge for 2 min at 14,000 r.p.m. (16,000 g), diluted 10-fold and titrated by plaque assay.

*Ae. aegypti* mosquitoes were permitted to ingest artificial blood meals containing equal parts of virus and washed human erythrocytes. Drops of the infectious meal were placed onto nylon netting covering the cages. The mosquitoes, which had been starved without sugar solution or water for 3 days, were allowed to feed directly from the drops. Mosquitoes were exposed to the artificial meal for 15 min, and replete females were selected and held in cages for 14 days. Mosquito head tissue was then examined by indirect immunofluorescence, and the body remnants were titrated.

**Indirect immunofluorescence assay (IFA).** Flasks (75 cm²) of Vero cells were infected at an m.o.i. of 0.5 with each virus. Cells were incubated at 37 °C for 48 h, trypsinized, placed on multi-spot slides, air-dried and fixed in cold acetone. The indirect IFA began by the addition of MAbs fixed in cold acetone. The indirect IFA began by the addition of MAbs diluted 1:10 in PBS and then making twofold dilutions to 1:20480. The dilutions were added to the slides and incubated at 37 °C for 30 min. Remaining MAb was removed by washing the mixture three times for 5 min each in PBS. Commercial goat anti-mouse IgG coupled to fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories) was then added at a dilution of 1:200 and incubated for 30 min at 37 °C. The slides were washed again with PBS, air dried and examined microscopically.

For the mosquito tissue IFA, mosquito heads were placed on ethanol-washed slides, crushed, fixed in cold acetone for 20 min and circled with a Tech pen. The heads were assayed indirectly by incubating with TC-83 hyperimmune ascitic fluid (HIAF) 1:100 in PBS, followed by goat anti-mouse FITC diluted 1:100 in PBS each at 37 °C for 30 min. Slides were then rinsed twice for 10 min each in PBS, air-dried and mounted for examination.

**Results**

**MAb maps of different variants of VEE**

Because we wanted to use IFA as our assay to detect virus antigen, the MARVs were tested after passage in Vero cells by indirect IFA against the MAb to which they were resistant, to determine their stability and IFA reactivity (Table 1). TC-83 was used as a positive virus control and TC-83 HIAF was used as a positive antibody control. The IFA results were similar to the ELISA results previously published (Johnson et al., 1990). IFA titres were considerably lower than ELISA titres due to the reduced sensitivity of the IFA. None of the variants was detected by its respective selecting antibody demonstrating that reversion had not occurred during passage.
Table 1. Reactivity of MARVs with MAbs using IFA

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Antibody</th>
<th>TC-83</th>
<th>3B4C-4</th>
<th>1A4D-1</th>
<th>1A3A-9</th>
<th>1A3B-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2*</td>
<td>3B4C-4</td>
<td>10240</td>
<td>&lt;10</td>
<td>1280</td>
<td>5120</td>
<td>1280</td>
</tr>
<tr>
<td>E2*</td>
<td>1A4D-1</td>
<td>&gt;20480</td>
<td>2560</td>
<td>&lt;10</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>E2*</td>
<td>1A3A-9</td>
<td>160</td>
<td>10</td>
<td>20</td>
<td>&lt;10</td>
<td>640</td>
</tr>
<tr>
<td>E2*</td>
<td>1A3B-7</td>
<td>1280</td>
<td>1280</td>
<td>320</td>
<td>640</td>
<td>&lt;10</td>
</tr>
<tr>
<td>6B6C-1 (SLE)†</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>TC-83 HIAF</td>
<td>320</td>
<td>80</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td></td>
</tr>
</tbody>
</table>

* Homologous antibody–virus reactions are underlined.
† MAb specific for flavivirus, Saint Louis encephalitis (SLE).

Virus replication in mammalian and insect cells

Both Vero and C6/36 cells were infected with either TC-83 or the MARVs and samples of the medium taken at 48 h were diluted 10-fold and tested by plaque assay (Fig. 1). In Vero cells, all of the viruses had titres of 1.5 x 10^6 to 1.5 x 10^7 p.f.u./ml. In C6/36 cells, however, TC-83 titres were 10^10 p.f.u./ml, and the titres for the MARVs were approximately 4 x 10^9 p.f.u./ml. MARV 1A3B-7 was an exception with a titre of 4 x 10^7 p.f.u./ml.

Growth curve assays were used to define the replication kinetics of the viruses in C6/36 cells (Fig. 2). TRD and TC-83 viruses attained titres of 3 x 10^9 p.f.u./ml and 7 x 10^9 p.f.u./ml, respectively in 24 h. MARV 1A4D-1 had a peak titre of 2 x 10^10 p.f.u./ml at 36 h, and MARVs 3B4C-4 and 1A3A-9 had titres of 5 x 10^8 p.f.u./ml and 7 x 10^8 p.f.u./ml, respectively by 72 h. MARV 1A3B-7 had a titre of 2 x 10^7 p.f.u./ml at 36 h.

Virus replication in Ae. aegypti mosquitoes

The ability of viruses to replicate in vivo in Ae. aegypti mosquitoes following intrathoracic inoculation was compared with their ability to replicate following oral infection. First, TC-83 and the MARVs were inoculated intrathoracically to bypass mesenteronal barriers. Mosquitoes were taken at timed intervals, including time zero, to measure the approximate amount of virus initially inoculated. The body remnants of each mosquito at each timed interval were titrated by plaque assay. TC-83 and all of the MARVs replicated to between 10^6 and 10^7 p.f.u./ml by 3 to 4 days post-infection (Fig. 3).
Mosquitoes were then infected orally by allowing them to feed on artificial blood meals to determine the ability of TC-83 and the MARVs to disseminate from the midgut to the head. The unfed portions of the meals were tested by plaque assay to determine the amount of virus each contained. After 14 days extrinsic incubation, the head tissue of each infected mosquito was tested for virus antigen using IFA (Table 2). The body tissues were each homogenized and titrated by plaque assay (Table 2). Only the MARV 1A3B-7, which had titres of $3 \times 10^5$ p.f.u./ml, showed a significant difference from TC-83, the titre of which was $2.6 \times 10^6$ p.f.u./ml ($P < 0.05$). The MARV 1A3B-7 also failed to disseminate from the midgut to the head.

### Discussion

We have demonstrated that a single amino acid change in the E2 glycoprotein of VEE can significantly alter the ability of virus to infect vectors productively. In C6/36 cells, TC-83 and three of the MARVs replicated efficiently. MARV 1A3B-7, however, showed a 1000-fold decrease in the amount of virus produced compared with that produced by TC-83. All of the viruses, including MARV 1A3B-7, replicated efficiently following intrathoracic inoculation of Ae. aegypti. However, when mosquitoes were challenged, MARV 1A3B-7 inefficiently disseminated from the mosquito midgut. In contrast, TC-83 and the other MARVs replicated to high titres in the midgut and disseminated to the head. These results are consistent with the studies by Sundin et al. (1987) who found that a G1 glycoprotein MARV exhibited reduced infectivity and decreased dissemination from the midgut in Ae. triseriatus.

MARV 1A3B-7 has an isoleucine to phenylalanine substitution at amino acid 207 in E2, which causes altered reactivity and growth compared with the parent strain TC-83. This region of E2 has been identified as the E2<sup>h</sup> epitope by testing a vaccinia virus recombinant of TRD-1A (VACC/TRD-1A), which encodes a glutamic acid to lysine substitution at amino acid 209 (Kinney et al., 1988). This VACC/TRD-1A recombinant also fails to react with anti-E2<sup>h</sup> MAb, while TC-83, TRD and the vaccinia virus recombinant of TC-83 reacted well. These data demonstrate that amino acid residue 209 is also associated with the E2<sup>h</sup> epitope of the E2 glycoprotein.

The E2<sup>h</sup> epitope is significant because it is involved in early virus–host cell interactions. If anti-E2<sup>h</sup> antibody is incubated with virus before infection of a cell monolayer, the virus is neutralized by 99 to 100% in both Vero and C6/36 cells (data not shown). The two early interactions between virus and host cell are attachment of the virus to the cell and fusion of the viral envelope to the host cell membrane allowing virus entry. We have previously shown that the anti-E2<sup>h</sup> MAb blocks attachment of VEE virus to Vero cells. It is possible that the amino acid change in MARV 1A3B-7 changes the E2 conformation, which may alter attachment to mosquito cells. However, attempts to test attachment of these viruses to C6/36 cells were unsuccessful. We are presently preparing monospecific antibodies using peptides derived from the E2 amino acid sequence to characterize more precisely any structural differences between TC-83 and MARV 1A3B-7 using competitive binding assays (Hunt et al., 1990).

The fusion properties of alphaviruses have been studied previously showing the E1 glycoprotein to be responsible for virus fusion of cell membranes (Kielland et al., 1990; Boggs et al., 1989). The amino acid change in MARV 1A3B-7, which alters productive vector infection, is in the E2 glycoprotein. Therefore, the mechanism of reduced infectivity is not directly related to a change in the E1 glycoprotein. It is possible, however, that a change in E2 could alter the E1 fusion characteristics. To understand the molecular basis of this altered infectivity, the fusion characteristics of MARV 1A3B-7 will need to be characterized.

This is the first time that altered vector infectivity has been reported for alphaviruses. While TC-83 is an effective immunogen for eliciting protective VEE virus antibody, it has been isolated from mosquito vectors following horse vaccination (Pedersen et al., 1972). Our ability to identify non-lethal changes in the E2 glycoprotein that block virus dissemination from mosquito midgut to the brain is an important observation for alphavirus vaccine design. The E2<sup>h</sup> variant is also attenuated in mice when compared to TC-83 virus.

### Table 2. Oral infection rates in Ae. aegypti mosquitoes following exposure to TC-83 and MAR

<table>
<thead>
<tr>
<th>Variant</th>
<th>Bloodmeal titre*</th>
<th>Percentage infected (n)†</th>
<th>Percentage disseminated infection (n)‡</th>
<th>Body remnant titre§</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-83</td>
<td>7.3</td>
<td>80 (10)</td>
<td>38 (8)</td>
<td>6.1 (0.3)</td>
</tr>
<tr>
<td>3B4C-4 variant</td>
<td>7.6</td>
<td>89 (9)</td>
<td>25 (8)</td>
<td>5.9 (1.1)</td>
</tr>
<tr>
<td>1A4D-1 variant</td>
<td>7.7</td>
<td>89 (9)</td>
<td>50 (8)</td>
<td>5.9 (0.6)</td>
</tr>
<tr>
<td>1A3A-9 variant</td>
<td>7.9</td>
<td>78 (8)</td>
<td>43 (7)</td>
<td>6.1 (0.8)</td>
</tr>
<tr>
<td>1A3B-7 variant</td>
<td>7.3</td>
<td>60 (10)</td>
<td>0 (6)</td>
<td>5.5 (0.3)</td>
</tr>
</tbody>
</table>

* Log<sub>10</sub> p.f.u./ml.
† Percentage of mosquitoes with virus in body sample (n, sample size). Mosquitoes were harvested after 14 days extrinsic incubation.
‡ Percentage of infected mosquitoes with virus in the head.
§ Average titre of body remnants following oral infection (n = 10). Expressed as log<sub>10</sub> p.f.u./mosquito (S.D.).
(Johnson et al., 1990). These observations suggest that it should be possible to produce a live attenuated alphavirus vaccine that will be unable to be transmitted by mosquito vectors.

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References


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