Molecular and biological characterization of a non-glycosylated isolate of St Louis encephalitis virus

V. Vorndam,1* J. H. Mathews,2 A. D. T. Barrett,3 J. T. Roehrig2 and D. W. Trent2

1 Center for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Dengue Laboratory, 2 Calle Casia, San Juan, Puerto Rico 00921-3200, 2 Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado, U.S.A. and 3 School of Biological Sciences, University of Surrey, Guildford GU2 5XH, U.K.

The glycosylation patterns of the envelope (E) glycoprotein of several naturally occurring strains of St Louis encephalitis (SLE) virus were investigated. SLE viruses were found that contained both glycosylated and non-glycosylated E proteins, and one isolate (Tr 9464) that lacks N-linked glycosylation sites on its E protein was identified. SLE virus monoclonal antibodies that define E protein B cell epitopes and demonstrate biological activities reacted essentially to the same extent with glycosylated and non-glycosylated virions. These results indicate that glycosylation is not essential for epitope conformation or recognition. However, failure to glycosylate the E protein was associated with possible morphogenetic differences as manifested by reduced virus yields and differences in specific infectivity.

Introduction

St Louis encephalitis (SLE) virus is a member of the Flaviviridae family of arboviruses (Karabatsos, 1980, 1985; Westaway et al., 1985). This virus occurs widely in the Western Hemisphere, where human disease is well documented (Monath, 1980). It periodically causes human epidemics in North America, most recently in 1975 and 1990 (Chamberlain, 1980; Centres for Disease Control, 1990, 1991). The structure of SLE and related flaviviruses has been extensively studied. The major component of the virion envelope is a glycoprotein (the E protein) with an M, of approximately 55K. This glycoprotein forms virion spike-like projections and is the primary determinant of adsorption, haemagglutination and antibody-mediated neutralization and enhancement reactions (reviewed by Monath, 1990). The antigenic structure of the E protein has been characterized and is composed of a complex mosaic of type-, complex- and group-specific reactivities (Trent, 1977; Peiris et al., 1982; Brandt et al., 1982; Gentry et al., 1982; Henchal et al., 1982; Roehrig et al., 1983; Heinz & Roehrig, 1990).

The role that carbohydrate plays in the biological functions and immunoreactivity of viral glycoproteins is poorly understood and differs among virus groups. Sequence analysis of the genomes of flaviviruses has revealed considerable heterogeneity in the number and usage of glycosylation sites. Viruses having two potential N-linked glycosylation sites on the E glycoprotein are the MSI-7 strain of SLE (Trent et al., 1987), dengue type 1 (Mason et al., 1987), dengue type 2 (Deubel et al., 1986; Hahn et al., 1988; Gruenberg et al., 1988), dengue type 3 (Osatomi et al., 1988; Osatomi & Sumioshi, 1990), dengue type 4 (Zhao et al., 1986) and American isolates of yellow fever (YF) virus (Ballinger-Crabtree & Miller, 1990). Other flaviviruses contain a single glycosylation site, including tick-borne encephalitis (TBE) virus (Mandi et al., 1988), Murray Valley encephalitis virus (Dalgaard et al., 1986), Japanese encephalitis (JE) virus (Sumiyoshi et al., 1987; McAda et al., 1987) and an African isolate of YF virus (Rice et al., 1985; Ballinger-Crabtree & Miller, 1990). Apparently unique among enveloped viruses, two flaviviruses, West Nile (WN) and Kunjin (KUN) lack N-linked glycosylation sites on their E proteins (Wengler et al., 1985; Coia et al., 1988), whereas the African strains of YF virus fail to utilize their single glycosylation site (Deubel et al., 1987; Cane & Gould, 1989). In this report we identify naturally occurring variants of SLE virus that contain both glycosylated and non-glycosylated E proteins and one isolate that completely lacks glycosylation sites on its E protein. Monoclonal antibody (MAb) analysis indicates that glycosylation is not essential for epitope conformation or biological activity.

Methods

Cells and mice. Vero cells, the human adenocarcinoma cell line SW-13, duck embryo and CER (hamster) cells were maintained in Eagle's MEM supplemented with fetal bovine serum and penicillin/
streptomycin. NIH outbred 3-week-old (< 20 g) white Swiss mice were used for in vivo experiments.

**Viruses and antisera.** Viruses were obtained from the collection of the Centers for Disease Control as frozen tissue culture fluid samples, and have been previously characterized (Monath et al., 1980). Polyclonal hyperimmune ascitic fluids (HIAF) were obtained from the Arbovirus Reference Branch of this laboratory.

The isolation and characterization of the SLE MSI-7 strain glycoprotein-specific MAbs used in this study have been described (Roehrig et al., 1983). These MAbs were adjusted to 1 mg/ml which was considered as the undiluted value for assay purposes.

Fluorescein-labelled goat anti-mouse IgG (H & L) was obtained from the Jackson ImmunoResearch Laboratories.

**Immunological assays.** Haemagglutination inhibition (HI) and neutralization (N) assays were performed as previously described (Mathews et al., 1985; Clarke & Casals, 1988). Indirect fluorescence assay (IFA) tests were performed on acetone-fixed or unfixed virus-infected SW-13 cells.

**Tunicamycin treatment.** Purified, unfractionated tunicamycin (Tm, lot 361-26E-250-A) was either a gift from Dr Robert Hamill of the Eli Lilly Research Laboratories or was purchased from Boehringer Mannheim Biochemicals. Purified homologue A1 was obtained from Boehringer Mannheim Biochemicals. SW-13 cells were infected at an m.o.i. ≥ 1. After 2 h adsorption at 37 °C the monolayers were washed three times with MEM and fed with media containing 1 or 2.5 μg/ml of Tm. The cells were further incubated for 24 h for IFA tests or until c.p.e. began to appear for the other studies.

**Preparation of viral components.** Confluent monolayers of SW-13 or CER cells were infected with 1 ml of viral suspension and maintained in medium containing 10% of the normal amount of leucine, total amino acids or glucose and 2% fetal bovine serum. Twelve h after infection tritiated leucine, mixed amino acids or sugar were added to achieve a final activity of 33 μCi/ml. For analysis of intracellular proteins, cells were harvested when they showed 10% c.p.e. by washing once in cold PBS and lysing in RIPA buffer (Brugge et al., 1978). Purified virions and viral RNA were prepared by precipitation, centrifugation and phenol–chloroform extraction as described by Trent & Grant (1980). Sequencing of genomic RNA was performed according to Johnson et al. (1986). Plaque assays were performed in primary duck embryo cells.

**Protein analysis.** PAGE was performed by the method of Laemmli (1970) using 15% acrylamide and the labelled proteins were detected by fluorography (Bonner & Laskey, 1974). For preparative electrophoresis purified virions were reacted with fluorescamine (Lambris et al., 1979) and the individual proteins were visualized using a fluoroscence light. Proteins were cut from the gel and eluted in water. The proteins were precipitated with 5 vol. of chilled acetone, resuspended in a small volume of 1% ammonium bicarbonate buffer and digested with 200 μg/ml TPKC-trypsin (Worthington) overnight at 37 °C. Tryptic peptides were analysed by HPLC using two tandem Ultrasphere columns and an acetonitrile gradient, as described by Kinney & Trent (1982).

**Results**

**Analysis of viral proteins**

We have analysed the structural proteins of SLE virus isolates from different geographical locations (Table 1) for variation in their relative mobility when subjected to PAGE. Purified virions were electrophoresed on continuous polyacrylamide gels under conditions that maximized separation of the E protein according to M_r.

The E proteins of five of the seven SLE virus isolates studied split into two molecular species (Fig. 1a). All the virions examined apart from strains BeAn 246262 and Tr 9464 contained two E proteins. These two strains contained a single E protein, which comigrated with either the larger or smaller of the E proteins of other SLE virus strains. Similar results were obtained when the viruses were grown in either CER or primary duck embryo cells (data not shown).

Glycosylation of proteins can affect their migration in acrylamide gels (Knipe et al., 1977; Westaway et al., 1977). Since the MSI-7 strain of SLE virus contains two potential glycosylation sites, we examined these two bands by incorporation experiments using radioactive sugars and tryptic peptide analysis to determine whether both molecular species were glycosylated. Strain 76V-1177, which contained both molecular species, was grown in the presence of [3H]glucosamine and [3H]-mannose and the structural proteins were electrophoresed in parallel with those of this strain grown in the presence of [3H]leucine (Fig. 1b). Radioactivity of the carbohydrate-labelled virus comigrated with the slower migrating E protein species. To confirm that these

![Table 1. SLE viruses used in this study](attachment:table1.png)

![Fig. 1. (a) SDS–PAGE of purified virions of seven different strains of SLE virus. Lane 1, MSI-7; lane 2, 65V-310; lane 3, Tr 9464; lane 4, BeAn 246262; lane 5, BeH 203235; lane 6, 76V-1177; lane 7, 69M-1143. (b) SDS–PAGE of SLE 76V-1177 virus isolate labelled with [3H]leucine (lane 1), [3H]glucosamine (lane 2) and [3H]mannose (lane 3).](attachment:figure1.png)
proteins were glycosylation variants with the same primary structure, proteins of the MSI-7 strain were labelled with [3H]leucine and separated by PAGE. The tryptic peptide profiles of these two proteins were found to be virtually the same (Fig. 2). The identity of proteins in these two bands was further confirmed by coprecipitation with the 1B2C-5 MAb specific for the E protein (data not shown). These data indicate that the faster migrating protein is a non-glycosylated variant of the larger, glycosylated protein. In keeping with the nomenclature of others, we have designated the larger species ‘E’ and the smaller non-glycosylated form ‘Eo’ (Knipe et al., 1977).

We examined SLE strains BeAn 246262, which lacks detectable Eo, and Tr 9464, which contains no E to determine whether they produce the missing protein but fail to incorporate it into mature virions. Primary duck embryo cells were infected with strains 76V-1177, BeAn 246262 or Tr 9464, and treated with actinomycin D and hypertonic saline to suppress cellular protein synthesis, and then pulse-labelled with [3H]leucine. Lysates of the infected cells were analysed by SDS-PAGE (Fig. 3). Cells infected with all three viruses contained intracellular E proteins in amounts corresponding to those present in extracellular virions. This indicated that there is apparently no degradation or selective incorporation of the E proteins during maturation. Strain Tr 9464 does not produce a glycosylated E protein, and is apparently assembled with a non-glycosylated Eo. Sequence analysis of the Tr 9464 E protein gene confirmed the lack of a glycosylation site (Table 2). Compared with the MSI-7 strain, there are amino acid substitutions at both glycosylation sites in the Tr 9464 genome and these eliminate the recognition sequences.

**Immunological characterization**

We previously characterized and mapped antigenic determinants on the E glycoprotein of SLE virus using MAbs (Roehrig et al., 1983). Four reactivity groups (type, complex, subcomplex and flavivirus group) were defined by IFA. Furthermore, analysis based on the biological activities of HI and N, using the MSI-7 strain, identified eight distinct epitopes (Mathews & Roehrig, 1984). To determine whether the biological activity of the various anti-epitope MAbs was affected by lack of glycosylation in the Tr 9464 strain, N and HI assays were performed and compared with those of the MSI-7 virus (Table 3). The MAb reactivities of the two viruses were qualitatively almost the same. Strain Tr 9464 was found to be positive by IFA with representative MAbs from all four reactivity groups. The E1c and E1d epitopes were not significantly altered in the Tr 9464 virus strain. The E4b MAb did not neutralize the Tr 9464 virus; however,
Table 2. Comparative amino acid sequences of MSI-7 and Tr 9464 virus glycosylation sites

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence</th>
<th>Strain</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-7</td>
<td>TDSTSHGYSEQIGKQQARF</td>
<td>Tr 9464</td>
<td>TDSTSHGYSEQIGKQQARF</td>
</tr>
<tr>
<td></td>
<td>470</td>
<td>630</td>
<td></td>
</tr>
<tr>
<td>MSI-7</td>
<td>MCDSATFSKNPDTGHGTVIVE</td>
<td>Tr 9464</td>
<td>MCDSATFSKNPDTGHGTVIVE</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>640</td>
<td></td>
</tr>
</tbody>
</table>

* The underlined sequence locates the N-linked glycosylation site.

demonstrable N titres with the E4b MAb have been difficult to reproduce with the MSI-7 virus in subsequent experiments. The five epitopes demonstrating HI activity on MSI-7 virus were also present on the Tr 9464 E protein. Although some differences are seen in endpoint titres with the HI MAbs, the loss of the capacity to glycosylate the E protein as a naturally occurring mutational event does not affect the E glycoprotein epitopes.

Following infection of SW-13 cells with the MSI-7 virus, there is differential expression of E protein epitopes as a function of time (Roehrig, 1986). This may be related to protein processing events and virion morphogenesis. To determine whether the lack of glycosylation on the Tr 9464 virus affected the temporal sequence of these events, SW-13 cells infected with either MSI-7 or Tr 9464 viruses were harvested at various times and tested by IFA endpoint analysis with MAbs representative of early (E1a, E3) and late (E1b, E4b) E glycoprotein epitopes (Fig. 4). Although the Tr 9464 epitopes appeared to be expressed somewhat more slowly than those of the MSI-7 virus, the same dichotomy of early and late epitope development could be detected in both of these two viruses.

Tm is a potent inhibitor of protein glycosylation (Duksin & Mahoney, 1982). Cells infected with either the MSI-7 or Tr 9464 strain of SLE virus were treated with Tm to determine whether there were changes in epitope expression after inhibition of glycosylation (Table 4). Six

Table 3. Comparison of N and HI activity between the MSI-7 and Tr 9464 SLE virus strains using anti-E protein MAbs

<table>
<thead>
<tr>
<th>Biological activity for SLE viruses§</th>
<th>MSI-7</th>
<th>Tr 9464</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>HI</td>
</tr>
<tr>
<td>3B4C-7 Type E1b</td>
<td>&lt; 50</td>
<td>20</td>
</tr>
<tr>
<td>1B2C-5 Type E1b</td>
<td>&lt; 50</td>
<td>8000</td>
</tr>
<tr>
<td>6B5A-2 Type E1a</td>
<td>64000</td>
<td>32000</td>
</tr>
<tr>
<td>44C4-4 Type E1a</td>
<td>800</td>
<td>20</td>
</tr>
<tr>
<td>1B5D-1 Subcomplex E2</td>
<td>&lt; 50</td>
<td>8000</td>
</tr>
<tr>
<td>2B5B-3 Supercomplex E3</td>
<td>&lt; 50</td>
<td>8000</td>
</tr>
<tr>
<td>2B6B-2 Group E4b</td>
<td>&lt; 50</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>6B6C-1 Group E4b</td>
<td>200</td>
<td>3200</td>
</tr>
</tbody>
</table>

* All purified MAbs were adjusted to 1 mg/ml.
† Type denotes SLE virus; Subcomplex denotes SLE and JE viruses; Supercomplex denotes SLE, JE, WN, Murray Valley and YF viruses; Group denotes all flaviviruses.
‡ Epitopes determined by cross-reactivity analysis and differing biological reactivity.
§ Seventy percent plaque reduction and HI endpoint titres.
Synthesis and $M_0$ of the non-glycosylated, non-structural proteins NS5 and NS3 were not affected by treatment with this concentration of Tm, as demonstrated by either immunoblotting (data not shown) or IFA (as evidenced by the high reactivity of the SLE HIAF). Cells infected with MSI-7 and treated with 1 µg/ml Tm demonstrated a fourfold reduction in binding of only the anti-E2 MAb. This MAb bound well to Tr 9464 virus. It also recognized all forms of the E protein in immunoblots (data not shown). Binding of all other MAbs to either strain of SLE virus was not affected by Tm treatment. These data further support results with the Tr 9464 strain, which indicate that the SLE viral E protein conformation is not significantly affected by a natural or artificially induced lack of glycosylation.

**Biological activity of SLE viruses**

PAGE analysis of virions produced in the presence of Tm revealed virions that contained only E0 (data not shown) and were less infectious than virus containing normal amounts of E. Analysis of the MSI-7 virus produced with and without Tm indicated that the molar ratio of leucine in the E, C and M proteins was the same ($E:C:M = 1:1.3:1.5$) as that measured by isotope incorporation. Therefore, the c.p.m./p.f.u. ratios in purified viral preparations could be directly compared. Control and Tm-treated virions of the MSI-7 strain labelled with [3H]leucine were purified by consecutive velocity and isopycnic density gradients, counted for radioactivity and titrated for infectivity. The relative infectivity of virus grown in the presence of Tm was approximately 7% of that of the control virus (Table 5). Because the Tr 9464 strain produces non-glycosylated virions, it may also be expected to have a high c.p.m./p.f.u. ratio. We examined Tr 9464 virions in the same manner and found that they had the same ratio of three structural proteins and a low infectivity of approximately 2% in comparison with untreated MSI-7 virions. This infectivity to protein

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>Tm†</th>
<th>MAb reactive with epitope</th>
<th>SLE HIAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-7 (fixed)</td>
<td>0</td>
<td>E1b 3200 25600 1600</td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>E1e 3200 25600 1600</td>
<td>10000</td>
</tr>
<tr>
<td>MSI-7 (unfixed)</td>
<td>0</td>
<td>E1a 3200 25600 800</td>
<td>12000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>E1a 3200 25600 800</td>
<td>12000</td>
</tr>
<tr>
<td>Tr 9464 (fixed)</td>
<td>0</td>
<td>E4b 12800 12800 12800</td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>E4b 12800 12800 12800</td>
<td>10000</td>
</tr>
<tr>
<td>Tr 9464 (unfixed)</td>
<td>0</td>
<td>E4 12800 12800 12800</td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>E4 12800 12800 12800</td>
<td>10000</td>
</tr>
</tbody>
</table>

* Virus-infected SW-13 cells were fixed with ice-cold acetone where noted.
† Micrograms of Tm used.
‡ Reciprocal IFA titres.
Discussion

Carbohydrate can play a fundamental role in the structure and function of viral proteins. Glycosylation of viral proteins begins as a cotranslational event and, depending on the number and types of oligosaccharides involved, can control the folding of the nascent protein, cleavage of polyproteins, addressing and transport through the cell and stabilization of the final product (Delwart & Panganiban, 1990). The primary factor involved in protein structure and function, the amino acid sequence, controls attachment of oligosaccharides and other protein modifications. The factor that determines the disposition and functionality of artificially non-glycosylated viral proteins appears to be the ability of the nascent polypeptide to fold itself into the proper conformation and to allow essential disulphide bonds to form (Vidal et al., 1989).

The dependence of individual viral proteins on carbohydrate varies among virus groups (Kaluza et al., 1980), and may not be consistent within virus groups (Gibson et al., 1979; Chatis & Morrison, 1981). Evidence concerning dependence of the flavivirus E protein conformation on glycosylation is limited and varied. JE virus, which has a single glycosylation site, has a reduced ability to produce extracellular E and NS1 proteins in the presence of Tm, indicating their dependence on glycosylation for proper expression (Mason, 1989). YF viruses differ in their reactivity to MAbs when grown in mammalian or mosquito cells (Barrett et al., 1990). However, the E protein of African strains of YF virus is not glycosylated (Ballinger- Crabtree & Miller, 1990), indicating that factors such as membrane lipid composition may affect E protein structure. TBE virus E protein has a single utilized glycosylation site, but enzymatic deglycosylation after synthesis failed to alter antigenic expression (Winkler et al., 1987), indicating that carbohydrate was not required for maintenance of the antigenic structure. On the other hand, Guirakhoo et al. (1989) reported that carbohydrates stabilize TBE E protein antigens against denaturing agents. At the end of this spectrum, the Tr 9464 strain of SLE, KUN, WN and YF viruses have evolved primary E protein structures that do not require carbohydrate for normal tertiary structure. This lack of dependence on glycosylation is not related to the flavivirus's internal maturation site, since Bunyamwera viruses, which also bud internally, require carbohydrate for proper protein transport (Cash et al., 1980). The only significant difference noted in this study between the Tr 9464 and MSI-7 strains was a reduced specific infectivity of the Tr 9464 virions. The survival of this strain in nature may be problematic unless it is mosquito-adapted, such as by prolonged transovarial transmission, or glycosylation is less important for attachment and penetration of mosquito cells. Different isolates of SLE virus vary significantly in their virulence for birds, mice and monkeys (Monath et al., 1980; Bowen et al., 1980). The BeAn 246262 strain, which has only glycosylated E (Fig. 1), is highly virulent, as are the MSI-7, BeH 203235 and 76V-1177 strains, which contain both E and E0. The Tr 9464 isolate was of intermediate or low virulence (data not shown). However, the 65V-310 and 69M-1143 isolates, which also contain both E and E0, were of intermediate and low virulence, respectively. Therefore, there is not a clear correlation between virulence characteristics and glycosylation pattern with SLE virus. Nevertheless, such naturally occurring variants, of low virulence and non-glycosylated E proteins, may be considered possible vaccine candidates.

The reason why virions in a population contain both glycosylated and non-glycosylated E proteins is not well understood. It may be hypothesized that the folding of the nascent polypeptide during synthesis occludes to a variable extent the relevant amino acid sequences, making them less accessible to the cellular glycosyltransferases, resulting in inefficient glycosylation. The Asibi strain of YF virus, for example, contains a potential glycosylation site that is not utilized. On the other hand, the South American strains of YF virus have a second site that is glycosylated (Ballinger-Crabtree & Miller, 1990). Another example of glycosylation heterogeneity is seen in SLE virus strains that contain mixed populations of E and E0. SLE strain BeAn 246262 E protein has an accessible glycosylation site and therefore contains little or no E0. On the other hand, most strains of SLE have partially occluded glycosylation sites and the virions contain a mixture of both E and E0.

Table 5. Relative infectivity of normal and Tm-treated virions

<table>
<thead>
<tr>
<th>Virus</th>
<th>C.p.m.</th>
<th>P.f.u.</th>
<th>C.p.m./p.f.u. x 10^-6</th>
<th>Relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-7</td>
<td>724</td>
<td>1.9 x 10^9</td>
<td>3.8</td>
<td>1.000</td>
</tr>
<tr>
<td>MSI-7 (Tm)</td>
<td>251</td>
<td>4.7 x 10^9</td>
<td>55.4</td>
<td>0.071</td>
</tr>
<tr>
<td>Tr 9464</td>
<td>351</td>
<td>2.1 x 10^9</td>
<td>167.1</td>
<td>0.023</td>
</tr>
</tbody>
</table>

* C.p.m. in 0-1 ml.
† P.f.u. in 0-1 ml.
‡ Ratio of c.p.m./p.f.u. of MSI-7 divided by the same ratio of Tm-treated MSI-7 or Tr 9464.
If viruses can evolve protein structures that do not require carbohydrate for proper morphogenesis and function, the purpose of their glycosylation is unclear. The answer may lie in the cellular processing and maturation of viral components. An increase in the efficiency of assembly or transport may select for viruses with glycosylated proteins, whether or not virus infectivity requires the E protein to be glycosylated.

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References


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