

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_r 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 (Mandl *et al.*, 1988), Murray Valley encephalitis virus (Dalgarno *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, 1958). 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 fluorescent 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 TPCK-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 dis-

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

Strain	Source	Date	Location
MSI-7	Sparrow	1975	Mississippi
65V-310	Heron	1965	Mexico
Tr 9464	Mosquito	1955	Trinidad
BeAn 246262	Opussum	1972	Brazil
BeH 203235	Human	1971	Brazil
76V-1177	Mosquito	1976	Ecuador
69M-1143	Raccoon	1969	Florida

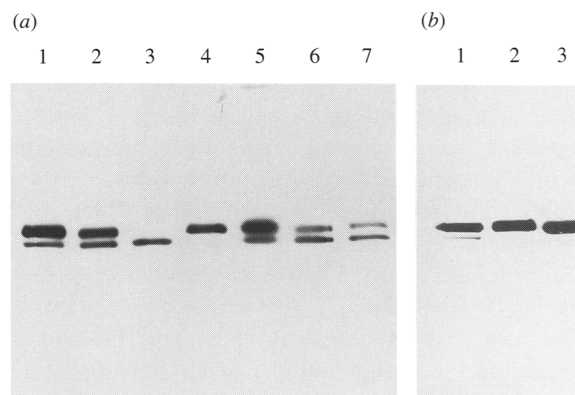


Fig. 1. (a) SDS-PAGE of purified virions of seven different strains of SLE virus. Lane 1, MSI-7; lane 2, 65V-310; lane 3, Tr 9464; lane 4, BeAn 246262; lane 5, BeH 203235; lane 6, 76V-1177; lane 7, 69M-1143. (b) SDS-PAGE of SLE 76V-1177 virus isolate labelled with [³H]leucine (lane 1), [³H]glucosamine (lane 2) and [³H]mannose (lane 3).

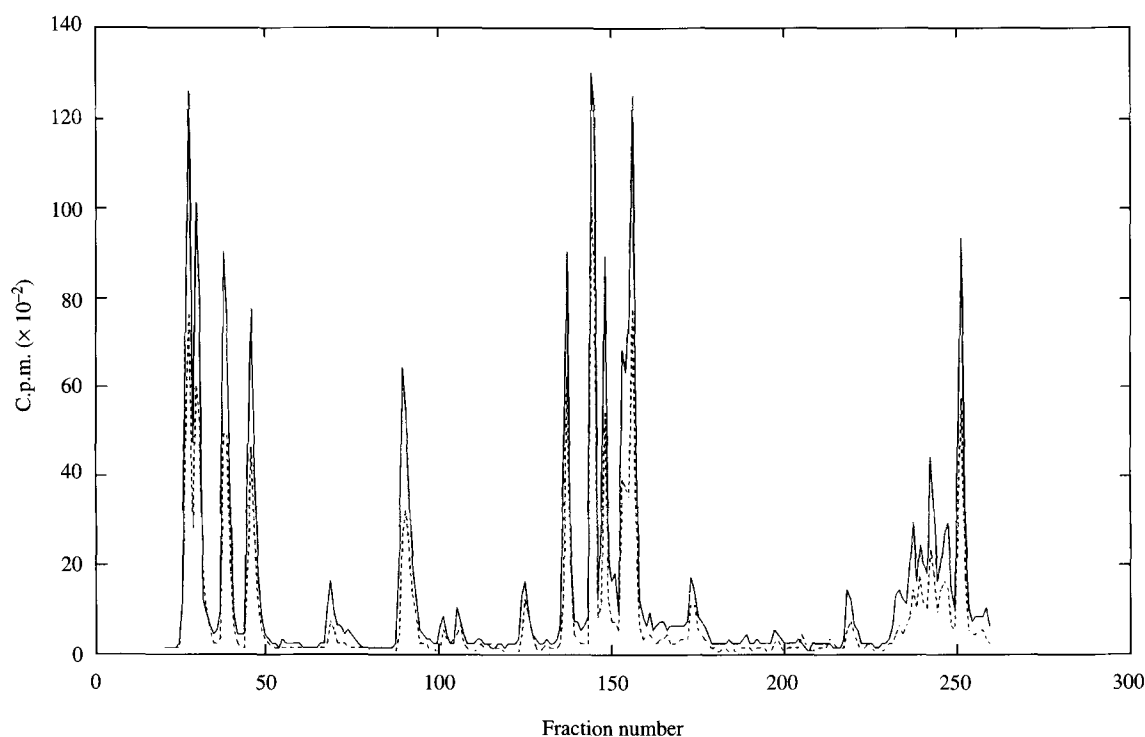


Fig. 2. Chromatographic profile of the tryptic peptides of the larger (solid line) and smaller (dotted line) M_r species resolved by SDS-PAGE of the E protein of the MSI-7 strain of SLE virus. Sequential runs have been superimposed.

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 'E₀' (Knipe *et al.*, 1977).

We examined SLE strains BeAn 246262, which lacks detectable E₀, 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 E₀. 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 E1^c and E1^d epitopes were not significantly altered in the Tr 9464 virus strain. The E4^b MAb did not neutralize the Tr 9464 virus; however,

Table 2. Comparative amino acid sequences of MSI-7 and Tr 9464 virus glycosylation sites*

Strain	Sequence	
	470	480
MSI-7	TDSTSHGNYSEQIGKNQAARF	
Tr 9464	TDSTSHGNYFEQIGKNQAARF	
	630	640
MSI-7	MCDSAFTFSKNPTDTGHGTVIVE	
Tr 9464	MCDSAFTFSKNPADTGHGTVIVE	

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

demonstrable N titres with the E4^b 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 (E1^d, E3) and late (E1^c, E4^b) 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

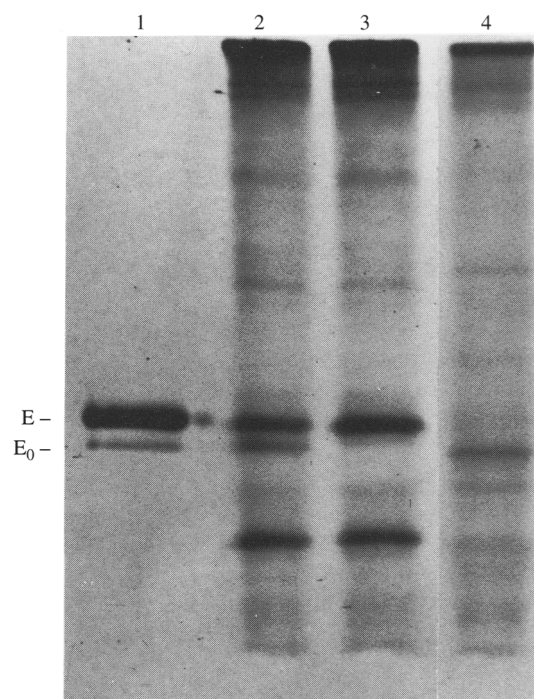


Fig. 3. SDS-PAGE analysis of the intracellular forms of the E glycoprotein of SLE virus strains: 76V-1177 virions (lane 1), 76V-1177 (lane 2), BeAn 246262 (lane 3), and Tr 9464 (lane 4).

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

MAb*	Specificity†	Epitope‡	Biological activity for SLE viruses§			
			MSI-7		Tr 9464	
			N	HI	N	HI
3B4C-7	Type	E1 ^a	< 50	20	< 50	< 10
1B2C-5	Type	E1 ^b	< 50	8000	< 50	2560
6B5A-2	Type	E1 ^c	64000	32000	> 10000	10240
4A4C-4	Type	E1 ^d	800	20	400	20
1B5D-1	Subcomplex	E2	< 50	8000	< 50	2560
2B5B-3	Supercomplex	E3	< 50	8000	< 50	2560
2B6B-2	Group	E4 ^a	< 50	< 10	< 50	< 10
6B6C-1	Group	E4 ^b	200	3200	< 50	10240

* 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.

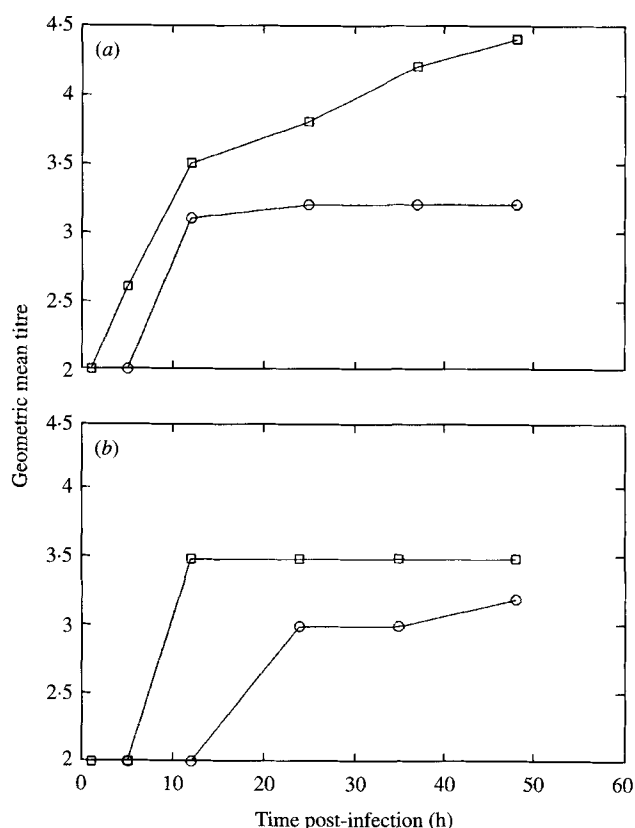


Fig. 4. Temporal appearance of the E protein epitopes of MSI-7 (a) and Tr 9464 (b) strains of SLE virus showing early epitopes (□) and late epitopes (○).

E protein-specific MAbs were titrated by IFA on acetone-fixed or unfixed SW-13 Tm-treated or control cells. The effect of Tm on glycosylation was monitored by immunoblotting virus-infected cell lysates with SLE HIAF or the E2 protein-specific MAb, 1B5D-1. Treatment of infected cells with 1 µg/ml of Tm converted 80 to 90% of the MSI-7 E protein to E₀, and had no effect on the M_r of the E₀ of Tr 9464 (data not shown).

Synthesis and M_r 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 epitope 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 4. Effect of Tm treatment on SLE virus E protein epitope expression

Antigen*	Tm†	MAb reactive with epitope						SLE HIAF
		E1 ^b	E1 ^c	E1 ^d	E2	E3	E4 ^b	
MSI-7 (fixed)	0	12800‡	3200	25600	3200	25600	1600	10000
	1.0	6400	3200	25600	800	12800	1600	10000
MSI-7 (unfixed)	0	3200	6400	12800	3200	3200	1600	10000
	1.0	3200	3200	12800	800	3200	1600	10000
Tr 9464 (fixed)	0	12800	12800	25600	1600	12800	12800	10000
	1.0	6400	6400	25600	1600	25600	12800	10000
Tr 9464 (unfixed)	0	1600	12800	12800	400	3200	6400	10000
	1.0	3200	6400	25600	800	6400	12800	10000

* Virus-infected SW-13 cells were fixed with ice-cold acetone where noted.

† Micrograms of Tm used.

‡ Reciprocal IFA titres.

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

Virus	C.p.m.*	P.f.u.†	C.p.m./ p.f.u. $\times 10^{-6}$	Relative infectivity‡
MSI-7	724	1.9×10^8	3.8	1.000
MSI-7 (Tm)	251	4.7×10^6	53.4	0.071
Tr 9464	351	2.1×10^6	167.1	0.023

* C.p.m. in 0.1 ml.

† P.f.u. in 0.1 ml.

‡ Ratio c.p.m./p.f.u. of MSI-7 divided by the same ratio of Tm-treated MSI-7 or Tr 9464.

ratio was similar to that of Tm-treated MSI-7 virions, suggesting glycosylation characteristics of the E protein may affect early stages of host cell interaction. MSI-7 and Tr 9464 were equally heat-stable (data not shown), indicating that virion morphogenesis and ultrastructure are not affected by glycosylation in these viruses.

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 some 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 glycosyl transferases, 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.

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.

A. D. T. Barrett was supported by a grant from the Wellcome Trust.

References

- BALLINGER-CRABTREE, M. E. & MILLER, B. R. (1990). Partial nucleotide sequence of South American yellow fever virus strain 1899/81: structural proteins and NS1. *Journal of General Virology* **71**, 2115–2121.
- BARRETT, A. D. T., MATHEWS, J. H., MILLER, B. R., MEDLEN, A. R., LEDGER, T. N. & ROEHRIG, J. T. (1990). Identification of monoclonal antibodies that distinguish between 17D-204 and other strains of yellow fever virus. *Journal of General Virology* **71**, 13–18.
- BONNER, W. M. & LASKEY, R. A. (1974). A film detection method for tritium-labelled protein and nucleic acid in polyacrylamide gels. *European Journal of Biochemistry* **46**, 83–88.
- BOWEN, G. S., MONATH, T. P., KEMP, G. E., KERSCHNER, J. K. & KIRK, L. J. (1980). Geographic variation among St. Louis encephalitis virus strains in the viremic responses of avian hosts. *American Journal of Tropical Medicine and Hygiene* **29**, 1411–1499.
- BRANDT, W. E., MCCOWN, J. M., GENTRY, M. K. & RUSSELL, P. K. (1982). Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants. *Infection and Immunity* **36**, 1036–1041.
- BRUGGE, J., ERICKSON, E., COLLETT, M. S. & ERICKSON, R. L. (1978). Peptide analysis of the transformation-specific antigen from avian sarcoma virus-transformed cells. *Journal of Virology* **26**, 773–782.
- CANE, P. A. & GOULD, E. A. (1989). Immunoblotting reveals differences in the accumulation of envelope protein by wild-type and vaccine strains of yellow fever virus. *Journal of General Virology* **70**, 557–564.
- CASH, P., HENDERSHOT, L. & BISHOP, D. H. L. (1980). The effects of glycosylation inhibitors on the maturation and intracellular polypeptide synthesis induced by snowshoe hare bunyavirus. *Virology* **103**, 235–240.
- CENTERS FOR DISEASE CONTROL (1990). St. Louis encephalitis – Florida and Texas, 1990. *Morbidity and Mortality Weekly Report* **39**, 756–759.
- CENTERS FOR DISEASE CONTROL (1991). St. Louis encephalitis outbreak – Arkansas, 1991. *Morbidity and Mortality Weekly Report* **40**, 605–607.
- CHAMBERLAIN, R. W. (1980). History of St. Louis encephalitis. In *St. Louis Encephalitis*, pp. 3–61. Edited by T. P. Monath. Washington, D.C.: American Public Health Association.
- CHATS, P. A. & MORRISON, T. G. (1981). Mutational changes in the vesicular stomatitis virus glycoprotein affect the requirement of carbohydrate in morphogenesis. *Journal of Virology* **37**, 37–316.
- CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *American Journal of Tropical Medicine and Hygiene* **7**, 561–573.
- COIA, G., PARKER, M. D., SPEIGHT, G., BYRNE, M. E. & WESTAWAY, E. G. (1988). Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. *Journal of General Virology* **69**, 1–21.
- DALGARNO, L., TRENT, D. W., STRAUSS, J. H. & RICE, C. M. (1986). Partial nucleotide sequence of Murray Valley encephalitis virus genome. *Journal of Molecular Biology* **187**, 309–323.
- DELWART, E. L. & PANGANIBAN, A. T. (1990). N-Linked glycosylation and reticuloendotheliosis retrovirus envelope glycoprotein function. *Virology* **197**, 648–657.
- DEUBEL, V., KINNEY, R. M. & TRENT, D. W. (1986). Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2, Jamaica genotype. *Virology* **155**, 365–377.
- DEUBEL, V., SCHLESINGER, J. J., DIGOUTTE, J.-P. & GIRARD, M. (1987). Comparative immunochemical and biological analysis of African and South American yellow fever viruses. *Archives of Virology* **94**, 331–338.
- DUKSEN, D. & MAHONEY, W. C. (1982). Relationship of the structural and biological activity of the natural homologues of tunicamycin. *Journal of Biological Chemistry* **257**, 3105–3109.
- GENTRY, M. K., HENCHAL, E. A., MCCOWN, J. M., BRANDT, W. E. & DALRYMPLE, J. M. (1982). Identification of distinct antigenic determinants on dengue 2 virus using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* **31**, 548–555.
- GIBSON, R., SCHLESINGER, S. & KORNFIELD, S. (1979). The non-glycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *Journal of Biological Chemistry* **254**, 3600–3607.
- GRUENBERG, A., WOO, W. S., BIEDRZYCKA, A. & WRIGHT, P. J. (1988). Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PuO-218 strains. *Journal of General Virology* **69**, 1391–1398.
- GUIRAKHOO, F., HEINZ, F. X. & KUNZ, C. (1989). Epitope model of tick-borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain, and conformational changes occurring at acidic pH. *Virology* **169**, 90–99.
- HAHN, Y. S., GALLER, R., HUNKAPILLAR, T., DALRYMPLE, J. M., STRAUSS, J. H. & STRAUSS, E. G. (1988). Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* **162**, 167–180.
- HEINZ, F. X. & ROEHRIG, J. T. (1990). Flaviviruses. In *Immunochimistry of Viruses II. The Basis for Serodiagnosis and Vaccines*, pp. 289–305. Edited by M. H. V. Van Regenmortel & A. R. Neurath. Amsterdam: Elsevier.
- HENCHAL, E. A., GENTRY, M. K., MCCOWN, J. M. & BRANDT, W. E. (1982). Dengue virus-specific and flavivirus group determinants identified by immunofluorescence with monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* **31**, 830–836.
- JOHNSON, B. J. B., KINNEY, R. M., KOST, C. L. & TRENT, D. W. (1986). Molecular determinants of alphavirus neurovirulence: nucleotide and deduced protein sequence changes during attenuation of Venezuelan equine encephalitis virus. *Journal of General Virology* **67**, 1951–1960.
- KALUZA, G., ROTT, R. & SCHWARZ, R. T. (1980). Carbohydrate-induced conformational changes of Semliki Forest virus glycoproteins determine antigenicity. *Virology* **102**, 286–299.
- KARABATSOS, N. (1980). General characteristics and antigenic relationships. In *St. Louis Encephalitis*, pp. 105–158. Edited by T. P. Monath. Washington, D.C.: American Public Health Association.
- KARABATSOS, N. (1985). *International Catalogue of Arboviruses and Certain other Viruses of Vertebrates*, 3rd edn. San Antonio: American Society of Tropical Medicine and Hygiene.
- KINNEY, R. M. & TRENT, D. W. (1982). Conservation of tryptic peptides in the structural proteins of viruses in the Venezuelan equine encephalitis complex. *Virology* **121**, 345–362.
- KNIPE, D. M., LODISH, H. F. & BALTIMORE, D. J. (1977). Localization of two cellular forms of vesicular stomatitis viral glycoprotein. *Journal of Virology* **21**, 1121–1127.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAMBRIS, J., PAPAMICHAIL, M. & FESSAS, P. (1979). Fluorescent labelling of proteins of lymphocyte plasma membranes. *Journal of Immunological Methods* **27**, 55–59.
- MCADA, P. C., MASON, P. W., SCHMALJOHN, C. S., DALRYMPLE, T. M., MASON, T. L. & FOURNIER, M. J. (1987). Partial nucleotide sequence of Japanese encephalitis virus genome. *Virology* **158**, 348–360.
- MANDL, C., HEINZ, F. & KUNZ, C. (1988). Sequence of the structural proteins of the tick-borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. *Virology* **166**, 197–205.
- MASON, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* **169**, 354–364.

- MASON, P. W., MCADA, P. C., MASON, T. L. & FOURNIER, M. J. (1987). Sequence of the dengue 1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. *Virology* **161**, 262–267.
- MATHEWS, J. H. & ROEHRIG, J. T. (1984). Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of St. Louis encephalitis virus by passive transfer with monoclonal antibodies. *Journal of Immunology* **132**, 1533–1537.
- MATHEWS, J. H., ROEHRIG, J. T. & TRENT, D. W. (1985). Role of complement and the Fc portion of immunoglobulin G in immunity to Venezuelan equine encephalomyelitis virus infection with glycoprotein-specific monoclonal antibodies. *Journal of Virology* **55**, 594–600.
- MONATH, T. P. (1980). Epidemiology. In *St. Louis Encephalitis*, pp. 239–312. Edited by T. P. Monath. Washington, D.C.: American Public Health Association.
- MONATH, T. P. (1990). Flaviviruses. In *Virology*, 2nd edn, pp. 955–1004. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.
- MONATH, T. P., CROPP, C. B., BOWEN, G. S., KEMP, G. E., MITCHELL, C. J. & GARDNER, J. J. (1980). Variation in virulence for mice and rhesus monkeys among St. Louis encephalitis strains of different origin. *American Journal of Tropical Medicine and Hygiene* **29**, 948–962.
- OSATOMI, K. & SUMIYOSHI, H. (1990). Complete nucleotide sequence of dengue type 3 virus genome RNA. *Virology* **176**, 643–647.
- OSATOMI, K., FUKU, I., TSURU, D., SHIBA, T., SAKAKI, Y. & SUMIYOSHI, H. (1988). Nucleotide sequence of dengue type 3 virus genome RNA encoding viral structural proteins. *Virus Genes* **2**, 199–208.
- PEIRIS, J. S. M., PORTERFIELD, J. S. & ROEHRIG, J. T. (1982). Monoclonal antibodies against the flavivirus West Nile. *Journal of General Virology* **58**, 283–289.
- RICE, C. M., LENCHER, E. M., EDDY, S. R., SHIN, S. J., SHEETS, R. L. & STRAUSS, J. H. (1985). Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**, 726–733.
- ROEHRIG, J. T. (1986). The use of monoclonal antibodies in studies of the structural proteins of togaviruses and flaviviruses. In *The Togaviridae and Flaviviridae*, pp. 251–278. Edited by S. Schlesinger & M. Schlesinger. New York: Plenum Press.
- ROEHRIG, J. T., MATHEWS, J. H. & TRENT, D. W. (1983). Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology* **128**, 118–126.
- SUMIYOSHI, H., MORI, C., FUKU, I., MORITA, K., KIHARA, S., KONDOU, J., KIKUCHI, Y., NAGARMATU, H. & IGARASHI, A. (1987). Complete nucleotide sequence of Japanese encephalitis virus genome RNA. *Virology* **161**, 497–510.
- TRENT, D. W. (1977). Antigenic characterization of flavivirus structural proteins separated by isoelectric focusing. *Journal of Virology* **22**, 608–614.
- TRENT, D. W. & GRANT, J. A. (1980). A comparison of New World alphaviruses in the western equine encephalitis complex by immunochemical and oligonucleotide fingerprint techniques. *Journal of General Virology* **47**, 261–282.
- TRENT, D. W., KINNEY, R. M., JOHNSON, B. J., VORNDAM, A. V., GRANT, J. A., DEUBEL, V., RICE, C. M. & HAHN, C. (1987). Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a, and ns2b. *Virology* **156**, 293–304.
- VIDAL, S., MOTTET, G., KOLAKOSKY, D. & ROUX, L. (1989). Addition of high-mannose sugars must precede disulfide bond formation for proper folding of Sendai virus glycoproteins. *Journal of Virology* **63**, 892–900.
- WENGLER, G., CASTLE, E., LEIDNER, U., NOWAK, T. & WENGLER, G. (1985). Sequence analysis of the membrane protein V3 of the flavivirus West Nile virus and of its gene. *Virology* **147**, 264–274.
- WESTAWAY, E. G., MCKIMM, R. W. & MCLEOD, L. G. (1977). Heterogeneity among flavivirus proteins separated by slab gels. *Archives of Virology* **53**, 305–312.
- WESTAWAY, E. G., BRINTON, M. A., GAIDAMOVICH, S. YA., HORZINEK, M. C., IGARASHI, A., KÄÄRIÄINEN, L., LVOV, K. D., PORTERFIELD, J. S., RUSSELL, P. K. & TRENT, D. W. (1985). Flaviviridae. *Inter-virology* **24**, 183–192.
- WINKLER, G., HEINZ, F. X. & KUNZ, C. (1987). Studies on the glycosylation of flavivirus E proteins and the role of carbohydrate in the antigenic structure. *Virology* **159**, 237–243.
- ZHAO, B., MACKOW, E., BUCKLER-WHITE, A., MARKOFF, L., CHANOCK, R. M., LAI, C. J. & MAKINO, M. (1986). Cloning full length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. *Virology* **155**, 77–88.

(Received 25 November 1992; Accepted 15 July 1993)