Homogeneity of the Structural Glycoprotein from European Isolates of Tick-borne Encephalitis Virus: Comparison with Other Flaviviruses

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(Accepted 21 July 1981)

SUMMARY

Isolates of tick-borne encephalitis (TBE) virus from Finland, Germany, Czechoslovakia, Switzerland and Austria were compared with strains of the Far Eastern subtype isolated in Russia as well as Louping ill virus and other flaviviruses belonging to a different serocomplex: West Nile, Murray Valley encephalitis and Rocio viruses. Analysis of the structural polypeptides by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed identical mol. wt. of the glycoprotein E (mol. wt. 55 000) and the core protein C (mol. wt. 15 000) for all the TBE virus strains analysed. However, the small envelope protein M from viruses isolated in Germany, Switzerland and Austria migrated slightly slower (apparent mol. wt. 7500) compared to M from viruses isolated in Finland, Czechoslovakia or the Far Eastern subtype strains (apparent mol. wt. 6500 to 7000). The structural glycoproteins were isolated from purified [35S]methionine-labelled virions and subjected to peptide mapping by limited proteolysis with α-chymotrypsin or V8 protease followed by SDS-PAGE of the resulting cleavage products. With both proteases a remarkably homogeneous pattern was obtained for all the European isolates with only very minor deviations from the common pattern in single cases. Similar but distinguishable patterns were obtained for the Far Eastern subtype strains and also Louping ill virus, which, in addition, differed in the mol. wt. of its core protein C (mol. wt. 16 000) and the small membrane protein M (mol. wt. 9000). These almost identical peptide maps observed with the TBE virus strains were in sharp contrast to the unrelated patterns obtained with the glycoproteins from West Nile, Murray Valley encephalitis and Rocio viruses. Although these viruses are serologically closely related and members of the same serocomplex of flaviviruses their glycoprotein peptide maps were completely different from one another. In a competitive radioimmunoassay all European TBE virus isolates showed identical immunological reactivity which further points to the great stability of this type of virus.

INTRODUCTION

Based on serological cross-reactions the flavivirus genus of togaviruses can be subdivided into several complexes (or subgroups) each containing a number of viruses or types (Porterfield, 1980; De Madrid & Porterfield, 1974). As also pointed out by Trent (1977) the different patterns of cross-reactions between flaviviruses may be explained by the presence of type-specific, complex reactive and group reactive antigenic determinants on the surface of the virus. One of these complexes is represented by the tick-borne encephalitis (TBE) complex comprising TBE, Langat, Negishi, Omsk Haemorrhagic fever, Kyasanur Forest disease and Louping ill viruses. Another tick-borne virus, Powassan, was found to be more closely related to viruses of the TBE complex than to other flaviviruses. It could, however, be clearly separated from the other members of the complex (Clarke, 1962) and in the study performed...
by De Madrid & Porterfield (1974) it did not cross-react with any other flavivirus in a micro-neutralization test. Negishi, Louping ill and TBE virus are very closely related and difficult to differentiate.

Using agar gel diffusion and antibody absorption experiments, individual strains of TBE virus could be further assigned to two subtypes (Clarke, 1964): the western subtype which occurs in Europe and is mainly transmitted by *Ixodes ricinus* and the Far Eastern subtype, which is prevalent in the eastern part of U.S.S.R. with *Ixodes persulcatus* as its main vector. However, the two subtypes overlap in the western U.S.S.R. and Far Eastern strains have also been isolated from *I. ricinus*. With the exception of the British Isles, Spain, Portugal and the Benelux countries the western subtype of TBE virus has been demonstrated to occur in every country of Europe where it represents by far the most important arthropod-transmitted virus causing human disease. In countries with endemic areas like Austria it represents a major public health problem due to several hundred hospitalized cases recorded per year. Immunization with a potent vaccine containing purified inactivated virus represents an effective and well-tolerated prophylactic means against the disease (Kunz et al., 1980a, b; Heinz et al., 1980).

Similar to other flaviviruses TBE virus contains only three structural proteins (*V*$_3$, *V*$_2$, *V*$_1$) (Heinz & Kunz, 1980) which are now designated E, C and M according to a recently introduced nomenclature of flavivirus proteins (Westaway et al., 1980). C is the only protein constituent of the virus core. The glycoprotein E and the non-glycosylated M together with lipid form the virus envelope.

Comparing the structural polypeptides of several flaviviruses, Shapiro et al. (1972) observed a slower migration rate in SDS–PAGE of *V*$_1$ (now designated M) from tick-borne compared to mosquito-borne flaviviruses and the authors suggested that this difference probably plays a role in determining host range. Another comparative study done by Westaway et al. (1977) revealed a strong heterogeneity among mol. wt. of E proteins from different flaviviruses which could, however, not be correlated with serological relationships.

By the isolation of biochemically defined subunits from TBE virus (Heinz & Kunz, 1980) and their immunological characterization (Heinz et al., 1981), it could be clearly established that experimental animals can be protected by immunization with preparations containing exclusively E and that corresponding antisera are able to neutralize the virus and passively protect animals from the disease. No protection could be conferred by the core or core antiserum. Therefore, the virus glycoprotein represents the most important antigenic determinant with respect to immunity against the disease.

As shown by Trent (1977) for a number of flaviviruses, the surface glycoprotein is also the most variable protein constituent leading to the establishment of distinguishable serogroups, whereas the core protein is not subject to variations and carries only group-reactive antigenic determinants. As antigenic variations depending on geographic distributions are not uncommon with other viruses we started an investigation to compare TBE virus strains isolated from ticks or vertebrates in different European countries in different years by the analysis of their structural polypeptides, peptide mapping of the virus glycoprotein and competitive radioimmunoassays of purified virions.

**METHODS**

**Virus strains.** All the strains of the western and eastern subtype of TBE virus as well as the other flaviviruses included in this study are listed in Table 1. The two isolates from Finland (Brummer-Korvenkontio et al., 1973) were provided by Dr M. Brummer-Korvenkontio, Institute of Virology, Helsinki, Finland. Dr R. Ackerman from the Neurological-Clinic, University of Cologne, F.R.G., sent us strain K-23 which he had isolated from a tick in
Comparison of TBE virus strains

Table 1. Strains of tick-borne encephalitis virus and other flaviviruses analysed

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>Strain designation</th>
<th>Source of isolation</th>
<th>Year of isolation</th>
<th>Number of mouse brain passages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TBE virus: western subtype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finland: Kumlinge island</td>
<td>A-52</td>
<td><em>Ixodes ricinus</em></td>
<td>1959</td>
<td>Unknown</td>
</tr>
<tr>
<td>Southeast Finland</td>
<td>S-10-12</td>
<td><em>Ixodes ricinus</em></td>
<td>1960</td>
<td>Unknown</td>
</tr>
<tr>
<td>West Germany: Karlsruhe</td>
<td>K-23</td>
<td><em>Ixodes ricinus</em></td>
<td>1975</td>
<td>4</td>
</tr>
<tr>
<td>Czechoslovakia:</td>
<td>Hypr</td>
<td>Human blood</td>
<td>1953</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Switzerland:</td>
<td>I-40</td>
<td><em>Ixodes ricinus</em></td>
<td>1972</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>I-Horse</td>
<td>Horse brain</td>
<td>1979</td>
<td>2</td>
</tr>
<tr>
<td>Austria: Burgenland</td>
<td>Neudörfi</td>
<td><em>Ixodes ricinus</em></td>
<td>1971</td>
<td>3</td>
</tr>
<tr>
<td>Lower Austria</td>
<td>St. Pölten</td>
<td><em>Ixodes ricinus</em></td>
<td>1971</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hochosterwitz</td>
<td><em>Ixodes ricinus</em></td>
<td>1971</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Scharl</td>
<td>Human brain</td>
<td>1956</td>
<td>3</td>
</tr>
<tr>
<td><strong>TBE virus: Far Eastern subtype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U.S.S.R.</td>
<td>Sofyn</td>
<td>Human brain</td>
<td>1937</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Bars</td>
<td>Human brain</td>
<td>1961</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Other flaviviruses

- Louping ill virus (strain Moredun: Scotland)
- West Nile virus (isolate from Nigeria; see Wengler et al., 1979)
- Murray Valley encephalitis virus (prototype of Yale Arbovirus Research Unit, New Haven, Conn., U.S.A.)
- Rocío virus (isolate from Brazil: H-34675)

Karlsruhe. Swiss isolates were obtained from Dr R. Wyler, Institute of Virology, University of Zürich, Switzerland (Wyler et al., 1973). Strains Neudörfi, St. Pölten and Hochosterwitz from Austria were isolated by Dr Ch. Kunz and plaque-purified in primary chick embryo cells by Dr J. Keppie, Microbiological Research Establishment, Porton Down, U.K. Strain Hypr from Czechoslovakia represented a high mouse brain passage of the original virus isolated in 1953 from the blood of a patient bitten by a tick in Brno, Moravia, and was obtained from the Institute of Virology, Slovak Academy of Sciences, Bratislava. The Far Eastern TBE subtype strain Sofyn, Louping ill virus and Murray Valley encephalitis virus were provided by Dr D. Clarke (The Rockefeller Foundation Virus Laboratories, New York, U.S.A.) and the second Far Eastern strain Bars by Dr M. P. Chumakov (Institute of Poliomyelitis and Viral Encephalitides, Moscow, U.S.S.R.). A plaque-purified stock of West Nile virus was obtained by Dr G. Wengler (Institute of Virology, University of Giessen, F.R.G.) and Rocío virus was sent by Dr Veronesi (Hospital das Clinicas, Faculdade de Medicina, Sao Paulo, Brazil).

Propagation and purification of the viruses. Stocks from the viruses listed in Table 1 were prepared as 10% (w/w) suspensions of infected suckling mouse brain which were used for the infection of primary chick embryo cell monolayers maintained in minimum essential medium (MEM) buffered with 15 mM-HEPES and 15 mM-EPPS at pH 7-6. After 40 h the supernatant was clarified at 10,000 g for 30 min at 4 °C and the virus was pelleted by ultracentrifugation at 50,000 g for 3 h at 4 °C. The virus was then resuspended in an appropriate volume of TAN buffer (0-02 M-triethanolamine, 0-13 M-NaCl, pH 8) and subjected to rate-zonal centrifugation in a 5 to 20% (w/w) sucrose density gradient (3 × 23 ml swinging-bucket rotor, MSE, 27000 rev/min, 4 °C, 110 min). The virus peak was located by scanning the gradient at 254 nm and subjected to equilibrium density gradient centrifugation in a 20 to 50% (w/w) sucrose gradient for 18 h at 4 °C in the same rotor. The virus peak was then dialysed against TAN pH 8 to remove excess sucrose and stored at −80 °C. The high purity of these virus preparations was verified by SDS-PAGE in the buffer system of Laemmli & Favre (1973). For the preparation of radioactively labelled virus, [35S]methionine (SJ. 204; Amersham International) at a final concentration of 5 μCi/ml was
added to a methionine-deficient medium containing 1 mg/ml bovine serum albumin (BSA). Purification was performed as described above omitting the equilibrium density gradient centrifugation step.

Isolation of the virus glycoprotein. Radioactively labelled purified viruses containing 0.5 mg/ml BSA (Serva, Heidelberg, F.R.G.) were treated with 1% Triton X-100 at room temperature for 1 h in a vol. of 0.5 ml. The sample was then layered over a 1.5 ml cushion of 40% (w/w) sucrose in TAN pH 8 and centrifuged for 3 h at 38000 rev/min at 4 °C in a 3 x 5 ml swinging-bucket rotor (MSE). As shown by SDS-PAGE this procedure results in a clean separation of the solubilized envelope proteins E and M (remaining in the solution above the cushion) from the intact virus core which can be recovered from the pellet.

Limited proteolysis of the glycoprotein. This was performed by the procedure described by Cleveland et al. (1977) for purified proteins. The envelope proteins containing 0.5% BSA were prepared as described above and then precipitated by the addition of TCA at 10% final concentration. After 30 min in an ice-bath the precipitate was collected by centrifugation at 3000 g for 5 min, washed twice in ice-cold acetone, dried and then solubilized by boiling for 3 min in 0.125 m-tris pH 6.8 containing 0.5% SDS and 10% glycerol. After cooling, a-chymotrypsin (from bovine pancreas, 45 units/mg, 17160 Serva) or V8 protease from Staphylococcus aureus (Miles Laboratories Ltd., Stoke Poges, Bucks., U.K.) were added to final concentrations of 400 ~g/ml and 1 mg/ml respectively and incubated for 30 min at 37 °C. The samples were then 2% with respect to SDS and 4% with respect to 2-mercaptoethanol and subjected to SDS-PAGE in 17 % gels according to Laemmli & Favre (1973). The gels were dried without further treatment and then subjected to autoradiography using Kodak X-Omat S film.

Competitive radioimmunoassay (RIA)

Antibody titration. One-hundred ~l of RIA buffer (0.02 m-tris, 0.13 m-NaCl, 0.5% BSA, 0.02% NaN₃, pH 7.5) were mock-reacted for 1 h at 37 °C with 50 ~l of various dilutions of a rabbit immune serum against live TBE virus (strain Neudörfl). Then, 50 ~l of purified TBE virus labelled with the Bolton–Hunter reagent (Amersham International) containing 5000 ct/min were added and incubated overnight at 4 °C. For the separation of bound from free label, 50 ~l of immunobeads (Bio-Rad) coated with goat anti-rabbit immunoglobulins were added according to the manufacturers instructions and incubated for 2 h at 37 °C. The beads were then pelleted by centrifugation at 2000 g for 5 min at room temperature and washed three times in RIA buffer before being counted. From the antibody titration curve obtained the dilution which resulted in 50% of the maximal binding was determined to be 1 in 100000.

Competitive test. One-hundred ~l of purified virus diluted in 10-fold steps in RIA buffer from 20 ~g/ml to 0.2 ng/ml were incubated for 1 h at 37 °C with 50 ~l antiserum (diluted 1 in 100000 to give 50% of maximal binding as determined by antibody titration). Then, exactly the same procedure was followed as described above for antibody titration.

Protein determination. Protein concentration were determined by a dye-binding assay after TCA precipitation according to Schaffner & Weissmann (1973) using BSA as a standard.

Preparation of immune serum. One ml of purified TBE virus at a protein concentration of 200 ~g/ml was emulsified in complete Freund's adjuvant and injected subcutaneously into a rabbit. After 4 and 8 weeks respectively, two more booster doses were given using the same virus preparation emulsified in incomplete Freund's adjuvant. Blood was taken after a further 2 weeks by ear vein puncture and the serum was stored at –20 °C.

RESULTS

Analysis of the structural polypeptides

Table 1 lists the strains of TBE virus (western and eastern subtype) isolated in different countries, and in addition to these, also the closely related Louping ill virus and flaviviruses
Comparison of TBE virus strains

not belonging to the TBE complex (West Nile, Murray Valley encephalitis (MVE) and Rocio viruses) which were included in the present study. The comparative analysis of the structural polypeptides of [35S]methionine-labelled viruses is shown in Fig. 1. The mol. wt. of the Austrian prototype strain (Neudörfl) determined on several different gels using bovine and ovine albumin, chymotrypsinogen, myoglobin, cytochrome c and aprotinin as markers were 55,000 for E, 15,000 for C and 7,500 for M. The glycoprotein (E) and the core protein (C) from all the other TBE virus strains and also those from the Far Eastern subtype (Sofyn) showed identical migration rates. However, the strains could be subdivided into two groups based on the mobility of the small envelope protein M which migrated definitely faster in the Far Eastern subtype as well as strains isolated in Finland and Czechoslovakia (estimated mol. wt. 6,500 to 7,000) compared to those from Austria, Germany and Switzerland (estimated mol. wt. 7,500).

Louping ill virus which serologically is very closely related and difficult to differentiate from TBE virus could easily be distinguished by its different polypeptide pattern. Although there was no apparent difference in the mol. wt. of the glycoprotein, both the core protein C as well as the small envelope protein M migrated definitely slower in the present gel system revealing mol. wt. of 16,000 and 9,000 respectively. The patterns of West Nile (49,000; 15,000; 7,000) and MVE viruses (56,000; 16,000; 7,000) which are grouped together into one serocomplex document the heterogeneity of polypeptide patterns which may exist between flaviviruses of the same serogroup (Westaway et al., 1977).
Peptide mapping of the envelope glycoprotein

The single glycoprotein of flaviviruses carries those antigenic determinants which induce protective immunity in the infected or immunized host. The identical migration rates of E from all the TBE virus strains tested do not necessarily mean complete identity of the native protein. For a more sensitive analysis we therefore isolated this glycoprotein and subjected it to peptide mapping by limited proteolysis using either α-chymotrypsin or V8 protease from *S. aureus* according to Cleveland *et al.* (1977). Before proteolysis the glycoproteins were boiled in the presence of SDS in order to convert them into a highly extended form, thus ensuring a maximum of potential cleavage sites being exposed to the action of the proteases. The procedure for the isolation of the glycoprotein from purified virus using Triton X-100 is fully described in Methods. As Triton X-100 solubilizes both envelope proteins also M is present in the starting material used for peptide mapping (Fig. 2). In addition to the viruses and strains shown in Fig. 1, a second strain of the Far Eastern subtype of TBE virus, termed Bars, and another mosquito-borne flavivirus, Rocio virus from Brazil, were included in this comparison. As in Fig. 1 again identical migration rates of E and the two M protein mobility groups of the TBE virus strains are discernible as well as the heterogeneity of E and M in the mosquito-borne flaviviruses.
Comparison of TBE virus strains

The mol. wt. range between E (55,000) and M (7,500) is sufficiently large for the separation of fragments generated by the proteases and their clear identification as derived only from E. In preliminary experiments the conditions of protease treatment were adjusted so that a maximum of different proteolytic fragments (with a detectable residual amount of undegraded E) was present between the mol. wt. range of 55,000 and 7,500. The pattern of fragments obtained with α-chymotrypsin, which predominately cleaves at tyrosine, phenylalanine and tryptophan is shown in Fig. 3. For all the TBE virus isolates from different European countries tested a remarkably identical pattern of cleavage fragments was obtained. Only strain 2 from Austria (St. Pölten) revealed a single significantly more intensive band at mol. wt. 30,000 which was highly reproducible with different virus preparations and could also be observed in stained gels. Again, the above mentioned mobility differences of M were apparent. The two Far Eastern (Russian) subtypes Sofyn and Bars had a very similar but distinguishable pattern from that of their western relatives in that the double band at mol. wt. approx. 20,000 migrated definitely faster in these strains. The bands obtained from the other closely related virus, Louping ill virus, were also very similar; however, a slight difference existed in the fragments at mol. wt. 40,000 and 35,000 and also in the band at mol. wt. 23,000, which was lacking. The excellent discriminating power of this method is exemplified by the
completely unrelated patterns obtained for the mosquito-borne flaviviruses West Nile, MVE and Rocio despite their grouping into the same serogroup due to a close serological relationship.

The potential of this mapping procedure can be amplified by the use of a second protease with different cleavage specificity. We used V8 protease from *S. aureus* which cleaves at aspartic and glutamic acid (Fig. 4). Similar to the results obtained with α-chymotrypsin the bands obtained from the western subtype isolates were identical, some probable minor aberrations showing up in the mol. wt. region 10000 to 14000 of the German and Czechoslovakian strain. Using this protease the Far Eastern strains could be differentiated from the western ones only by slight differences at mol. wt. 25000. Also, Louping ill virus again was very similar but the double band of the largest cleavage product (mol. wt. 40000) migrated definitely faster than its counterpart from TBE strains. Practically no identical bands were seen in the E fragments obtained from West Nile, MVE and Rocio viruses.

**Immunological comparison by competitive RIA**

The immunological comparison was based on a competitive RIA in a homologous antigen–antiserum system and consisted of the reaction between 125I-labelled purified virus
Comparison of TBE virus strains

Fig. 5. Immunological comparison of different TBE virus isolates and other flaviviruses by competitive RIA as described in Methods. (a) Labelling by the chloramine-T method; (b) labelling with the Bolton–Hunter reagent. , All the TBE virus isolates (western subtype) listed in Table 1; ■, eastern subtype strain Sofyn; ▲, Louping ill virus; ▼, West Nile virus; □, MVE virus.

(Austrian strain Neudörfl) and its homologous rabbit antiserum. This interaction was competed with complete purified virus derived from the different isolates and virus types. Complete virus was used as competitor in order to analyse the antigenic structure of E in its most native conformation as it is present on the surface of the virion. Therefore, no detergent was used in the RIA buffer. It cannot be excluded that the labelling procedure by itself modifies certain antigenic determinants and thereby may obscure antigenic differences existing in the competing virus. To circumvent this potential difficulty we employed two different iodination procedures: (i) the chloramine-T method by which iodine is transferred predominantly to tyrosine residues and (ii) labelling by the Bolton–Hunter reagent which reacts with free lysine and terminal amino groups. The results of these assays are presented in Fig. 5. Irrespective of whether the antigen was labelled with chloramine-T or the Bolton–Hunter reagent completely identical competition curves were obtained for all the TBE virus strains from different European countries and listed in Table 1. For the sake of clarity only one single curve has been drawn for all these strains. The Far Eastern subtype strain Sofyn and also Louping ill virus competed slightly less effectively and therefore could be differentiated from the western strains of TBE virus. Very little competition was obtained for the flaviviruses not belonging to the TBE complex, West Nile and MVE viruses. These data are consistent with the presence of type-specific, subgroup-specific and flavivirus group-reactive determinants on the surface of the virion.

DISCUSSION

A comparison of the polypeptide patterns of TBE virus strains isolated between 1956 and 1979 in various European countries revealed identical mol. wt. for the glycoprotein as well as the core protein. However, based on the mobility of the small envelope protein M in SDS gels the strains could be separated into two groups. Those from Austria, Switzerland and Germany with a calculated mol. wt. for M of 7500 and those from Finland and Czechoslovakia with mol. wt. of 6500 to 7000. This difference of mobility in SDS–PAGE does not necessarily indicate different mol. wt. since especially with small proteins and peptides the migration rate may not only be determined by the mol. wt. but also by the charge and SDS-binding capacity of the protein itself (Swank & Munkres, 1975). Furthermore, single amino acid substitutions may also change the migration of proteins in SDS–PAGE (De Jong et al., 1978). Nevertheless, as a convention the present results will be expressed as different calculated mol. wt.: 7500 versus 6500.

The members of the eastern subtype (Sofyn and Bars), which previously have been shown to be serologically distinguishable from the western subtype, also carry the faster migrating M
similar to the Finnish and Czechoslovakian isolates of the western subtype. The other closely related virus, Louping ill, has a clearly distinguishable polypeptide pattern. Although E has the same apparent mol. wt. both the core protein C and M migrate definitively slower with mol. wt. of 16,000 and 9000. This finding clearly separates Louping ill from TBE virus. The mosquito-borne flaviviruses (West Nile, MVE and Rocio) showed the previously reported mol. wt. heterogeneity of the structural proteins of flaviviruses (Westaway et al., 1977).

Based on data obtained by co-electrophoresis of viruses in a phosphate-buffered SDS gel system, Shapiro et al. (1972) discussed a possible distinction between mosquito-borne and tick-borne flaviviruses by a slower migration of V₁ (now designated M) from tick-borne viruses compared to that from mosquito-borne viruses. This property has been proposed to play a role in defining host range and thus being a biochemical marker for biological differences. Based on our results obtained in a discontinuous buffer system we would however suggest that this generalization should not be made, since we observed variations with respect to mobility in both virus groups, and at least with TBE viruses the mobility of M also seems to be a strain-specific, probably geographically defined, marker. However, as already mentioned, the migration behaviour of such small proteins may be influenced by factors other than mol. wt. and may also be different in gels using continuous versus discontinuous buffer systems.

The pattern of polypeptides and their mol. wt. are only a very crude measure for the comparison of virus strains. One of the most powerful techniques for the comparative analysis of RNA viruses with respect to variability at the genome level is oligonucleotide fingerprinting of the virus RNAs. As with other viruses, serological distinctiveness between flaviviruses is reflected by differences in oligonucleotide fingerprints as shown by Vezza et al. (1980) for the four serotypes of dengue virus. In addition, the RNA of serologically indistinguishable viruses may show different oligonucleotide fingerprints and Trent et al. (1979) established a genetic classification of Saint Louis encephalitis virus isolates based on similarities of the RNase T₁-resistant oligonucleotides, which could be correlated with area of isolation, epidemiological parameters and virulence for mice.

Flaviviruses contain a single 42S RNA species which codes for all the virus structural and non-structural proteins (for review, see Russell et al., 1980) and it is therefore not possible to assign differences in oligonucleotide fingerprints to individual proteins. In the present study we were primarily interested in whether variations occur in the virus structural glycoprotein, since this is the major and most likely single determinant for the induction of protective immunity in the infected host. Furthermore, the surface glycoprotein seems to be subject to the highest selective pressure in the wild and variations are expected to occur first of all in this protein as it is also characteristic for the glycoproteins of influenza virus (Webster & Laver, 1975), paramyxoviruses (Hall et al., 1980; Nagai et al., 1980) or α-viruses (France et al., 1979). Non-glycosylated internal proteins on the other hand seem to conserve a remarkable structural homology between strains differing in surface glycoproteins, a situation also found with mosquito-borne flaviviruses, in which serological distinctiveness in the glycoprotein and even subtype variations contrast with the presence of equal amounts of only group-reactive determinants on the internal core protein (Trent, 1977).

We therefore decided to analyse the glycoproteins isolated from purified virions by limited proteolysis according to Cleveland et al. (1977) with proteases of different cleavage specificities. As this method was applied to the polypeptide denatured by SDS, differences in primary structure should be revealed irrespective of an external or internal location on the native protein. Completely unrelated peptide maps were obtained for TBE, West Nile, MVE and Rocio viruses. This excellent discriminating power is even more impressive if one takes into account that, based on a close serological relationship, West Nile MVE and at least provisionally also Rocio virus (Karabatsos, 1978) are grouped together into the same
serocomplex of flaviviruses. In sharp contrast to this distinctiveness were the uniform patterns obtained for the TBE virus isolates from all over Europe and only in single cases (e.g. Austrian strain St. Pölten) slight deviations in an otherwise identical pattern were noted. In the Far Eastern subtype strains and in Louping ill viruses minor variations in the very similar cleavage patterns were observed. Because we used SDS-solubilized proteins these slight changes may indicate amino acid substitutions anywhere in the polypeptide chain at protease-sensitive sites. As native protein molecules seem to contain only a limited amount of antigenic determinants, each involving only six to eight amino acids located preferentially at very exposed sites (Crumpton, 1974; Atassi, 1980), immunological changes are expected to occur only by amino acid substitutions within these determinants or at other sites, provided that this exchange is accompanied by a conformational change involving active antigenic sites. For an immunological comparison in a competitive RIA we therefore used complete purified virions as competitors which carry the glycoprotein in its most native conformation as an integral part of the virus membrane. No difference in reactivity whatsoever could be found between the TBE virus isolates, not even in those cases where slight deviations in the partial proteolysis maps had been observed, which indicates that these changes did not influence immunologically active sites. Slightly weaker competition was observed for Louping ill virus and the eastern subtype virus. Thus, together with their distinguishable peptide patterns after limited proteolysis and the different mol. wt. of C and M found in Louping ill virus, we confirm their separation from the western subtype of TBE virus as distinct viruses.

Although the western subtype strains were isolated over a period of 25 years from different species in very distant geographic areas they proved to have a remarkable immunological and biochemical homology of their glycoprotein. It can therefore be concluded that TBE virus represents an extremely stable member of the flavivirus genus. This contrasts to a similar study performed with mosquito-borne flaviviruses. Trent (1977) compared isolates of Japanese encephalitis virus by solid-phase RIA and did find variations in the type-specific determinant which confirmed previous findings using antibody absorption and CF and HI tests (Okuno et al., 1968). Further investigations will be necessary to analyse whether stability on the one hand and variability on the other are general properties of tick-borne and mosquito-borne flaviviruses.

REFERENCES


(Received 12 May 1981)