Formation of Polymeric Glycoprotein Complexes from a Flavivirus: Tick-borne Encephalitis Virus

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SUMMARY

Treatment of tick-borne encephalitis (TBE) virus with Triton X-100 (TX-100), octylglucoside (OG) or cetyltrimethylammonium bromide (CTAB) caused dissociation of the virus envelope into dimers or monomers of the glycoprotein V₃. By centrifugation into detergent-free sucrose density gradients, these subunits were found to reassociate and to form haemagglutinating homogeneous glycoprotein complexes sedimenting at 15 to 16, 16 to 18 and 11 to 12 S after TX-100, OG and CTAB treatment, respectively. Glycoprotein complexes obtained after TX-100 solubilization contained less than 1% lipid and detergent by weight.

INTRODUCTION

Tick-borne encephalitis (TBE) virus is a member of the flavivirus subgroup of togaviruses. Investigations with many serologically distinct flaviviruses have revealed that these viruses contain only three structural proteins: V₁, V₂, V₃, which show slight type-dependent variations in their mol. wt., ranging from 8000 to 9000 for V₁, 14000 to 16000 for V₂ and 50000 to 60000 for V₃ (Shapiro et al. 1972; Westaway, 1975; for review, see Schlesinger, 1977). The most recent data on the chemical composition of a flavivirus (Saint Louis encephalitis virus) are as follows: 8% RNA, 66% protein, 17% lipid and 9% carbohydrate (Porterfield et al. 1978). By the use of different solubilization and separation procedures V₂ was found to be located in the spherical core together with single-stranded RNA of messenger polarity. V₃ is the only structural glycoprotein, and together with V₁ and lipid, forms the lipoprotein envelope. The role of V₃ in the structure of flaviviruses is not yet completely clear since, depending on the detergent used, it has been reported to be recovered either in the fraction containing the cores or in the solubilized envelope (Westaway & Reedman, 1969; Trent & Qureshi, 1971). The virus glycoprotein V₃ is the most important antigen for protection from disease and induces neutralizing and haemagglutination inhibiting antibodies (Kitano et al. 1974; Della-Porta & Westaway, 1977; Heinz & Kunz, 1977; Trent, 1977). In a previous paper we have provided evidence that the TBE virus glycoprotein has a hydrophobic tail which is embedded in the lipid bilayer and therefore represents an amphiphilic protein (Heinz & Kunz, 1979).

One of the critical prerequisites determining the immunogenicity of proteins seems to be their presentation in a multimeric form (Unanue, 1972). This has also recently been demonstrated for exactly defined different physical forms of the amphiphilic membrane proteins of Semliki Forest virus (Morein et al. 1978). Octameric protein complexes and virosomes prepared from the envelope proteins were equally effective, whereas monomeric spikes resulted in much lower protection against challenge. In this paper, the reassociation of the envelope protein(s) of TBE virus into homogeneous multimeric protein complexes after solubilization with different detergents is described.
METHODS

Virus growth and purification. The TBE virus strain used throughout this study (termed Neudörfl) was isolated from a tick in Austria by intracerebral inoculation of SPF mice and has been cloned by Dr J. Keppie (Microbiological Research Establishment, Porton Down, Salisbury, Wilts.) in SPF chick embryo cells. A third mouse brain passage of this clone served as seed virus for the infection of chick embryo cells for virus propagation. Propagation of the virus in monolayers of primary chick embryo cells was performed as described by Heinz & Kunz (1977). Virus proteins were labelled by adding 5 µCi/ml ³H-leucine (L-4, 5-³H-leucine, 40 to 60 Ci/mmol) and ³H-lysine (L-4,5-³H-lysine, 15 to 40 Ci/mmol) immediately after infection to the maintenance medium lacking these amino acids. For labelling with ¹⁴C-choline, the growth medium was removed from the cells 3 h before infection and maintenance medium containing 1 µCi/ml ¹⁴C-choline [(methyl) ¹⁴C-choline chloride, 40 to 60 mCi/mmol] was added. For infection of cells, this medium was removed and added back after an adsorption period of 2 h. All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks. The virus harvested 40 h p.i. was concentrated by ultracentrifugation and purified by two cycles of sucrose density gradient centrifugation as described previously (Heinz & Kunz, 1977).

Detergent treatment and formation of protein complexes. Virus samples (200 µg/ml) in TAN (0.05 M-triethanolamine; 0.1 M-NaCl; pH 8.0) were treated with Triton X-100 (TX-100; Serva, Heidelberg, Germany), octylglucoside (OG; Calbiochem, Lahn, Germany) or cetyltrimethylammonium bromide (CTAB; Serva) for 1 h at 4 °C at concentrations indicated in Results and the figure legends. For reassociation of solubilized envelope proteins we used a similar method to that described by Simons et al. (1978) for the formation of water-soluble aggregates from amphiphilic membrane proteins. Samples of detergent-treated virus (0.6 ml) were layered on to sucrose density gradients in TAN, pH 8.0, which were composed of 12 ml 10 to 50 % (w/w) sucrose and a top layer of 0.3 ml 5 % (w/w) sucrose containing half the amount of detergent used for dissociation of the virus. Centrifugation was carried out in a 6 x 14 ml Ti-SW rotor (MSE, Crawley, Sussex) at 35,000 rev/min at 4 °C for 24 h. The gradients were fractionated by upward displacement using an ISCO model 640 density gradient fractionator with continuous monitoring of absorbance at 280 nm. For determination of radioactivity, aliquots from each density gradient fraction were prepared for liquid scintillation counting using Instagel from Packard Instruments. Spillover of ¹⁴C into the ³H channel was corrected by the use of the external standard method and previously established quench curves.

Haemagglutination (HA) titrations. HA activity of samples from the density gradients was determined at pH 6.4 by serial twofold dilutions in microtitre plates using goose erythrocytes according to Clarke & Casals (1958).

Chemical crosslinking. Samples of virus (200 µg/ml) treated with detergents (1 h at room temperature) or samples from density gradients (approx. 40 µg/ml) were treated with dimethylsuberimidate (DMS) at a final concentration of 12 mg/ml for 30 min at room temperature. Then the samples were precipitated by 10 % TCA at 0 °C, washed twice in ice-cold acetone and resuspended in sample buffer for SDS-PAGE analysis.

SDS-PAGE. Continuous SDS-PAGE in phosphate buffer, staining with Coomassie brilliant blue and destaining were done essentially as described by Maizel (1971).

Other methods. Protein concentrations were determined according to Schäffer & Weismann (1973) using bovine serum albumin as a standard. The sedimentation coefficients of the protein complexes were determined as described by Martin & Ames (1961), using human IgG and IgM as markers.
RESULTS

Formation of envelope protein complexes

TBE virus, double-labelled with $^{14}$C-choline and $^3$H-leucine-lysine was treated with TX-100, OG or CTAB at different concentrations for 1 h at room temperature and then subjected to centrifugation into a detergent-free sucrose density gradient as described in Methods (Fig. 1). After dissociation with 0.2 % TX-100 (detergent to protein ratio, 10:1) (Fig. 1a) the cores, containing $V_3$ as the only protein constituent (Fig. 2d) were recovered in the pellet. All the other $^3$H-leucine-lysine radioactivity was found in a single peak which exactly correlates with HA activity and represents a complex of both envelope proteins $V_3$ and $V_1$ (Fig. 2b). Most of the $^{14}$C-choline radioactivity remained on top of the gradient and the lipid contamination of the envelope protein complexes was determined (three determinations) to be 8 $\mu$g lipid per mg protein. This value has been confirmed by re-centrifugation of the isolated complexes. The specific activities of the virus preparations used for these determinations were 4030 $^3$H-d/min/$\mu$g protein and 2220 $^{14}$C-d/min/$\mu$g lipid. The calculations were based on the assumption that flaviviruses contain 17 % lipid and 66 % protein, as determined for Saint Louis encephalitis virus (Porterfield et al. 1978).

Using radioactively labelled TX-100 [phenyl-$^3$H(N)-, sp. act. 1'58 mCi/mg, New England Nuclear, Boston, Mass., U.S.A.], the TX-100 content of the protein complexes was estimated to be 7 $\mu$g TX-100 per mg protein (two determinations). Also, at higher TX-100 concentrations (1 %; Fig. 1b) both envelope proteins were recovered almost quantitatively in a single, HA-associated peak. Another mild non-ionic detergent, OG, yielded somewhat different results in that more than 10 times greater concentrations were necessary to obtain delipidated envelope protein complexes. Using 1 % OG (Fig. 1c), good separation of the envelope from the core occurred, but the envelope protein complexes, recovered in the middle of the gradients, contained almost half of the total $^{14}$C-choline counts and at 2 % OG a certain amount of lipid was still present in this peak. More complete delipidation was achieved at 5 % OG (Fig. 1d) but the lipid contamination was still approx. 0.03 mg lipid per mg protein (two determinations). Using 5 % OG, a second smaller but definite $^3$H peak was detected in fraction 2 which contained the small envelope protein $V_1$ exclusively (Fig. 2c). Therefore, in contrast to TX-100, delipidation of the protein complexes by OG also caused removal of $V_1$. Another interesting finding was that the peak of HA activity was always shifted slightly to the denser end of the protein peak. The cores obtained were not as pure as those obtained from the TX-100 gradients and contained small amounts of residual $V_3$ even at 5 % OG (Fig. 2e).

Treatment with 0.2 % of the cationic detergent CTAB caused almost complete dissociation of both the virus envelope and the core (Fig. 1f). Presumably due to denaturation, reassociation of $V_3$ into larger complexes cannot take place and, therefore, all the solubilized proteins, $V_3$, $V_2$ and $V_1$ are recovered from the top fractions of the gradient. When milder conditions were used (0.1 %; Fig. 1e) dissociation was incomplete and not as clear-cut as after TX-100 treatment. The ‘cores’ recovered from the pellet contained $V_3$ in addition to $V_3$ (Fig. 2f) but analysis of the top fractions of the gradient revealed the presence of all virus proteins including $V_3$, thus also indicating partial breakdown of the virus core.

Under these conditions an appreciable amount of $V_3$ was apparently present in a state that allowed reaggregation which resulted in the formation of a sharp HA-associated peak containing approx. 2 % lipid (two determinations). The only polypeptide detectable in this complex was $V_3$. After dissociation with TX-100 or OG, 15 to 25 % (and after CTAB treatment, 5 to 15 %) of the initial HA units applied were recovered from the gradients. As sodium deoxycholate is frequently used for membrane solubilization procedures and
Fig. 1. Formation of TBE virus glycoprotein complexes, after dissociation with different detergents, by centrifugation into detergent-free sucrose density gradients as described in Methods. (a) 0.2% TX-100; (b) 1% TX-100; (c) 1% OG; (d) 5% OG; (e) 0.1% CTAB; (f) 0.2% CTAB. — — . 3H-leucine-lysine; — — — , 14C-choline; , HA titre; , , 3H-leucine-lysine in pellet; , 14C-choline in pellet. Sedimentation is from left to right.
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has also been applied in studies with flaviviruses (Westaway & Reedman, 1969; Trent & Qureshi, 1971; Della-Porta & Westaway, 1977) this detergent was also used in our initial experiments. However, only very broad peaks of envelope protein aggregates were obtained and these, compared to the other detergents used, carried low or no HA activity. Deoxycholate, therefore, seemed to be less appropriate for the formation of defined and biologically active envelope protein complexes from TBE virus.

Chemical crosslinking of envelope protein complexes

The reassociation of glycoprotein molecules by the method described above from a monomorphic state (as they appear in the presence of different detergents) into a multimeric state could also be demonstrated by crosslinking with DMS followed by SDS-PAGE analysis. This is shown in Fig. 3. Crosslinking of untreated TBE virus results in a typical PAGE pattern, with decreasing amounts of V2 polymers and V3 polymers without evidence for the presence of heteropolymers. This has also been shown by two-dimensional SDS-PAGE analysis of protein complexes obtained with a cleavable crosslinker (Heinz & Kunz, 1980). In the presence of 1% TX-100 or 2 to 5% OG, the crosslinking pattern of V2 is unchanged (most of it is found as a high mol. wt. complex on top of the gel) indicating that the core is intact. Under the same conditions V3 dimers (and small amounts of higher

Fig. 2. SDS–PAGE in 7.5% gels of samples from the density gradients shown in Fig. 1. (a) Complete virus; (b) fractions 9 and 10 from Fig. 1(a); (c) fraction 2 from Fig. 1(d); (d) pellet from Fig. 1(a, b); (e) pellet from Fig. 1(d); (f) pellet from Fig. 1(e).
Fig. 3. SDS-PAGE in 4% gels after crosslinking virus samples in the presence of 1% TX-100, 5% OG, or 1% CTAB (c, e, g, respectively) and of glycoprotein complexes obtained after centrifugation into detergent-free sucrose density gradients (d, f, h, respectively) (two peak fractions from the density gradients were used for crosslinking after dialysis against TAN, pH 8.0). (a) Uncrosslinked virus; (b) crosslinked virus. Approx. 40 µg protein per slot was loaded and the gels were stained with Coomassie brilliant blue R-250.

Fig. 4. Sedimentation of TBE virus glycoprotein complexes in sucrose density gradients. These complexes were obtained as described in Results after dissociation with (a) TX-100; (b) OG; (c) CTAB. Sedimentation is from left to right. The gradients were composed of 5 to 20% (w/w) sucrose in TAN (pH 8.0) with a cushion of 50% (w/w) sucrose at the bottom of the tubes. Centrifugation was carried out in a 6 x 14 ml Ti-SW rotor (MSE) for 14 h at 30000 rev/min at 4°C.
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polymers) are present, indicating that even at high concentrations of these non-ionic detergents V₃ dimers are still stable. CTAB causes almost complete dissociation of the core and the envelope into monomers of all three structural proteins.

The reassociation of the virus glycoprotein by the use of detergent-free density gradients is evident from the crosslinking pattern of the delipidated protein complexes obtained (Fig. 3d, f, h). V₃ polymers similar to those seen in untreated virus are present (Fig. 3b). Only after dissociation with TX-100 is the smallest virus protein V₁ present in these aggregates, whereas it is left in the lipid and detergent-containing top layer after dissociation with 5% OG or 0.1% CTAB.

Sedimentation behaviour of envelope protein complexes

To test for the homogeneity of the protein complexes obtained, these were analysed by sedimentation in 5 to 20% sucrose density gradients (Fig. 4). The preparations obtained with TX-100, OG or CTAB showed very homogeneous sedimentation behaviour with S values around 15 to 16, 16 to 18 and 11 to 12, respectively. From the absence of radioactivity on top of the 50% sucrose cushion or in the pellet, it can be concluded that no further aggregation or precipitation had occurred.

DISCUSSION

Many different methods have been described for the isolation of immunologically active subunits from flaviviruses or flavivirus-infected cells (Kitano et al. 1974; Stohlman et al. 1976; Trent et al. 1976; Della-Porta & Westaway, 1977; Trent, 1977). The physical state of these antigens used for the characterization of their role in flavivirus immunology has been determined only occasionally. The tremendous difference between the immunogenicity of monomeric compared to multimeric protein complexes from Semliki Forest virus has been demonstrated by Morein et al. (1978) and there is at least one report on flaviviruses which is concerned with a similar problem. Using different DOC concentrations Della-Porta & Westaway (1977) prepared from Kunjin virus 'small envelope fragments' which the authors suggest to be no more than monomers or dimers of the envelope glycoprotein, and 'large envelope fragments' of 100 to 120S. Only the large complexes elicited a similar antibody response as complete virions whereas small fragments or a detergent-solubilized extract of infected cells were unable to stimulate a detectable antibody response. For further studies on the immunologic characteristics of TBE virus subunits we have therefore established conditions for the preparation of homogeneous polymeric envelope protein complexes. The method applied, i.e. solubilization of the virus envelope followed by reaggregation of the amphiphilic glycoprotein by sedimentation into a detergent-free density gradient, proved to be very efficient, but definite detergent-dependent differences were observed. Using 0.2% TX-100 for dissociation of the virus, protein complexes could be prepared which contained both envelope proteins V₃ and V₁ and less than 1% lipid and detergent. As we have shown previously (Heinz & Kunz, 1979) hydrophobic amino acids are enriched in the smallest virus protein V₁, and its presence in these aggregates is likely to be due to hydrophobic interaction with the lipophilic tail of V₃. The results obtained with the second non-ionic detergent differed in two important respects. Much higher concentrations (5%) were necessary for the delipidation of envelope protein complexes (residual lipid content about 3%) and under these conditions the interaction of V₁ with V₃ is also abolished. Association forces between V₃ molecules, therefore, seem to be stronger than those between V₃ and V₁ and this conclusion is also suggested by experiments with the cationic CTAB. Using conditions mild enough to allow reassociation of envelope proteins, V₃ is found exclusively in the aggregates formed, whereas V₁ remains on top of the gradients together with lipid, V₃ from
disrupted cores and that portion of V₃ which is not able to reassociate. All the protein complexes described carried HA activity. As shown by sedimentation analysis, these preparations proved to be homogeneous and no further aggregation or precipitation could be detected. Their sedimentation coefficients, however, were different, depending on the detergent used and values of 15 to 16, 16 to 18 and 11 to 12S have been determined for complexes obtained after TX-100, OG or CTAB solubilization, respectively. The immunological characterization of these envelope protein complexes from TBE virus is currently under investigation.

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REFERENCES


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