Isolation of Subviral Components from Transmissible Gastroenteritis Virus

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SUMMARY

Exposure of purified transmissible gastroenteritis virus, a porcine coronavirus, to non-ionic detergents resulted in the removal of the surface projections and > 98% of the virus lipid. Virus RNA was associated with a subviral particle which had a sedimentation coefficient of 650S, compared with 495S for the intact virion, and which banded in Cs2SO4 gradients at 1.295 g/ml. Negatively stained preparations of subviral particles were shown by electron microscopy to contain spherical particles of 60 to 70 nm diam., similar in appearance to those derived from oncarnaviruses.

Polyacrylamide gel electrophoresis of the polypeptides from isolated subviral particles showed that these structures contained three of the four major virus structural proteins, the arginine-rich polypeptide VP2 and the two membrane glycopolypeptides VP3 and 4. The detergent-liberated surface projections, composed of a single species of sulphated glycopolypeptide, VP1, were isolated by rate-zonal centrifugation through sucrose gradients followed by precipitation with ammonium sulphate in the presence of bovine serum albumin.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV), a member of the coronavirus group (Tajima, 1970), is bounded by a host derived lipid bilayer (B. V. Pike & D. J. Garwes, in preparation) from which radiate large club-shaped projections. A single species of glycopolypeptide is associated with the projection while three major polypeptides, of which two are glycosylated, make up the remainder of the virus structural protein (Garwes & Pocock, 1975).

The structural components of TGEV are of interest antigenically since it is not known how many antigens the virus contains nor which are involved in virus neutralisation and piglet immunity. Recently it was reported that three immunoprecipitating antigens could be extracted from TGEV-infected piglet small intestine (Bohac & Derbyshire, 1975), but the relationship of these to the virion and to immunity has yet to be established.

We have been investigating methods for dissociating and characterizing the TGEV structural elements and this report describes the removal and isolation of the surface projections from an RNA-containing subviral particle.
METHODS

**Virus.** TGEV was grown in primary pig kidney cells with medium ELG or EG+AA (Garwes & Pocock, 1975) or in secondary adult pig thyroid cells with Medium 199/gal (Pocock & Garwes, 1975) and purified by rate zonal centrifugation as previously described (Garwes & Pocock, 1975).

**Radioactive labelling.** Virus RNA was labelled by maintenance of infected cells in medium containing 5-3H-uridine (0.4 µM, sp. act. = 26 Ci/mmol). The structural polypeptides were labelled with 35S-L-methionine (0.01 mM, sp. act. = 2 Ci/mmol), 4,5-3H-L-leucine (0.05 mM, sp. act. = 0.2 Ci/mmol), U-14C-L-arginine monohydrochloride (0.016 mM, sp. act. = 0.3 Ci/mmol), 6-3H-D-glucosamine (2.7 µM, sp. act. = 7.3 Ci/mmol) or 35S-sulphate (0.8 mM, sp. act. = 0.125 Ci/mmol). Virus for lipid analysis was grown in pig kidney cells in medium ELG containing 0.15 µCi/ml of 14C-palmitic acid, prepared as described by Robbins & Macpherson (1971).

All radioactive precursors were purchased from the Radiochemical Centre, Amersham, Bucks. Radioactivity was determined with a Packard model 2425 liquid scintillation spectrometer using a Triton X-100/toluene scintillant.

**Rate zonal and isopycnic equilibrium centrifugation.** Purified radioactive virus was treated either with 1% Nonidet P40 (NP40, B.D.H. Chemicals Ltd, Poole, Dorset) at 20 °C for 15 min or with water under similar conditions and was then centrifuged through linear sucrose and caesium sulphate gradients as described in the text. Solution density of the caesium sulphate fractions was determined by measurement of refractive index.

**Analysis of virus polypeptides.** Samples of radioactive virus and virus components were dissociated and reduced with 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol at 100 °C for 2 to 5 min and electrophoresed through 5% polyacrylamide gels with 0.1 M-sodium phosphate buffer, pH 7.2, as described by Garwes & Pocock (1975), except that 0.135% ethylene diamine was substituted for the 0.135% N, N'-methylene bisacrylamide used previously. After fractionation of the gel, 1 mm slices were dissolved in 0.2 ml 1 M-piperidine and the radioactivity was measured after the addition of 3 ml Triton X-100/toluene scintillant.

**Lipid analysis.** Preparations of TGEV labelled with 14C-palmitic acid were analysed for lipids by the method described by Gray (1967). The virus preparations were dried under a stream of nitrogen and extracted with a small volume of chloroform:methanol (2:1) at 4 °C for 18 h followed by a fresh volume at 20 °C for 5 min. The extracts were pooled, reduced to approx. 25 µl by evaporation and chromatographed on 20 cm x 20 cm silica gel coated plates (Type 5737, E. Merck, Darmstadt) using chloroform:methanol:water (65:25:4) in the first direction and tetrahydrofuran:methanol:water (10:6:4:1) in the second direction. The plates were dried over P2O5 for 18 h between the two chromatographic directions. The lipids were located by autoradiography with Kodirex AP54 film (Kodak Ltd, London), the spots were scraped from the glass and the radioactivity was measured by liquid scintillation counting.

**Electron microscopy.** Samples for electron microscopic examination were applied to carbon-collodion coated grids that had been treated with bacitracin to facilitate even spreading (Gregory & Pirie, 1973) and were negatively stained with 2% (w/v) sodium phosphotungststate, pH 7.2. The preparations were examined with a Philips EM300 microscope at instrumental magnifications of 15000 to 75000 x at an accelerating voltage of 80 kV.
Subviral components of TGEV

RESULTS

The effect of NP40 on the sedimentation coefficient of TGEV

Purified TGEV, labelled with ³H-uridine, was incubated in either distilled water or 1 % NP40 and then centrifuged through linear 15 to 30 % sucrose gradients. NP40-treated TGEV consistently sedimented slightly faster than untreated controls (Fig. 1) and this finding could be reproduced with virus treated with 1 % Triton X-100 (Rohm and Haas, Philadelphia, U.S.A.).

The sedimentation coefficients of TGEV before and after treatment with NP40 were determined by the method of Martin & Ames (1961). By comparison with vesicular stomatitis virus, which has a sedimentation coefficient of 625 S (Bradish, Brooksby & Dillon, 1956) the complete and NP40-treated forms of TGEV were shown to sediment with values of 495 S and 650 S respectively.

Isopycnic centrifugation

In order to determine the density of the 650 S particle, ³H-uridine labelled TGEV was centrifuged to equilibrium in Cs₂SO₄ gradients ranging in density between 1.1 and 1.7 g/ml.
Untreated virus was located as a major band of density 1.21 g/ml (Fig. 2) with smaller amounts in the 1.54 g/ml and 1.63 g/ml regions of the gradient. After NP40 treatment, the density of the virus particle increased to 1.295 g/ml and there was a small increase in the amount of radioactivity in the 1.54 and 1.63 g/ml areas (Fig. 2).

**Infectivity**

The preparations of TGEV used in this work contained between $10^7$ and $10^8$ p.f.u./ml as determined by plaque assay on secondary adult pig thyroid cells; in this system 1 TCD$_{50}$ corresponds to 1 to 2 p.f.u.

Following treatment with 1% NP40 and subsequent recovery by sedimentation, no infectivity could be demonstrated in the 650S preparations. Cells incubated with such preparations for 24 to 48 h at 37 °C showed no cytopathic changes and produced no detectable virus. Infectivity of the 650S component could not be enhanced by treatment of the cell...
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Fig. 3. Polyacrylamide gel electrophoresis of 35S-methionine labelled TGEV isolated from sucrose gradients following rate zonal sedimentation of (a) untreated and (b) NP40-treated virus. Migration is from left to right and the arrows denote the location of bromophenol blue used as a marker.

cultures with DEAE-dextran at concentrations up to 250 μg/ml, a procedure that has proved useful for the demonstration of infectivity in RNA extracted from many viruses.

Polypeptide analysis of the 650S particle

Sedimentation of 35S-methionine labelled TGEV through 15 to 30% sucrose for 1 h at 70,000 g followed by analysis of the structural polypeptides by polyacrylamide gel electrophoresis showed that the RNA-containing material that sedimented with a value of 650S after detergent extraction contained all the major virus polypeptides except VP1 (Fig. 3). The VP1 glycopolypeptide had been previously shown to be associated with the surface projections of TGEV (Garwes & Pocock, 1975). Detergent treatment of TGEV appeared, therefore, to remove the surface projection and produce a subviral particle.

Isolation of the surface projections

Centrifugation of NP40-treated virus through a 6 ml 15 to 30% sucrose gradient for 16 h at 100,000 g caused the RNA-containing subviral particles to pellet and a band of protein to move approx. half-way down the gradient (Fig. 4a). Attempts to precipitate this band of material by either 80% (v/v) ethanol or 80% (v/v) acetone were unsuccessful; radioactivity remained
Fig. 4. Analysis of components from NP40-treated TGEV. (a) 3H-leucine labelled virus was treated with 1% NP40, layered over a 6 ml linear 15 to 30% sucrose gradient and centrifuged at 100,000 g for 16 h. Following fractionation into 0.2 ml portions, radioactivity was determined in each fraction and the pelleted material, containing 4500 ct/min, was resuspended in 0.2 ml water. Sedimentation is from right to left. (b) The pelleted material from the sucrose gradient shown in (a) was analysed by polyacrylamide gel electrophoresis. (c) The radioactivity from fractions 14 and 15 of the sucrose gradient shown in (a) was precipitated with 80% saturated ammonium sulphate in the presence of 1 mg/ml bovine serum albumin and analysed by polyacrylamide gel electrophoresis. The direction of migration in the electropherograms (b) and (c) is from left to right and the location of the bromophenol blue marker is shown by the arrows.

in the supernate after either treatment at 4°C overnight followed by centrifugation at 20,000 g for 30 min. The material was precipitated, however, by 5% (w/v) trichloroacetic acid or by ammonium sulphate, with bovine serum albumin present at 1 mg/ml to act as carrier. Fig. 5 illustrates the recovery of 3H-leucine labelled material from the sucrose gradient band after the addition of several concentrations of ammonium sulphate. Good recovery was achieved with 80% saturated ammonium sulphate and we have routinely used this concentration overnight at 4°C to precipitate the radioactive protein band.

Analysis of the polypeptide composition of the pelleted subviral particles and of the protein band after precipitation demonstrated that the particles contained polypeptides VP2 and
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Fig. 5. Recovery of radioactivity from the sucrose gradient band by ammonium sulphate precipitation. Material from fractions 14 and 15 of the sucrose gradient shown in Fig. 4(a) was mixed with bovine serum albumin at 1 mg/ml and then samples were mixed with saturated ammonium sulphate solution to give the concentrations shown. The preparations were centrifuged at 15000 g for 20 min after 18 h at 4 °C, the sedimented material was dissolved and its radioactivity was determined. The data are expressed as the percentage of the total radioactivity in each sample recovered in the precipitated material.

Table 1. Lipid analysis of untreated and Nonidet-extracted TGEV

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Identification</th>
<th>Radioactivity recovered (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated virus</td>
</tr>
<tr>
<td>1</td>
<td>Phosphatidylethanolamine</td>
<td>8060</td>
</tr>
<tr>
<td>2</td>
<td>Phosphatidylcholine</td>
<td>23755</td>
</tr>
<tr>
<td>3a</td>
<td>Lysolecithin 1</td>
<td>12749</td>
</tr>
<tr>
<td>3b</td>
<td>Sphingomyelin 1</td>
<td>1325</td>
</tr>
<tr>
<td>4</td>
<td>Phosphatidylycerine</td>
<td>1689</td>
</tr>
<tr>
<td>5</td>
<td>Phosphatidylinositide</td>
<td>2382</td>
</tr>
<tr>
<td>6</td>
<td>Monohexoseceramide</td>
<td>7016</td>
</tr>
<tr>
<td>7</td>
<td>Dihexoseceramide</td>
<td>1363</td>
</tr>
<tr>
<td>8</td>
<td>Trihexoseceramide</td>
<td>1867</td>
</tr>
<tr>
<td>9</td>
<td>Tetrahexoseceramide</td>
<td>1577</td>
</tr>
<tr>
<td>10</td>
<td>Aminoglycolipid</td>
<td>791</td>
</tr>
</tbody>
</table>

* No spot detectable on autoradiogram.

VP3 + 4, as reported above, while the glycopolyptide of the surface projections, VP1, was associated with the protein band (Fig. 4b and c). The radioactivity that remained near the top of the sucrose gradient (Fig. 4) was probably derived from degraded virus as poly-peptides VP2 and VP3 + 4 were present as non-sedimentable material.

Virus lipid analysis

To determine how much virus lipid was removed by NP40 treatment, TGEV labelled with 14C-palmitic acid was left untreated or held in 1% NP40 for 30 min at 20 °C, then layered over 10% sucrose in water and sedimented at 150000 g for 2 h. The pellets were extracted with chloroform:methanol (2:1) and analysed for lipids by two-dimensional thin-layer chromatography. The results are presented in Table 1; identification of the various lipids was made by comparison with unlabelled standards (B. V. Pike & D. J. Garwes, in pre-
Fig. 6. Morphology of TGEV after treatment with 1% NP40. (a) and (b) Untreated virions, (c) and (d), NP40-treated virus, after sedimentation through 10% sucrose to remove lower mol. wt. components. The samples were negatively stained with 2% (w/v) sodium phosphotungstate, pH 7.2.

The only lipid spots that were detectable after NP40 treatment of the virus were phosphatidyl choline, monohexoseceramide and the lysolecithin/sphingomyelin mixture. The radioactivity recovered from these spots was approx. 1 to 2% of that from the equivalent spots derived from untreated virus and it may be concluded therefore, that, under the conditions used, NP40 removes more than 98% of the virus lipid.

Electron microscopy

The electron micrographs illustrated in Fig. 6 show intact, untreated virions and the subviral particles isolated from detergent-treated virus by sucrose density sedimentation. The diam. of TGEV was generally in the range of 70 to 90 nm, excluding the surface projections, or 100 to 120 nm including the corona (Fig. 6a). Intact virus preparations showed particles distributed evenly on the grid with no tendency to aggregate, as seen in the lower-power field in Fig. 6b. The subviral particles were 60 to 70 nm across with an occasional structure of twice that diam. (Fig. 6c and d). All preparations of subviral particles appeared aggregated on the grid, the number of particles in each clump varying between 5 and approx. 80. There was no evidence of surface projections on the particles but the surface of the particles appeared irregular or pitted. Many particles seemed incomplete or partly degraded but it was not possible to see an obvious thread-like substructure as described by Johnson-Lussenburg (personal communication) for the human respiratory coronavirus 229E.
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Fig. 7. The relative proportion of arginine in the structural polypeptides of TGEV. Purified virus, grown in the presence of either $^3$H-leucine (●○●○) or $^{14}$C-arginine hydrochloride (○---○), was dissociated in 1% SDS and 1% 2-mercaptoethanol at 100 °C and analysed by polyacrylamide gel electrophoresis. Migration is from left to right.

Virus & polypeptide VP2

The most likely location for VP2, the only non-glycosylated major structural polypeptide, is internal in association with the virus RNA. In oncornaviruses the internal RNA-associated structural polypeptide has been shown to be unusually rich in arginine (Fleissner, 1971) and it was of interest to see whether any of the TGEV polypeptides were rich in this amino acid. Fig. 7 shows the pattern derived from TGEV grown in the presence of $^3$H-leucine and $^{14}$C-arginine. It can be seen that the relative proportion of arginine in VP2 was considerably greater than in either VP1 or VP3+4, in contrast to the patterns produced with virus labelled with either $^3$H-leucine (Fig. 7) or $^{35}$S-methionine (Garwes & Pocock, 1975). These data cannot be used to calculate the absolute leucine:arginine ratios since the pool sizes and specific activities for these amino acids in the cells have not been determined but the data suggest that the arginine content of VP2 was 2 to 3 times that expected from its leucine content when compared with the proportions in the other structural polypeptides.
Fig. 8. Detection of sulphate in the structural polypeptides of TGEV. Virus was grown in the presence of either \( ^3\)H-leucine (○—○) or \(^{35}\)S-sulphate (●—●). Following purification, the virus was dissociated in 1% SDS and 1% 2-mercaptoethanol at 100°C and analysed by polyacrylamide gel electrophoresis. Migration is from left to right.

**Sulphation of the virus glycoproteins**

Recently, Compans & Pinter (1975) reported the presence of sulphate groups on the surface glycoproteins of influenza virus. Analysis of TGEV grown in the presence of \(^{35}\)S-sulphate and \(^3\)H-leucine (Fig. 8) demonstrated that the surface glycoprotein projections of this coronavirus are sulphated whereas the VP\(_3 + 4\) membrane glycopolypeptides contain no detectable sulphate. The \(^{35}\)S-labelled material located at the top of the gel presumably corresponds to the mucopolysaccharide described by Compans & Pinter in their preparations of influenza virus.

**DISCUSSION**

We have shown that removal of lipid from purified TGEV by non-ionic detergents detached the surface projections from an RNA-containing subviral particle. The buoyant density of the subviral particle was higher than that of the intact virus, consistent with the removal of lipid, and it had a higher sedimentation coefficient. Electron microscopic examination of negatively stained subviral particle preparations showed spherical bodies, smaller than the virion, and polypeptide analysis revealed all the virus structural proteins except for the surface projections. Whether the subviral particles as isolated represent the unchanged internal structure of the TGE virion or whether the material undergoes a morphological rearrangement to a more stable form following removal of the lipid envelope cannot be determined from the data presented.

In a recent report, the Western Hemisphere Committee on Animal Virus Characterisation (1975) recommended that the genus coronavirus be classified in the Family Paramyxoviridae.
Treatment of paramyxoviruses with non-ionic detergents leads to the release of the helical nucleocapsid which has a sedimentation coefficient considerably lower than that of the virion and in this characteristic, as well as in the difference in the structure of the nucleic acid (Garwes, Pocock & Wijaszka, 1975) the coronaviruses and the paramyxoviruses are markedly dissimilar. Our data on the structure and biochemistry of TGEV would support the recent proposal that coronaviruses should be classified in the monogeneric family, Coronaviridae (Tyrell et al. 1975).

The electron micrographs of TGEV subviral particles presented above resemble those of cores derived from type B and type C oncornaviruses (Nowinski, Sarker & Fleissner, 1973). While the size and general appearance of these cores are similar to TGEV it is not possible to compare their composition as there is a great deal of variation in the findings for different members of the oncornaviruses. Treatment of avian myeloblastosis virus with Nonidet produced cores containing predominantly the gs 1 polypeptide (Bolognesi et al. 1972) whereas Rous sarcoma virus cores, also produced by Nonidet, were reported to contain gs 1 and gs 4 (Coffin & Temin, 1971) or predominantly gs 4, the arginine-rich structural polypeptide (Nowinski et al. 1973).

The finding that the TGEV polypeptide VP2 is rich in arginine suggests that it may be associated with the RNA. If this is the case, then the remaining core polypeptides VP3 +4 may form the outer boundary of the spherical structure, a role consistent with their carbohydrate content. Attempts to isolate a ribonucleoprotein complex composed of TGEV RNA and VP2 only have so far proved unsuccessful.

After removal of the virus lipid with NP40, the TGEV surface projections have been isolated by rate zonal centrifugation followed by precipitation with ammonium sulphate in the presence of bovine albumin as carrier. Our inability to precipitate the isolated projections with ethanol or acetone suggests that the glycopolypeptide may be associated with lipid-detergent complexes. That the surface projections are antigenically intact following the isolation procedure described is indicated by our finding that the intramammary inoculation of purified projections into the pregnant sow resulted in the formation of virus neutralising antibody in serum and colostrum (D. J. Garwes, Cartwright & Lucas, unpublished observation).

It is of interest that the surface projection glycopolypeptide VP1 is sulphated, whereas the VP3 +4 membrane glycopolypeptides are not. Compans & Pinter (1975) showed that all the glycopolypeptides of influenza virus were sulphated, but as the function of the sulphate groups is not known, the significance of our finding with TGEV remains obscure.

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