The Polypeptide Structure of Transmissible Gastroenteritis Virus

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SUMMARY

The polypeptides of purified preparations of the coronavirus responsible for transmissible gastroenteritis of pigs have been examined by polyacrylamide gel electrophoresis. Four major polypeptides, VP1 (mol. wt. 200 000), VP2 (50 000), VP3 (30 000) and VP4 (28 500) and two minor polypeptides, VP1a (105 000) and VP1b (80 500) have been reproducibly demonstrated in the virion, of which VP1, VP3 and VP4 contain carbohydrate. Treatment of the virion with the proteolytic enzyme bromelain removes the surface projections and VP1, thus identifying this glycopolypeptide as the major structural component of the projection.

INTRODUCTION

Transmissible gastroenteritis (TGE) is a highly infectious disease of pigs, causing a high mortality rate in young piglets. Since its first description by Doyle & Hutchings (1946) there has been much research into the disease and its prevention (reviewed by Woode, 1969) but vaccine development has been only partially successful. Piglet immunity depends upon passive transfer of maternal antibody in colostrum and milk; the most effective vaccines produced are those incorporating attenuated virus, but these provide only approx. 60 to 65% survival, as against 25 to 30% for litters from non-immune sows (Tamoglia, 1972). There is thus a clear need for a better understanding of the causal virus of this disease.

TGE virus was shown by Tajima (1970) to be a member of the coronavirus group (Almeida et al. 1968). Little is known about the structure of these viruses; the only study so far reported has been on the protein composition of the human coronavirus OC43 (Hierholzer et al. 1972). Unlike TGE virus, mouse brain adapted OC43 has a haemagglutinin activity and this character was used to purify the virus by adsorption to and elution from human erythrocytes. OC43 was shown to have six or seven structural polypeptides of which four were glycoproteins.

The present study on the structure of TGE virus was initiated in an attempt to characterize the viral components involved in the immune response. This report deals with the polypeptide structure of the virion and the identification of the glycopolypeptide that is located in the surface projections.

METHODS

Cell cultures. Primary pig kidney (PK) cells, obtained from hysterotomy-derived piglets, were grown as monolayers in 4 oz flat glass bottles in ELG medium (Earle's salts; 0.5% lactalbumin hydrolysate; 0.1% galactose; the antibiotics penicillin, streptomycin and
Table 1. The concentration of amino acids in medium EG + AA

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration* (mm)</th>
<th>Amino acid</th>
<th>Concentration* (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>2.2</td>
<td>Lys</td>
<td>3.4</td>
</tr>
<tr>
<td>Arg</td>
<td>0.9</td>
<td>Met</td>
<td>0.6</td>
</tr>
<tr>
<td>Asp</td>
<td>3.0</td>
<td>Phe</td>
<td>1.1</td>
</tr>
<tr>
<td>Cys</td>
<td>0.9</td>
<td>Pro</td>
<td>2.7</td>
</tr>
<tr>
<td>Glu</td>
<td>5.9</td>
<td>Ser</td>
<td>2.6</td>
</tr>
<tr>
<td>Gly</td>
<td>1.1</td>
<td>Thr</td>
<td>1.6</td>
</tr>
<tr>
<td>His</td>
<td>0.6</td>
<td>Try</td>
<td>0.5</td>
</tr>
<tr>
<td>Ile</td>
<td>2.1</td>
<td>Tyr</td>
<td>0.9</td>
</tr>
<tr>
<td>Leu</td>
<td>3.8</td>
<td>Val</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* These concentrations are equivalent to those typically present in 0.5% lactalbumin hydrolysate (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.).

mycostatin at 100 units/ml, 100 µg/ml and 25 units/ml respectively, supplemented with 10% lamb serum and buffered with 0.033% sodium bicarbonate at pH 7.2; Luther, 1972). Secondary adult pig thyroid (APT/2) cells were grown as monolayers in 50 mm tissue culture plastic Petri dishes in medium 199/gal (Rutter & Luther, 1973).

**Virus growth.** The FS 772/70 cloned strain of TGE was obtained from Miss S. Cartwright, Central Veterinary Laboratory, Weybridge, and was used at its 125th passage through PK cells.

Confluent monolayers of PK cells were drained and infected with 1 ml of a stock virus at an input multiplicity of approx. 1 p.f.u./cell. After 1 h adsorption at 37 °C, 9 ml of maintenance medium (ELG; 2.5% lamb serum; 0.11% sodium bicarbonate; 0.02 M-HEPES [N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid, Sigma London Chemical Co. Ltd]), pH 6.8, was added and incubation was continued for 36 h at 37 °C, at which time about half the cells had rounded up and detached from the glass. For stock virus preparations, the cultures were frozen and thawed once and cell debris was removed by low speed sedimentation. Virus titres, as determined by plaque assay in APT/2 cells, were between $2 \times 10^6$ and $1 \times 10^7$ p.f.u./ml.

**Radioactive labelling.** Virus containing radioactive amino acids was grown in PK cells as described above but with the following modifications; the virus inoculum was removed after 1 h adsorption period, the cell sheet was washed with saline to remove amino acids present in the inoculum, 10 ml of medium EG + AA was added and incubation at 37 °C was continued for 36 h. Medium EG + AA was similar to the virus growth medium described above, but the 0.5% lactalbumin hydrolysate was replaced by a synthetic mixture of amino acids, formulated to be comparable with those found in lactalbumin hydrolysate, as detailed in Table 1. In use, the leucine or methionine was omitted from the mixture and replaced by L-[4,5-3H]-leucine (0.02 mM: sp. act. = 1 Ci/mmol) or L-[35S]-methionine (0.01 mM: sp. act. = 2 Ci/mmol) respectively.

For radioactive labelling of virus glycoproteins, virus was grown as for stock but with the addition to the virus growth medium of either D-[1-3H]-fucose (1.5 µM: sp. act. = 13.4 Ci/mmol) or D-[6-3H]-glucosamine (2.7 µM: sp. act. = 7.3 Ci/mmol).

Radioactivity was measured using NE 233 and NE 250 scintillants (Nuclear Enterprises Ltd, Edinburgh) in a Packard model 2425 scintillation spectrometer.

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks.

**Virus purification.** Infected cultures were harvested after 36 h incubation, frozen and thawed once to detach the cells from the glass and the suspension was subjected to high
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frequency sound for 30 s at 4 °C using 100 W from a Soniprobe Type 7530A (Dawe Instruments Ltd, London). The suspension was clarified by centrifuging at 15000 g for 20 min and solid ammonium sulphate was slowly added to the supernate to 40% saturation at 4 °C. After at least 6 h at 4 °C, the precipitate was removed by centrifuging at 15000 g for 20 min and dissolved in 0·14 M-NaCl, 0·01 M-tris, pH 7·2 (T-S), to one tenth of the original volume. This was layered over a stepped sucrose gradient, consisting of 1 vol. 60% sucrose, 4 vol. 10% sucrose and 2 vol. of virus, and centrifuged at 70000 g for 1 h. The gradient was fractionated and the 10%/60% interphase, containing all the virus, was diluted with an equal volume of 20% sucrose, pH 8·0, and left for 2 h to dissociate the virus from cell debris. This suspension was layered over a linear 20% to 45% sucrose gradient in 10 m-m-tris, pH 8·0, and centrifuged at 70000 g for 1·5 h. After fractionation, the virus band was located by infectivity, radioactivity or optical density, diluted with two vol. of water and pelleted at 150000 g for 2 h. After resuspension in a small vol. of water, the preparation was used as ‘purified virus’. Sedimentations at less than 20000 g were carried out in angle rotors in an M.S.E. Highspeed 18 centrifuge and those above 20000 g employed swing-out rotors in an M.S.E. Superspeed 65 centrifuge (Measuring and Scientific Equipment Ltd, Crawley), all at 4 °C.

Electron microscopy. Virus samples were examined after negative staining with 2% (w/v) potassium phosphotungstate, pH 7·0, by the agar diffusion technique of Anderson & Doane (1972). A Philips EM 300 electron microscope was used at instrumental magnifications of 70000 to 114000 times and at an accelerating voltage of 80 kV.

Treatment of virus with bromelain. Purified radioactive virus was incubated at 37 °C for 1 h in 50 mM-dithiothreitol, pH 7·2, with or without bromelain (Sigma London Chemical Co. Ltd) at 1·3 mg/ml, as described by Compans (1971).

After incubation, each sample was layered over a 6 ml linear 15% to 30% sucrose gradient and centrifuged at 70000 g for 45 min at 20 °C. Following fractionation, the virus was located by radioactivity assay and the fraction containing the most radioactivity was used for electron microscopy and polypeptide analysis. The bromelain-treated virus was not pelleted at this stage as this was found to cause complete disruption of the virion.

Polypeptide analysis. Virus samples were heated at 100 °C for 2 min in the presence of 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptopoethanol, cooled and electrophoresed through polyacrylamide gels at 12·5 mA/gel. The gels contained 5% acrylamide, 0·135% N,N'-methylene bisacrylamide, 0·1% SDS, 0·1 M-sodium phosphate, pH 7·2, 0·075% tetramethylethylendiamine and were polymerized with 0·075% ammonium persulphate; running buffer was composed of 0·1 M-sodium phosphate, pH 7·2, and 0·1% SDS. Electrophoresis was continued until the tracking dye, bromophenol blue, was approx. 1 cm from the bottom of the 7 cm gel. The gels were frozen and cut into 1 mm slices with a Mickle Gel Slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey); each slice was soaked overnight in a scintillation vial containing 10 ml scintillant composed of 10% (v/v) Soluene-100 (Packard Instrument Ltd, Caversham) and 0·2% water in NE 233 and the radioactivity was counted. The mobilities of the viral polypeptides were measured relative to the tracking dye and compared with the mobilities of proteins of known mol. wt. that had been reduced, electrophoresed as above and stained with Coomassie brilliant blue. The proteins used as standards were obtained from Sigma London Chemical Co. Ltd, and comprized bovine haemoglobin (17000), porcine pancreatic trypsin (24000), porcine gastric pepsin (34500) and bovine serum albumin, monomer (69000) and dimer (138000).
Table 2. The recovery of virus infectivity during purification

<table>
<thead>
<tr>
<th></th>
<th>Infectivity (p.f.u./ml)</th>
<th>Volume (ml)</th>
<th>% recovery</th>
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<tbody>
<tr>
<td>Tissue culture fluid</td>
<td>6 x 10^7</td>
<td>100</td>
<td>(100)</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td>4.6 x 10⁸</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>10 %/60 % sucrose interphase</td>
<td>1.4 x 10⁹</td>
<td>1.5</td>
<td>35</td>
</tr>
<tr>
<td>Pelleted virus from 20 to 45 % sucrose gradient</td>
<td>1.5 x 10⁹</td>
<td>1.0</td>
<td>25</td>
</tr>
</tbody>
</table>

RESULTS

Virus purification

TGE virus purified by the method described above retains its infectivity and its morphological integrity.

The data presented in Table 2 are from a representative experiment. There is a progressive loss of infectious virus at the various steps of the purification process but whether this results from damage to the virions or from an incomplete recovery of the particles cannot be determined. It seems likely, however, that it is the latter since the purification steps are designed to be selective for virus and some particles will necessarily be discarded with the cellular debris and medium contaminants.

The particles shown in Fig. 1 (a) and (b) were prepared from unpurified virus samples while those in Fig. 1 (c) and (d) were purified as described and are typical of virus treated in this way. There was no obvious loss of surface projections from the ‘corona’ and the membrane remained intact, as judged by the exclusion of negative stain from the centre of the particle.

Following ammonium sulphate precipitation, used primarily to reduce the volume of the preparation, the virus pellet was resuspended in one-tenth of the starting volume. It was found that the use of a smaller volume was unsuitable, since the density of the resulting solution was too high to layer onto 10 % sucrose and dialysis cannot be used as virus adsorbs onto the cellulose membrane.

Similarly, the virus collected at the 10 %/60 % sucrose interphase cannot be dialysed to reduce specific gravity and must be diluted in order to layer the virus onto the top of the linear sucrose gradient. If virus is recovered from the 10 %/60 % sucrose interphase fraction by sedimentation into a pellet, it was found that after resuspension there was a 1 to 2 log¹₀ loss of infectivity and substantial removal of surface projections from the virions. Once the residual host membrane material was removed at the rate zonal sedimentation step, the virus could be pelleted and resuspended without damage.

Virus polypeptide analysis

The polypeptides of purified TGE virus were analysed by polyacrylamide gel electrophoresis in SDS – sodium phosphate, as described in Methods. The electropherograms shown in Fig. 2 (a) and (b) were produced from virus that had been grown in the presence of [³H]-leucine and [³⁵S]-methionine respectively. The patterns are essentially similar and show three major bands, VPI, VP2 and VP3 +4 and several minor peaks of which only two VP1a and VP1b were reproducibly demonstrable in all virus preparations examined. The separation of VP3 from VP4 has proven difficult by this method of electrophoresis but the presence of VP3 as a peak or shoulder on the trailing side of VP4 has been seen in every
The polypeptides of TGE virus

preparation of purified TGE virus that we have examined, totalling more than 20 polyacrylamide gels. Electrophoresis of the virus polypeptides in polyacrylamide gels containing 7.5% or 10% total amide did not improve the resolution of VP3 and VP4.

The approximate mol. wt. of the virus polypeptides have been determined by comparison with the relative mobility of proteins of known mol. wt. These data, illustrated in Fig. 3, provide mol. wt. values, averaged from at least 12 determinations, of: VP1 200000; VP1a 105000; VP1b 805000; VP2 50000; VP3 30000; VP4 28500.

The effect of bromelain on virus structure

Incubation of TGE virus at 37 °C for 1 h in the presence of bromelain, followed by rate zonal sedimentation produced particles lacking the surface projection corona but having an apparently undamaged membrane (Fig. 1f). Virions treated similarly but without enzyme in the incubation buffer remained morphologically intact (Fig. 1e) as did virus in preparations in which the bromelain was replaced by trypsin, pepsin or rennin. Treatment of [3H]-leucine-labelled virus with bromelain and re-isolation from a sucrose gradient followed by SDS polyacrylamide gel electrophoresis showed that VP1 was digested from the virion (Fig. 4) indicating that VP1 is a structural polypeptide of the surface projection. It is possible
that the minor polypeptide VP1a is also removed but since this polypeptide is only present in small amounts it is difficult to be certain of the result.

**Virus glycoproteins**

The pattern of polypeptides from virus labelled with [3H]-glucosamine is shown in Fig. 5. The arrows indicate the expected location of the polypeptides. The label was found in two regions of the electropherogram, 70% being associated with VP1 and the remainder distributed in the VP3 + 4 region; polypeptides VP1a, 1b and 2 were not labelled. Bromelain treatment of [3H]-glucosamine-labelled TGE virus (as described above) resulted in the total loss of VP1 but left the radioactivity of VP3 + 4 (Fig. 5), confirming the result found with the amino acid label. These data indicate, therefore, that the surface projections of TGE virus contain a single species of glycopolypeptide. The incorporation of [3H]-fucose into the virus gave electropherograms with a similar pattern of radioactivity to those shown for [3H]-glucosamine; the distribution of label was, however, 84% in VP1 and 16% in VP3 + 4.
The polypeptides of TGE virus

Fig. 3. Estimation of mol. wt. of TGE virus structural polypeptides by comparison of their mobilities in SDS-polyacrylamide gel relative to the tracking dye, bromophenol blue, with those of protein standards (as described in Methods).

Fig. 4. SDS-polyacrylamide gel electrophoresis of [3H]-leucine-labelled virus incubated at 37 °C in the absence (●—●) or presence (□—□) of bromelain for 1 h and re-isolated by rate zonal sedimentation.
DISCUSSION

We have shown that purified particles of transmissible gastroenteritis virus contain four major polypeptide species, VP1, VP2, VP3 and VP4 and possibly two minor species, VP1a and VP1b, having apparent mol. wt. of approx. 200,000, 50,000, 30,000 and 28,500 and 105,000 and 80,500 respectively. The largest polypeptide VP1, contains carbohydrate and is located in the virus surface projections; a smaller amount of carbohydrate is associated with VP3 and VP4.

In the early stages of development of the purification procedure, we experienced difficulty in preserving virus infectivity and morphological integrity. It became clear, however, that the main causes of these problems were pelleting and resuspension of virus contaminated with host membrane fragments, dialysis of virus to remove solutes, such as ammonium sulphate and sucrose and the use of phosphate buffers. Examination of resuspended pellets of unpurified or partially purified virus by electron microscopy showed many particles missing some or all of the surface projections and with stain penetrating into the virion, suggesting damage to the lipid bilayer. Once purified, resuspension of pelleted virus caused no such damage and this may indicate that shearing of the virus projections attached to host receptors could be the cause of the problem. Why infectivity is lost when virus is held in a cellulose bag during dialysis is not understood but presumably the virions are adsorbed onto the cellulose membrane. We have found that dilution is acceptable in place of dialysis to lower specific gravity between the purification steps. The nature of the interaction between TGE virus and phosphate ions, resulting in lowered infectivity and instability of virus structure (unpublished data), is presently being investigated in this laboratory; the sub-
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Substitution of tris or HEPES in place of phosphate in virus buffer, however, considerably helps in the virus purification procedure.

We have demonstrated four major and possibly two minor polypeptides in the TGE virion but there are problems in deciding whether a small peak in an electropherogram is of viral origin or whether it represents a small proportion of contaminating host material. It is doubtful, also, whether virus structural elements that are present as only one or two molecules per virion could be detected by the method described above. At most, it can be said that TGE virus preparations purified as described contain these polypeptides and that certainty as to their viral origin will come from further work on their location and function within the virion.

The mol. wt. assigned to the TGE virion polypeptides cannot be considered as certain since the presence of carbohydrate on the polypeptides of VP1, VP3 and/or VP4 may affect the apparent mol. wt. determined by relative mobility in polyacrylamide gel. The only major polypeptide, therefore, that can be sized by this method is VP2 at a mol. wt. of 50,000.

VP1 is a high mol. wt. glycopolypeptide associated with the viral projection. Its glycoprotein nature is consistent with the findings with the surface projections of other RNA viruses containing lipid including the human coronavirus OC43 (Hierholzer et al. 1972). Bromelain treatment of OC43, however, was shown to remove two glycopolypeptides, mol. wt. 104,000 and 150,000, from the virion leaving two other glycopolypeptides, 191,000 and 60,000, on the particle. It is quite clear from these data, as well as from the different mol. wt. of the six or seven polypeptides of OC43, that the protein compositions of TGE virus and the human coronavirus are dissimilar.

The location of the other structural glycopolypeptides of TGE virus, VP3 and VP4, is uncertain. The presence of carbohydrate on these molecules would suggest that they are associated with the outer membrane of the virus but they are neither removed nor measurably digested by bromelain and they remain on the virus 'core' when the lipid bilayer is stripped off by non-ionic detergents, a procedure that quantitatively releases VP1 (unpublished data). The only protein that can be definitely assigned to the external surface of this virus, therefore, is the projection with its structural glycopolypeptide VP1 and this may constitute the only external protein antigen of TGE. Work is in progress to investigate the function of the surface projection and its role in host immunity and to determine whether any other structural elements that might be involved in the immune response are present on the outer surface of the virion.

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REFERENCES


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