Effect of Sulphhydryl Reagents on the Biological Activities, Polypeptide Composition and Morphology of Haemagglutinating Encephalomyelitis Virus

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SUMMARY

The effect of sulphydryl reagents on haemagglutinating encephalomyelitis virus (HEV), a coronavirus of pigs, was investigated. Using increasing concentrations of dithiothreitol (DTT), 50% of the virus infectivity and haemagglutination (HA) activity could be removed by 1.5 mM and 4 to 5 mM respectively. The effect of DTT concentrations on the polypeptide composition was also examined. Of the three external glycoproteins gp 125 was found to be the most susceptible, 50% being removed by incubation of the virus with 5 to 6 mM-DTT. Of the other two glycoproteins gp 180 was unaffected by DTT concentrations up to 100 mM and the amount of gp 100 gradually declined at concentrations above 20 mM. The rates of removal of the virus HA activity and gp 125 suggested that this polypeptide was an essential part of the virus haemagglutinin. The lack of evidence for any interpeptide disulphide bonds suggested that the loss of these glycoproteins was due to an alteration in their conformation brought about by the cleavage of intrapeptide disulphide bonds. The loss of protein from the surface of the virus resulted in a change in the virus morphology with the appearance of thin fibrous projections instead of the characteristic petal-like coronavirus projections.

INTRODUCTION

Haemagglutinating encephalomyelitis virus (HEV) or vomiting and wasting disease virus has been shown to be a member of the coronavirus group (Greig et al. 1971; Philip et al. 1971), a group which contains viruses of similar morphology to infectious bronchitis virus of chickens (IBV; Almeida et al. 1968). Recently Pocock & Garwes (1977) reported the separation by electrophoresis of five major polypeptides from purified preparations of HEV, only one of which was non-glycosylated. They also described the separation of two subviral complexes by treatment of the virus with non-ionic detergent. One of the complexes contained the virus RNA and two polypeptides, p 56 and gp 26.5; the other, comprising three glycoproteins gp 180, gp 125 and gp 100, exhibited the virus haemagglutination (HA) activity.

Lukert (1972) reported that the attachment of IBV to tissue culture cells was very sensitive to low concentrations of many sulphhydryl reagents. Inhibition of Sendai virus HA by these reagents has also been reported (Neurath et al. 1973) and shown to be due to the cleavage of an interpeptide disulphide bond (Ozawa et al. 1976). The two peptides comprising the influenza virus haemagglutinin have been reported by Laver (1971) to be connected by a
Table 1. The effect of pre-treatment of virus or cells with 10 mM-DTT on the infectivity of two porcine coronaviruses

<table>
<thead>
<tr>
<th>Sample pre-treated</th>
<th>Titre (p.f.u./ml)</th>
<th>% change v. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV</td>
<td>&lt;0.5×10⁵</td>
<td>&gt;99.98%</td>
</tr>
<tr>
<td>APT/2 cells</td>
<td>5.3×10⁵</td>
<td>+112%</td>
</tr>
<tr>
<td>Control</td>
<td>2.5×10⁵</td>
<td>—</td>
</tr>
<tr>
<td>TGE</td>
<td>2.5×10⁵</td>
<td>-43.9%</td>
</tr>
<tr>
<td>APT/2 cells</td>
<td>2.4×10⁵</td>
<td>-42.1%</td>
</tr>
<tr>
<td>Control</td>
<td>5.7×10⁵</td>
<td>—</td>
</tr>
</tbody>
</table>

disulphide bond. It was therefore important to determine the presence of such bonds between the polypeptides of HEV in order to increase our knowledge of the external structure of the virus. This report describes the effect of sulphydryl reagents on the integrity of HEV in an attempt to elucidate which of the virus polypeptides is the haemagglutinin.

METHODS

Virus. The FS 255 strain of HEV was grown and assayed in secondary adult pig thyroid (APT/2) cell cultures (Pocock & Garwes, 1977). To label the virus polypeptides with radioisotope the virus was grown in the presence of L-4,5-³H-leucine (0.02 mm; sp.act. = 1 Ci/mmol). Virus was purified by ammonium sulphate precipitation followed by rate zonal centrifugation in sucrose gradients as previously described (Pocock & Garwes, 1977). Transmissible gastroenteritis virus (TGE) strain FS772/70 was also grown and assayed in APT/2 cell cultures (Pocock & Garwes, 1975).

Treatment of virus with dithiothreitol (DTT). Virus samples to be treated with DTT were suspended in phosphate buffered saline (PBSa). Following the addition of DTT to give the required concentration, the mixtures were incubated at 37 °C for 1 h. The virus to be used for polypeptide analysis was then centrifuged through a layer of 10% sucrose at 100 000 g for 1 h. The resultant pellets were resuspended in sterile distilled water and used for subsequent analysis.

Virus polypeptide analysis. The virus polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Garwes et al. (1976). Virus samples were dissociated with 1% SDS at 100 °C with or without the presence of 2 mercaptoethanol (2-ME) as indicated.

Electron microscopy. Samples of virus were examined after negative staining with potassium phosphotungstate by the method of Bridger & Woode (1976).

RESULTS

Effect of DTT on infectivity

The results of an initial experiment in which either unpurified virus or the tissue culture cells were pre-treated with 10 mM-DTT for 1 h at 37 °C are shown in Table 1. Pre-treatment of HEV with this concentration removed virtually all of the infectivity whereas pre-treatment of the cells prior to virus inoculation enhanced rather than reduced their susceptibility, indicating that DTT modified the virus. Also shown in Table 1 are the results of a similar experiment carried out with the other known porcine coronavirus, TGE. In this case, although a slight reduction in titre was observed, it appeared to be an effect on the tissue culture cells rather than the virus.
Fig. 1. The effect of increasing concentrations of DTT on (a) HEV infectivity and (b) HEV HA. Samples of virus were incubated for 1 h at 37 °C with the required concentration of DTT. For the infectivity experiments the titre of the virus after treatment ($V_c$) was compared with a sample incubated in the absence of DTT ($V_0$).

The result of treating HEV with increasing amounts of DTT is shown in Fig. 1(a) and indicates that the effect on infectivity was proportional to DTT concentration; for a 50% reduction 1.5 mM was required.

**Effect of DTT on HA**

The effect of increasing concentrations of DTT on HEV HA is shown in Fig. 1(b). As the amount of DTT was increased, the HA activity was reduced logarithmically. From the results of several experiments it was found that between 4 and 5 mM-DTT was required for a 50% reduction in the HA titre.

**The separation of HEV polypeptides by SDS-PAGE under non-reducing conditions**

To determine whether the loss of infectivity or HA activity was due to the cleavage of interpeptide disulphide bonds, SDS-PAGE of purified 3H-leucine labelled virus was performed in the presence or absence of 2-ME. Under non-reducing conditions or treatment with up to 1% 2-ME, the type of pattern obtained is shown in Fig. 2(b). The profile revealed the presence of the five major polypeptides associated with HEV (from left to right) gp 180, gp 125, gp 100, p 56 and gp 26-5 as described by Pocock & Garwes (1977). As high mol. wt. protein complexes, indicative of the presence of interpeptide disulphide bonds, were not observed when non-reducing conditions were used, it was concluded that this type of bond was not important for the structural integrity of HEV.

When the concentration of 2-ME was increased to 2%, electropherograms similar to...
Fig. 2. SDS-PAGE of ³H-leucine labelled HEV disrupted at 100 °C by 1 % SDS in the presence (a) or absence (b) of 2 % 2-ME. Electrophoresis proceeded from left to right and the arrows denote the position of the marker dye, bromophenol blue.

Table 2. The effect of sulphydryl reagents on the electrophoretic mobility of HEV gp 180

<table>
<thead>
<tr>
<th>Treatment before electrophoresis</th>
<th>Pre-treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1 % SDS</td>
<td>0·310*</td>
</tr>
<tr>
<td></td>
<td>(190000)†</td>
</tr>
<tr>
<td>1 % SDS : 2 % 2-ME</td>
<td>0·237</td>
</tr>
<tr>
<td></td>
<td>(230000)</td>
</tr>
<tr>
<td>% decrease in mobility</td>
<td>30·8</td>
</tr>
</tbody>
</table>

* Mobility of gp 180 relative to bromophenol blue.
† Apparent mol. wt.

Fig. 2(a) were obtained showing a reduced mobility for gp 180. As the numerical data in Table 2 indicates, this reduction was of the order of 30 % with a concomitant increase in the apparent mol. wt.

When the virus was pre-treated with 100 mm-DTT for 1 h at 37 ºC and then centrifuged through 10 % sucrose, the resultant pelleted virus contained gp 180 which had a similar mobility, under non-reducing conditions, to that of control virus (Table 2). Again, a 30 % reduction in the mobility of gp 180 was observed when 2 % 2-ME was used in the dissociating solution before electrophoresis.
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Effect of DTT on the removal of surface glycoproteins from HEV

The result of pre-treatment of \(^{3}\)H-leucine labelled HEV with 100 mM-DTT, a concentration at which infectivity and HA can no longer be detected, followed by centrifugation through 10% sucrose before SDS-PAGE is shown in Fig. 3. The electropherogram in Fig. 3(a) was obtained with HEV which had been treated with PBSa as control and revealed the presence of the five major HEV polypeptides. Fig. 3(b) shows the effect of pre-treatment of the virus with 100 mM-DTT. Although the amounts of gp 180, p 56 and gp 26:5 were similar to the control values, essentially all of gp 125 and between 60 and 70% of gp 100 was removed by this treatment. Under similar conditions but with increasing concentrations of DTT it was possible to determine the amounts of gp 180, gp 125 and gp 100, relative to p 56, which remained associated with the virus (Fig. 4). As expected from the results of the previous experiment, the amount of gp 180 remained constant at all DTT concentrations tested. Concentrations of up to 20 mM-DTT had no effect on the amount of gp 100, but at higher concentrations some of this protein was removed, although, as stated earlier, 30 to 40% remained associated with the virus at 100 mM. The third glycoprotein, gp 125, was removed logarithmically as the virus was treated with increasing concentrations of DTT. Pre-treatment of the virus with 5 to 6 mM-DTT reduced the amount of gp 125 to 50% of the...
Fig. 4. The effect of pre-treatment of HEV with increasing concentrations of DTT on the ratio of the amount of each of the external glycoproteins (gp x) to the amount of p 56 associated with the virus. Following incubation with the required DTT concentration the ³H-leucine labelled HEV was pelleted through 10% sucrose, disrupted with 1% SDS and 2% 2-ME at 100 °C and electrophoresed. At each DTT concentration the number of counts associated with gp 180 (■—■); gp 125 (●—●) and gp 100 (▲—▲) was compared to the number of counts associated with p 56.

control value. The glycoprotein gp 26-5 remained unaffected by all concentrations of DTT used in these experiments (data not shown).

Effect of DTT on virus morphology

Fig. 5(a) shows an HEV particle from a control preparation which displays the characteristic coronavirus morphology (Greig et al. 1971). Most particles showed typical pleomorphism with diam. ranging from 80 to 100 nm covered with bulbous projections 15 to 20 nm in length. Occasional particles displayed atypical projection morphology (Fig. 5 b). Instead of the projections being bulbous or petal-like, they appeared thin and fibrous but having a similar length. Estimates from a control preparation revealed the presence of no more than 5% of these particles with atypical projections. Examination of virus pre-treated with concentrations of DTT up to 10 mm showed the virus morphology to be similar to that of the control preparation. With higher concentrations, increasing numbers of particles with atypical projections were seen. Fig. 5(b) and (c) show two particles from a preparation treated with 50 mm-DTT. At this concentration no particles were observed with typical coronavirus projections.

DISCUSSION

The effect of various concentrations of DTT on the biological activities, morphology and polypeptide composition of HEV was examined. The results indicated that both the infectivity and HA activity of the virus were destroyed by DTT. The infectivity was found to be
Effect of sulphydryl reagents on HEV

Fig. 5. The effect of DTT on the morphology of HEV. Samples of virus were treated for 1 h at 37 °C with either distilled water (a & b) or 50 mM-DTT (c & d) prior to staining with potassium phosphotungstate.

more sensitive than the HA to the action of the sulphydryl reagent, 1.5 mM-DTT being required to reduce the infectivity by 50% whereas between 4 and 5 mM was required to diminish the HA by a similar amount. The infectivity of TGEV, the other porcine coronavirus so far isolated, was found to be insensitive to 10 mM-DTT.

Ozawa et al. (1976) described the importance of an interpeptide disulphide bond between two external glycoproteins of Sendai virus which when cleaved by DTT destroyed both the haemagglutination and neuraminidase activities of the virus. The presence of such bonds can be shown by the appearance of high mol. wt. complexes in polyacrylamide gels when the virus has been dissociated under non-reducing conditions. When such conditions were applied to HEV the electrophoretic pattern was identical to that obtained from virus dissociated in the presence of 1% 2-mercaptoethanol, indicating the absence of interpeptide disulphide bonds. When the concentration of this sulphydryl reagent was increased to 2%, a reduction in the mobility of the high mol. wt. glycoprotein gp 180 was observed. This reduction in mobility and hence increase in the apparent mol. wt. of the glycoprotein can be explained by the cleavage of an inaccessible intrapeptide disulphide bond leading to a greater unfolding of the polypeptide. Pre-treatment of the virus with 100 mM-DTT did not appear to cleave this bond although it is possible that reformation could have occurred after removal of the DTT prior to disruption of the virus with SDS.

Even though interpeptide disulphide bonds could not be detected between any of the HEV polypeptides, treatment of the virus with 100 mM-DTT resulted in the removal of 100% of
gp 125 and between 60 and 70% of gp 100. Under increasing DTT concentration the removal of gp 125 was found to be logarithmic. Between 5 and 6 mM-DTT was required to remove 50% of this polypeptide, a similar value to that needed to reduce the virus HA by the same amount. The rates of loss of gp 125 and HA activity with increasing DTT concentration, as determined from the log plots shown in Fig. 1(b) and Fig. 4, were similar which suggested that gp 125 is an essential part of the HEV haemagglutinin. The amount of gp 100 associated with the virus remained constant with treatments of up to 20 mM-DTT but higher concentrations caused the removal of some of this polypeptide. Gp 180, p 56 and gp 26-5 were unaffected by any of the DTT concentrations used in these experiments. The removal of polypeptides from the surface of the virus could be due to a change in conformation of the protein following the cleavage of intrapeptide disulphide bonds. The variation in the concentration of DTT required for the removal probably reflects the accessibility of the disulphide bonds to the reagent.

Little or no effect on the polypeptide composition of the virus was observed with DTT concentrations as low as 1.5 mM, the concentration at which 50% of the infectivity was lost. The effect on infectivity was probably due to the cleavage of a readily accessible intrapeptide bond resulting in an alteration of the polypeptide conformation such that the protein can no longer perform its normal function. It was not possible from these experiments to determine which step in the virus replication cycle was blocked by this treatment, or which polypeptide was involved.

As expected, the loss of protein from the surface of the virus resulted in an altered morphology. The major variation between control and DTT-treated virus occurred in the morphology of the characteristic coronavirus projections. Because of the number and bulbous nature of these projections covering the control particles, it was difficult to obtain exact detail of the surface structure of the virus. The fibrous projections seen on DTT-treated particles may therefore have resulted from the removal of protein with the consequent loss of their bulbous form, or their presence could indicate that more than one type of projection occurs, the bulbous type being sensitive to, hence removed by DTT treatment, the other fibrous type being insensitive becomes visible in the electron microscope. From this investigation it was not possible to determine which of these alternatives was correct.

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REFERENCES


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