Identification of an Outer Capsid Glycoprotein of Human Rotavirus by Concanavalin A

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SUMMARY

An outer capsid glycoprotein of human rotavirus (serotype 1) was localized and characterized by use of lectins, electron microscopy and a modified Western blotting technique. Lectins with specificity for fucose, galactose, glucose and mannose were mixed with purified single- or double-shelled human rotavirus. Aggregates observed by electron microscopy were obtained with double-shelled virus and concanavalin A, the only tested lectin with mannose specificity. By SDS-polyacrylamide gel electrophoretic analysis nine structural polypeptides could be identified. Five of these polypeptides were components of the inner capsid (VP1, VP2, VP3, VP4, VP6) and four components of the outer capsid (VP5, VP7, VP8, VP9). When using a modified Western blotting technique employed for glycoprotein detection, only VP7 was found to be glycosylated. This glycoprotein could be identified in rotavirus from human stools as well as from cell cultures. Heterogeneity in molecular weight of VP7 was observed in different isolates. An unexpected heterogeneity of VP7 was seen within a human rotavirus strain. An un-plaqued stock of virus was found to exhibit two distinguishable glycoprotein bands (VP7, VP7a). After 10 passages in MA-104 cells the same strain was found to exhibit only one glycoprotein band.

INTRODUCTION

Glycoproteins have been identified in only a few non-enveloped viruses (Ishibashi & Maizel, 1974). Therefore, recognition that the non-enveloped rotaviruses encode for two glycoproteins, one structural and one non-structural, is of special interest (Ericson et al., 1983; Estes et al., 1983). The structural glycoprotein of human rotavirus has only been subjected to some characterization (Rodger et al., 1977) whereas simian and bovine rotaviruses are well characterized (Ericson et al., 1983; Estes et al., 1982; Killen & Dimmock, 1982; Matsuno & Mukoyama, 1979). Available data indicate that the glycoprotein, which is the type-specific neutralization antigen (Bastardo et al., 1981; Killen & Dimmock, 1982), contains an N-linked oligosaccharide with a high-mannose oligosaccharide moiety (Ericson et al., 1982).

Characterization of glycoproteins has generally been accomplished by radiolabelling of the carbohydrates, whereas detection of carbohydrates can be done by periodic acid–Schiff staining. A modification of the latter technique was used by Rodger et al. (1977) for detection of a glycoprotein of human rotavirus. A limitation of the staining technique is its low sensitivity and that no detailed characterization of the carbohydrate moiety on the glycoprotein is possible. Due to the difficulty of cultivating human rotavirus in vitro, a promising approach for characterization of the glycoproteins of the virus might be to utilize lectins.

The aim of the present study was to localize and characterize the glycoprotein on cultivable and non-cultivable human rotavirus by use of lectins, electron microscopy and a modified Western blotting technique.
Cells and viruses. Foetal rhesus monkey cells, MA-104, were grown in Eagle’s MEM with 10% foetal calf serum. For large-scale production, culture bottles of 2 l vol. were seeded with MA-104 cells.

A cultivable human rotavirus strain, serotype 1 (I3160/81), was obtained from T. Flewett (Birmingham, U.K.). Simian rotavirus SA-11 was obtained from Institut für Medizinische Mikrobiologie und Virologie, Düsseldorf, F.R.G. Both strains were treated with 20 μg/ml trypsin (Difco) for 30 min and inoculated to MA-104 cells. After an adsorption period of 1 h at 37 °C, MEM containing 5 μg/ml trypsin without serum was added. The cells were harvested 2 to 3 days post-infection and frozen and thawed twice. The cell debris was discarded after centrifugation at 2000 g for 10 min.

Purification of rotavirus. Cell supernatants and stool specimens prepared as 10% suspensions were extracted twice with Freon 113 (trifluorotrichloroethane). The virus present in the aqueous phase after centrifugation for 20 min at 2000 r.p.m. was subsequently centrifuged for 2 h at 100000 g through 1 ml 35% sucrose and 1.5 ml of 1.33 g/ml CsCl onto a CsCl (1.40 g/ml) cushion. The virus band visible on the cushion was removed and mixed with 20 °g/ml Triton X-100.

GluNAc), concanavalin A (Glu, Man).

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Electron microscopy of lectin–virus interactions. Lectins were dialuted in a buffer containing 25 mM-Tris–HCl, 5 mM-CaCl2, 5 mM-MgCl2, 70 mM-NaCl, pH 7-2. To a 10 μl lectin suspension 50 μl purified human rotavirus (110 μg/ml) was added. After 1 h incubation at room temperature a drop of the mixture was added to a carbon–Formvar-coated grid; 0.5 min later the grid was stained with a drop of 2% phosphotungstic acid pH 7.2. The excess fluid was removed with filter paper before the grid was examined for 5 min for the presence of rotavirus aggregates in a Philips 200 electron microscope. Specimens were scored as positive if virus aggregates containing more than 20 particles were seen.

Polyacrylamide gel electrophoresis (PAGE). Samples were analysed in SDS–polyacrylamide gels (13% polyacrylamide, 0.39% bisacrylamide) by using the discontinuous Tris–glycine buffer system of Laemmli (1970). Proteins were dissociated for 5 min at 100 °C in a sample buffer containing 10 mM-Tris–HCl, 2-5% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue pH 6-8. Polypeptides were stacked at 10 mA/gel and the electrophoresis was continued at 30 mA until the bromophenol blue dye ran out of the gel. Gels were fixed and stained with 0.2% Coomassie Brilliant Blue in 50% methanol/10% acetic acid and destained in 30% methanol/10% acetic acid.

Electrophoretic transfer of separated proteins to nitrocellulose sheets. The electrophoretically separated proteins were transferred immediately to nitrocellulose sheets (BA85; Schleicher & Schüll) by 25 mM-Tris/192 mM-glycine/20% methanol (pH 8-3) essentially as described by Towbin et al. (1979). The transfer was performed for 3–5 h at room temperature at 0.22 A in a Bio-Rad Trans-Blot system. The transfer was monitored by staining the nitrocellulose sheet with amido black (Towbin et al., 1979).

Detection of concanavalin A-binding glycoproteins. After transfer to the nitrocellulose sheet the protein binding sites were blocked by soaking the sheet in a buffer containing 10 mM-Tris–HCl, 100 mM-NaCl, 10 mM-Mg2+, 10 mM-Ca2+, pH 7-35, and 2-5% bovine serum albumin (BSA) for 60 min at room temperature. The BSA was used to periodate-oxidized in order to remove any lectin-binding activities (Glass et al., 1981). The following steps were essentially those described by Clegg (1982). The nitrocellulose sheet was incubated for 60 min at room temperature with 10 μg/ml concanavalin A (Pharmacia), 0-05% Triton X-100 in buffer. The sheets were then washed (5 x 5 min) in buffer with Triton X-100 and incubated with the glycoprotein horseradish peroxidase (25 μg/ml; Sigma) in the same buffer for a further 60 min. Excess enzyme not bound to concanavalin A was removed by washing (5 x 5 min) in buffer. The glycoprotein bands were detected by incubation with freshly prepared 0.05% 4-chloro-1-naphthol (Sigma) and 0.01% H2O2. Concanavalin A-binding glycoproteins appeared blue within 1 to 5 min.

3H]Glucosamine labelling of human rotavirus. A confluent MA-104 monolayer in a 800 cm2 roller bottle was infected with 1 ml trypsin-activated human rotavirus strain I3160/81 (105.0 TCID50/0.1 ml). After 1 h adsorption at 37 °C the inoculum was removed and replaced with Eagle’s MEM containing 5 μg/ml trypsin. At 6 h post-infection, the medium was changed to 60 ml Eagle’s MEM containing 5 μg/ml trypsin, 8 mM-fructose, 1/5 of normal glucose concentration and 1-5 mM D-[1,6-3H]glucosamine hydrochloride (New England Nuclear). At 30 h post-infection double-shelled particles were purified. The 14C-methylated protein markers (Amer sham) were myosin, mol. wt. 200000 (200K), phosphorylase b (92-5K), BSA (69K), ovalbumin (46K), carbonic anhydrase (30K) and lysozyme (14.3K).
Fig. 1. (a, b) Electron microscopy of purified single-shelled (a) and double-shelled (b) human rotavirus. (c) Interaction between purified double-shelled human rotavirus strain 13160/81 and concanavalin A (110 µg/ml). (d) Same rotavirus in the absence of concanavalin A. Bar markers represent 100 nm (a, b) and 200 nm (c, d).
Fig. 2. SDS-PAGE of 160 µg purified double-shelled (b) and 115 µg single-shelled (c) human rotavirus strain I3160/81. (a) Molecular weight standards: phosphorylase (94K), albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20.1K), and ß-lactalbumin (14.4K) (Pharmacia). The nomenclature of VP1 to VP9 refers to the structural polypeptides, with VP1 representing the largest polypeptide.

RESULTS

Localization of an outer capsid glycoprotein of human rotavirus by concanavalin A and electron microscopy

The ability of different lectins to interact with purified single- and double-shelled human rotavirus isolated from stools and cell cultures was studied by electron microscopy. Lectins with different sugar specificities were tested in concentrations from 200 to 12.5 µg/ml. Only concanavalin A was able to aggregate rotavirus (Fig. 1c), and this aggregation only occurred
Identification of a human rotavirus glycoprotein

Fig. 3. Detection of a structural glycoprotein of human rotavirus by concanavalin A in a modified Western blotting technique. The viral polypeptides were separated by SDS–PAGE and transferred to a nitrocellulose sheet at 0·22 A for 3·5 h. Amido black staining of molecular markers (a) and of the polypeptides of double-shelled human rotavirus strain 13160/81 (b) is shown. Glycoprotein detection (c to h): human rotavirus purified from different stools (c, d); simian rotavirus SA-11 (e); occurrence of one (f) and two (g) structural glycoproteins VP7, VP7a in different passages of a human isolate (strain 13160/81); ovalbumin (h), a 43K glycoprotein containing a high-mannose oligosaccharide (Kornfeld & Kornfeld, 1980).

Fig. 4. SDS–PAGE of [3H]glucosamine-labelled double-shelled human rotavirus (strain 13160/81). Positions and corresponding molecular weights of the 14C-methylated protein markers are shown on the right.

with double-shelled particles. No aggregation was obtained by other lectins. By using α-methyl-D-mannoside (200 mM), a sugar inhibitor for concanavalin A binding, the aggregation was prevented as well as dissociated.

**SDS–PAGE of human rotavirus polypeptides**

The polypeptides of purified single- and double-shelled human rotavirus (strain 13160/81) were separated by SDS–PAGE and designated VP1 to VP9 (Fig. 2) as described by Estes et al.
Single-shelled virions were found to contain five structural polypeptides with approximate mol. wt. of 118000 (VP1), 92000 (VP2), 88000 (VP3), 84000 (VP4) and 41000 (VP6). Double-shelled virions in addition contained four polypeptides, with mol. wt. of 60000 (VP5), 38000 (VP7), 30000 (VP8) and 26000 (VP9). The Coomassie Brilliant Blue-stained SDS gel (Fig. 2) shows that VP2 and VP6 are the major structural polypeptides of single-shelled virions, whereas VP5 and VP7 are the major polypeptides of the outer capsid. In order to detect VP8 and VP9 it was necessary to use more than 100 μg virus/slot.

**Detection of a glycosylated polypeptide of human rotavirus using concanavalin A in a modified Western blotting technique**

To characterize further the polypeptides of the outer capsid (VP5, VP7, VP8, VP9), concanavalin A was employed in a modified Western blotting technique used for glycoprotein detection (Clegg, 1982; Glass *et al.*, 1981). For identification of any glycosylated polypeptide(s) of human and simian rotavirus the viral polypeptides were first separated by SDS–PAGE and then transferred to a nitrocellulose sheet. The glycoproteins were then identified by concanavalin A and peroxidase as described in Methods.

One structural glycoprotein of human rotavirus was identified (Fig. 3). The glycoprotein corresponded in size to VP7 and could be detected both in rotavirus material purified from stools and from cell cultures. The mol. wt. of the glycoprotein was found to vary between 36000 and 38000 with different isolates (Fig. 3). No correlation was seen between the molecular weight of VP7 and the origin (cell culture or stool) of the strains.

The specificity of the method was monitored by inhibiting the binding of concanavalin A to the glycoprotein in the presence of 200 mM-α-methyl-D-mannoside. Radiolabelling of human rotavirus with [3H]glucosamine confirmed the presence of one structural glycoprotein (Fig. 4). One structural glycoprotein, with a molecular weight of approximately 37000 (VP7) was detected in simian rotavirus SA-11 (Fig. 3c); this finding is in agreement with results previously reported (Ericson *et al.*, 1983; Estes *et al.*, 1982).

Diversity of VP7 electrophoretic mobility was seen also within the same virus strain. An unaquailed stock of the human rotavirus strain I3160/81 was found to exhibit two distinguishable glycoprotein bands with approximate molecular weights of 38000 and 37000. However, after ten passages the isolate was found to exhibit only one glycoprotein band (Fig. 3). The occurrence of two glycoprotein bands designated VP7, VP7a was due neither to overloading of the slot in the PAGE experiment, nor to virus contamination as controlled by RNA electrophoresis.

**DISCUSSION**

The present study reports localization and characterization of an outer capsid glycoprotein (VP7) of human rotavirus from stools or from cell cultures by use of lectins, electron microscopy and a modified Western blotting technique.

The ability of lectins with different sugar specificities to interact with single- and double-shelled human rotavirus was monitored by electron microscopy. Aggregates were seen only with double-shelled virus and concanavalin A, the only lectin with mannose specificity. It is proposed from the sugar specificity of concanavalin A that the oligosaccharide on the glycoprotein contains mannose and/or glucose. It is also proposed that the oligosaccharide does not contain N-acetyl glucosamine in the terminal position, nor fucose or galactose as lectins with this sugar specificity had no effect on the virus. The results presented indicate the presence of a high-mannose oligosaccharide on the glycoprotein. The glycoprotein of simian rotavirus SA-11 has previously been reported to contain a high-mannose oligosaccharide (Ericson *et al.*, 1982).

The identification of nine structural polypeptides of human rotavirus is in agreement with results by Offit *et al.* (1983) and Dyall-Smith & Holmes (1981). The fourth outer capsid protein (VP9) of human rotavirus, in the present study a 26000 mol. wt. protein and in studies by Offit *et al.* (1983) a 27000 mol. wt. protein, has not been identified from virus purified from stools (Rodger *et al.*, 1977; Espejo *et al.*, 1980). Detection of the smallest polypeptide (VP9) is possibly a reflection of the large quantity of double-shelled virus used in PAGE. Due to the presence of
trypsin during cultivation, the protein could also be a cleavage product from a larger protein. An example of the latter is the cleavage of the 88K protein of SA-11 to 60K plus 28K (Estes et al., 1981).

The rotavirus genome has been reported to code for one structural glycoprotein (Ericson et al., 1983), which was confirmed by this study. The glycoprotein was found to be identical to VP7, and could be identified in rotavirus from human stools as well as from cell cultures. The molecular weight of VP7 was found to be similar in two of the three human rotaviruses (Fig. 3), although their RNA patterns were all different. In addition to VP7 the fourth outer capsid protein (VP9; Dyall-Smith & Holmes, 1981) and a protein designated VP10C (McCrae & McCorquodale, 1982) have been suggested to be glycosylated. In this study only one glycoprotein band was found by the concanavalin A–peroxidase method and by autoradiography. In a previous study the concanavalin A–peroxidase method also gave results consistent with results of autoradiography using purified isotope-labelled virus (Clegg, 1982).

A diffuse protein band located between VP7 and VP8 was seen using an unplaqued stock of rotavirus. The polypeptide, designated VP7a according to the nomenclature of Estes et al. (1981, 1982), was found to be glycosylated. It has previously been reported that simian rotavirus SA-11 can give one or two glycoprotein bands (Estes et al., 1981, 1982). Two glycoprotein bands of VP7 have also been reported for calf rotavirus (Killen & Dimmock, 1982; McCrae & McCorquodale, 1982). The occurrence of two glycoprotein bands of VP7 within an unplaqued stock of human rotavirus could be due to a mixture of several different phenotypes as suggested by Estes et al. (1982). As our previous stock was not plaque-purified this can not be excluded. VP7a could also be a proteolysis product of VP7, although that is not the case for VP7a of simian rotavirus SA-11 (Estes et al., 1981).

The combined use of concanavalin A and the modified Western blotting technique is a promising method for characterization of glycoproteins of non-cultivable viruses. Here, this technique was used for the first time with a non-enveloped virus. It is simple to perform and gave reproducible results. Concanavalin A may in addition be used for purification of the rotavirus glycoprotein.

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