Polypeptides of Bovine Rotavirus

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SUMMARY

Polyacrylamide gel electrophoresis of bovine rotavirus or neonatal calf diarrhoea virus (NCDV) grown in cell culture resolved eight species of polypeptide. The inner shell particles contained five polypeptides and the outer shell three polypeptides. A major polypeptide of the outer shell was glycosylated. The infectivity of NCDV was enhanced by treatment with trypsin in vitro. All eight polypeptides were affected by trypsin treatment as judged by diminished intensity of polypeptide bands by radiography and several new bands appeared. The intracellular synthesis of NCDV polypeptides was studied by pulse and pulse-chase experiments. Infected cells contained all eight virus capsid proteins and, in addition, three presumably virus-specific polypeptides which were non-capsid polypeptides (NCVP). There was no evidence that any of these polypeptides was processed after synthesis. It is suggested, therefore, that all these polypeptides are primary gene products.

INTRODUCTION

Bovine rotavirus, like other rotaviruses isolated from various animals including man, is an agent of gastroenteritis in newborns (Kraft, 1957; Malherbe & Strickland-Cholmley, 1967; Mebus et al. 1969; Bishop et al. 1973; Flewett et al. 1973, 1975; Rodger et al. 1975a; Bryden et al. 1976; McNulty et al. 1976; Tzipori et al. 1976). During the acute phase of infection, particles of these viruses are excreted into faeces in high concentration (Davidson et al. 1975). The abundance of virus particles in faeces has made it possible to study the morphology, chemical composition and immunological properties of these viruses extensively, in spite of the difficulty in growing some members of this group in cell cultures. It is now generally accepted that rotaviruses from different animal species are similar in morphology, consisting of double shells (Flewett et al. 1973; Holmes et al. 1975; Bridger & Woode, 1976).

With NCDV obtained from faeces, the outer and inner shells were reported to contain four and five species of polypeptides respectively (Rodger et al. 1977). We have reported that the established line of monkey kidney cells, MA-104, provides a convenient system for both efficient growth and plaque assay of NCDV (Matsuno et al. 1977). The present communication deals with the polypeptide analysis of NCDV grown in these cells and the intracellular synthesis of structural and non-structural polypeptides.

METHODS

Cells and virus. MA-104 (macacus Rhesus monkey kidney) cells were grown in monolayers in tissue culture flasks or dishes in Eagle's minimal essential medium (MEM), containing 10% foetal calf serum. Propagation and plaque assay of NCDV (Lincoln...
strain) have been described previously (Matsuno et al. 1977). Virus was plaque-purified three times and passaged once or twice in the above cell line. When cytopathic effect (c.p.e.) involved approx. 75% of the monolayer, the infected cultures were frozen and thawed three times and stored at \(-70\) °C.

**Isotopic labelling and purification of virus.** Confluent monolayers of MA-104 cells were infected at a multiplicity of 10 to 30 p.f.u./cell. After 1 h adsorption at 37 °C, the inoculum was removed and the medium containing a radioactive precursor was added. The media used were MEM with amino acids reduced to 1/10 of the usual concentration and \(^{14}\text{C}-\)amino acid mixture (1 \(\mu\)Ci/ml), MEM with glucose reduced to 1/3 of the usual concentration and \(^{14}\text{C}-\)glucosamine (2 \(\mu\)Ci/ml), or methionine-deficient MEM with \(^{35}\text{S}\)-methionine (5 \(\mu\)Ci/ml). At 16 h after infection, the cells and medium were frozen and thawed three times. Cell debris was removed by centrifugation at 3000 g for 30 min and the virus was sedimented by centrifugation for 3 h at 60000 g. The pellet was resuspended in phosphate-buffered saline (PBS) and caesium chloride was added to a final density of 1.36 g/ml. Centrifugation for 16 h at 35000 rev/min in a Beckman SW 50.1 rotor at 4 °C resulted in the formation of two bands at the buoyant density of 1.36 g/ml and 1.38 g/ml. By electron microscopy, these two bands were found to contain virions (Fig. 1a) and inner shell particles (Fig. 1b), respectively.

**Electron microscopy.** Virion and inner shell particle preparations were negatively stained with 2% potassium phosphotungstate (pH 7.2) and examined with a JEM 100 V electron microscope.

**Isotopic labelling of intracellular virus proteins.** Confluent monolayers of MA-104 cells were infected with virus at a multiplicity of 30 p.f.u./cell. Before labelling with \(^{35}\text{S}\)-methio-
nine, monolayers were pre-incubated for 30 min in methionine-free MEM. Labelling was carried out at 20 μCi/ml of 85S-methionine in methionine-free MEM. In pulse-chase experiments the labelling medium was removed after the pulse and monolayers were washed three times with Eagle's MEM and incubated in MEM containing the standard amount of unlabelled methionine.

At the end of pulse or chase period the monolayers were washed three times with ice-cold PBS. The cells were scraped off with a rubber policeman and suspended in 1 ml of PBS. The suspension was frozen at -70 °C.

**Treatment of virions with trypsin.** Purified virions were treated with 10 μg/ml of acetyltrypsin for 30 min at 37 °C. After incubation, the samples were pelleted by centrifugation at 60000 g for 3 h at 4 °C and resuspended in either gel sample buffer for SDS-polyacrylamide gel electrophoresis or PBS for infectivity titration. Infectivity was determined by plaque assay on MA-104 cells as described (Matsuno et al. 1977) except that the agar overlay did not contain trypsin, but contained 300 μg/ml of DEAE dextran.

**Polyacrylamide gel electrophoresis (PAGE).** Polypeptides were examined by electrophoresis in polyacrylamide slab gel with SDS and discontinuous buffer system. Either 8 % gels containing 4 M-urea or 13 % gels without urea were used (Lamb & Choppin, 1977). Samples precipitated with 8 vol. of acetone at -20 °C were dissolved in 6 M-urea, 1 % SDS, 1 % 2-mercaptoethanol, 0.005 % bromophenol blue and boiled for 2 min. Mol. wt. of polypeptides were estimated by the method described (Shapiro et al. 1967). As a reference, *Escherichia coli* RNA polymerase subunits, β' (165000), β (155000), and α (39000) and bovine serum albumin (68000), were used.

Electrophoresis was carried out at a constant voltage of 40 V for 16 h. Fuji 400 Safety Screen X-ray film was exposed to the dried gel.

**Chemicals and isotopes.** D-14C(U)-glucosamine (200 mCi/mmol), L-14C(U)-amino acid mixture, and L-35S-methionine (250 to 600 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Reagents for polyacrylamide gel electrophoresis were obtained from the Wako Pure Chemical Corp., Tokyo, Japan. Trypsin, acetylated, was obtained from Sigma Chemical Corp., St Louis, Mo., U.S.A. The standard proteins were purchased from Boehringer, Mannheim, Germany.

**RESULTS**

**Polypeptide composition of virion and inner shell particle**

Radiolabelled virions of NCDV grown in MA-104 cells were purified and analysed by polyacrylamide gel electrophoresis (Fig. 2). Electrophoresis of disrupted virions labelled with 14C-amino acid mixture in 8 % gels containing urea resolved eight species of polypeptides. Polypeptide bands were numbered in the order of increasing electrophoretic mobility, following Rodger et al. (1975b). A polypeptide not described by these authors was found between VP6 and VP7 on 8 % gels but not on 13 % gels and was designated tentatively as VP6a (Fig. 2a). On the other hand VP4 and VP9 contained in virions purified from calf stools (Rodger et al. 1975b, 1977) were absent from our preparations in both gel systems. Inner shell particles, as described in the Methods, were shown to consist of five polypeptides (VP1, VP2, VP3, VP6 and VP6a) (Table 1). By subtraction, the outer shell was presumed to consist of three other polypeptides (VP5, VP7 and VP8; Table 1). Labelling with 14C-glucosamine showed that VP7, the major polypeptide of the outer shell, was a glycopeptide [Fig. 2(4)].
Fig. 2. PAGE of NCDV virions and inner shell particles labelled with $^{14}$C-amino acid or $^{14}$C-glucosamine. Migration is from top to bottom. (1) Purified virions. (2) Purified virions were incubated for 30 min at 37 °C with trypsin, 10 μg/ml. (3) Inner shell particles. (4) $^{14}$C-glucosamine labelled virions. (a) 8 % polyacrylamide gel containing 4 M-urea was used. (b) 13 % polyacrylamide gel was used.

**Enhancement of infectivity by the treatment of NCDV with trypsin in vitro**

We have previously reported that the efficient plaque formation of NCDV on MA-104 cells required the addition of trypsin in the agar overlay medium (Matsuno et al. 1977). Whether the enhanced plaque formation was due to a direct effect of trypsin on the virion or not was examined. NCDV grown in MA-104 cells in the absence of trypsin was treated with trypsin in vitro, and the infectivity before and after the treatment was compared. Trypsin was not incorporated into the agar overlay medium in order to avoid a possible activation of inactive virus particles on the agar plate for infectivity titration. After the publication of our previous report (Matsuno et al. 1977), we found that NCDV did form plaques in the absence of trypsin as efficiently as in its presence, if the concentration of DEAE dextran in the overlay was increased to 300 μg/ml (unpublished observation). The treatment with 10 μg/ml of acetyl-trypsin for 30 min at 37 °C resulted in a 64-fold increase in infectivity ($3.39 \times 10^9$ to $2.18 \times 10^{11}$ p.f.u./ml). It was concluded, therefore, that the enhanced plaque formation was due to the activation of non-infectious virus particles by proteolytic activity of trypsin. We examined the change in polypeptide composition of the virion accompanying the enhancement of infectivity. Treatment with trypsin diminished the intensity of all eight polypeptides in the radio-autogram regardless of whether they were located internally or outside the virion and 7 to 8 new bands appeared (Fig. 2(2)]. One of them migrated to a position corresponding to that of VP4, and the other to that of VP9, described for virions purified from calf stools (Rodger et al. 1975b, 1977).
**Bovine rotavirus polypeptides**

Table 1. *Molecular weights of the polypeptides of NCDV* *

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Mol. wt.</th>
</tr>
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<tbody>
<tr>
<td>VP 1</td>
<td>130,000</td>
</tr>
<tr>
<td>2</td>
<td>115,000</td>
</tr>
<tr>
<td>3</td>
<td>97,000</td>
</tr>
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<td>5</td>
<td>68,000</td>
</tr>
<tr>
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<td>34,000</td>
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<td>7</td>
<td>28,000</td>
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<tr>
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</tr>
<tr>
<td>2</td>
<td>17,500</td>
</tr>
<tr>
<td>3</td>
<td>13,500</td>
</tr>
</tbody>
</table>

* The resolving gel contained 8% acrylamide and 0.213% N,N'-methylenebisacrylamide containing 4 M-urea.

† The polypeptides were numbered by the system of Rodger *et al.* (1975a) as shown in Fig. 2.

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**Fig. 3**
Fig. 3. Single cycle growth curve of the NCDV in MA-104 cells. The multiplicity of inoculation was 30 p.f.u./cell.

**Fig. 4**
Fig. 4. The synthesis of polypeptides in NCDV-infected MA-104 cells. Infected and uninfected cells were labelled with $^{35}$S-methionine for 20 min at various times after infection and processed for electrophoresis and autoradiography. Polypeptide separation is on 8% slab gels containing 4 M-urea and migration is from top to bottom. U, uninfected.
Fig. 5. Pulse-chase experiments in NCDV-infected MA-104 cells. Infected cells were labelled at 8 h after infection with $^{35}$S-methionine for 20 min and the labelling medium was then removed and replaced with excess MEM. At the times indicated after removal of the label, cultures were processed for electrophoresis and autoradiography.

**Synthesis of NCDV-specific polypeptides in infected cells**

When MA-104 cells were infected with NCDV at the multiplicity of 30 p.f.u./cell, infectious progeny virus appeared at 4 h and increased exponentially to a peak titre at 10 to 12 h (Fig. 3).

To study the synthesis of virus polypeptides during the virus growth cycle, a series of cultures was infected with NCDV at the same multiplicity and labelled with $^{35}$S-methionine for 20 min at various time intervals after infection (Fig. 4). At 2 h, no virus polypeptides were discerned beyond background incorporation into cellular protein. At 4 h, all virion polypeptides other than VP5 became visible. In addition, three polypeptides that had not been seen in the virion, NCVP1, 2, and 3, were labelled in infected cells (Table 1). By 6 h, all eight capsid polypeptides and the three NCVPs were clearly shown. All these polypeptides appeared to be synthesized at a nearly constant rate from 6 h onwards.

In order to eliminate the possibility that any of the NCVPs were derived from precursors by proteolytic cleavage, cultures were chased after 20 min pulse-labelling at 8 h (Fig. 5). The PAGE patterns did not change appreciably during a chase period of up to 2 h. A chase as short as 5 min after the pulse resulted in a similar pattern (data not shown). The three NCVPs were apparently not derived from other polypeptides but were probably synthesized as such.
DISCUSSION

The polypeptide composition of NCDV virions we present is different in some respects from the one published previously (Rodger et al. 1975b). Two polypeptide bands (VP4 and VP9) were missing in our electropherograms and, instead, a new band (VP6a) was present. Such a difference might have resulted from the use of different gel systems, as evidenced by the fact that VP6a was resolved in 8 % gels containing urea but not in 13 % gels without urea. But it is equally possible that some of the different PAGE patterns resulted from the different sources of virus. While we have studied virions grown in cell cultures, previous investigators made use of virions derived from calf stools. It is possible that the latter preparations had been subjected to limited proteolysis in the intestinal tract. When cell culture-grown virions were treated with trypsin, all virion polypeptides were more or less affected and some presumably split products were electrophoresed with similar migration patterns to the missing polypeptides.

Since our finding of enhanced plaque formation by trypsin, other investigators have confirmed the enhancing effect of trypsin for the growth of NCDV under fluid medium (Babiuk et al. 1977; Almeida et al. 1978). The present study showed that trypsin activated NCDV in vitro. However, enhancement of infectivity by trypsin treatment was not traced to a modification of a specific polypeptide component and its mechanism remains unsolved. It is also possible that trypsin inactivated an inhibitor which prevented the attachment of virions to the cells. However, our unpublished finding that the treatment of virus with trypsin did not alter the virus adsorption to the cell argues against this possibility.

In addition to eight capsid polypeptides, our study revealed the presence of three polypeptides which were not found in the virion. Since they remained unchanged during the chase period, they were unlikely to be derived from the capsid polypeptides but were probably new species that were synthesized in the cell but not incorporated into the virion. Nine species of reovirus-specified polypeptides have been detected in infected cells. Eight of these are primary gene products (designated λ1, λ2, μ0, μ1, δ1, δ2, δ2A, and δ3), while one (μ2) is derived from μ1 by cleavage. Six of the primary gene products are capsid polypeptides, while two, namely μ0 and δ2A, are not (Zweerink et al. 1971). Similarly, there was no indication that the eight capsid polypeptides of NCDV were modified after synthesis. It is suggested, therefore, that the eleven polypeptides which we found were all primary products coded for by the NCDV genome.

The number of gene products found in the present study is in agreement with the number of dsRNA segments in NCDV, reported to be 11 to 12 (Newman et al. 1975; Rodger et al. 1975b; Kalica et al. 1976). Most, if not all, of these RNA segments are therefore likely to be monocistronic messages.

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REFERENCES


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