Isolation from Chickens of a Rotavirus Lacking the Rotavirus Group Antigen

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SUMMARY

A virus, designated 132 virus, was isolated from the faeces of chickens in chick embryo liver cell cultures. The morphology and morphogenesis of 132 virus were indistinguishable from that of rotaviruses. The nucleic acid of 132 virus had the nuclease resistance of double-stranded RNA, and was separated by polyacrylamide gel electrophoresis into 11 segments with mol. wt. ranging from $2.07 \times 10^6$ to $0.20 \times 10^6$. SPF chickens were susceptible to oral infection with 132 virus, which replicated in the villous epithelial cells of the small intestine. 132 virus was therefore a rotavirus by morphological, biochemical and biological criteria. However, by immunofluorescence it was not possible to demonstrate an antigenic relationship between 132 virus and known avian and mammalian rotaviruses, indicating that 132 virus does not possess the group antigen shared by all previously characterized rotaviruses. This finding has implications for the diagnosis of rotavirus infections by serological tests.

INTRODUCTION

Rotavirus infections have been associated with enteritis and diarrhoea in a variety of mammalian (Flewett & Woode, 1978; McNulty, 1978) and avian (McNulty et al., 1979a, 1980) species. Rotaviruses possess a double-stranded RNA genome consisting of 11 segments and are now classified as a separate genus in the Reoviridae family (Matthews, 1979). Although different serotypes of rotavirus have been recognized, all the rotaviruses isolated so far, whether of mammalian or avian origin, share a common group antigen which can be detected by immunofluorescence or complement fixation tests (Kapikian et al., 1976; Woode et al., 1976a; Thouless et al., 1977; McNulty et al., 1979a, 1980). In this paper we report the isolation of a virus, designated 132 virus, which has morphological, biochemical and biological characteristics of rotaviruses yet lacks the rotavirus group antigen.

METHODS

Isolation of 132 virus. Approx. 15% suspensions of faeces were made in Eagle's (BHK) medium containing 1000 units penicillin/ml and 1000 μg streptomycin/ml. These were centrifuged at 3000 g for 30 min and the supernatant was used to infect chicken embryo liver (CEL) cell cultures.

Prior to inoculation, faecal suspensions were treated for 1 h at 37 °C with trypsin at a final concentration of 5 μg/ml and the same concentration of the enzyme was incorporated into the maintenance medium (McNulty et al., 1979a, 1980). Normally cultures were harvested 24 h after infection and growth of virus was assessed by immunofluorescent staining. However, from the 9th to the 19th passage of 132 virus in CEL cultures, 48 h passage periods were used with fresh trypsin (5 μg/ml final concentration) added to the medium at 24 h. For serial
passage the whole culture was stored at \(-70 \, ^\circ\text{C}\) until fresh cells were available, then thawed and inoculated as above.

**Preparation of chicken embryo liver cell cultures.** CEL cultures were prepared from the livers of 12- to 14-day-old embryos. Embryonated hens' eggs were obtained from the laboratory's specific pathogen-free (SPF) flock. This flock is kept in houses with a positive pressure filtered air ventilation system and is regularly tested for freedom from infection with a number of micro-organisms, including rotaviruses. Using the indirect immunofluorescence test (McNulty et al., 1979a) antibody to rotavirus or 132 virus has never been detected in this flock and rotavirus has never been observed by direct electron microscopic examination of faeces from the flock. Livers were trypsinized using 0.05 \% trypsin and the cells grown in 125 \times 15 \, \text{mm} \) roller tubes containing 22 \times 7 \, \text{mm} \) glass coverslips. M199 medium containing 10\% foetal calf serum was used for growth and Earle's lactalbumin with no serum as maintenance medium.

**Direct electron microscopic examination of faeces and intestinal contents.** This was done using Method C of McNulty et al. (1979b). Briefly this involved fluorocarbon extraction of faecal filtrates, followed by concentration of the virus in the resulting aqueous phase by centrifugation at 91000 \, g \) for 1 \, h \) at 4 \, ^\circ\text{C}. Pellets were resuspended in distilled water, mounted on carbon-coated grids and stained with methylamine tungstate.

**Antisera.** Hyperimmune antisera to the Northern Ireland 75-447 isolate of calf rotavirus (McNulty et al., 1977), Ch 1 chicken rotavirus (McNulty et al., 1980), Ty 1 turkey rotavirus (McNulty et al., 1980) and 132 virus were prepared in adult SPF hens housed in isolators. The viruses were propagated in cell cultures using trypsin treatment, partially purified and concentrated by sonication, treatment with 0.25 \% SDS and pelleting through 30\% sucrose (Todd et al., 1980). Birds were inoculated intramuscularly in several sites with a mixture of virus and Freund's complete adjuvant and boosted by an intravenous inoculation of virus alone after an interval of 4 to 6 weeks. One week later the hens were bled and the titre of the serum determined by indirect immunofluorescence (McNulty et al., 1979a, 1980). If necessary, a further intravenous inoculation was given. Birds were bled out 1 week after the final inoculation.

Convalescent antisera to Ch 1 chicken rotavirus and Ty 2 and Ty 3 turkey rotaviruses were prepared in SPF chickens (McNulty et al., 1980). Convalescent antiserum to the Compton UK isolate of bovine rotavirus (Bridger & Woode, 1975) and hyperimmune antiserum to the Compton SW 20/21 isolate of pig rotavirus (Woode et al., 1976b) were kindly supplied by Dr J. C. Bridger, ARC Institute for Research on Animal Diseases, Compton, Berks, U.K. These antisera were prepared in gnotobiotic calves and pigs and had indirect immunofluorescence titres of 1280 and 81920 respectively when tested against homologous virus (J. C. Bridger, personal communication).

**Indirect immunofluorescence.** Frozen sections of intestinal tract or coverslip cultures of CEL cells infected with 132 virus or Ch 1 chicken rotavirus were fixed in acetone for 10 \, \text{min} \) at room temperature. The Nebraska isolate of calf rotavirus was kindly supplied by Dr E. P. Bass, Norden Laboratories, Lincoln, Nebraska, U.S.A. and was grown in primary calf kidney cells. Sections or coverslips were stained with doubling dilutions of antisera ranging from 1/20 to 1/10000 for 1 \, h \) at 37 \, ^\circ\text{C} \) or overnight at 4 \, ^\circ\text{C}. Following a wash in PBS, counterstaining was performed as above with the appropriate anti-species globulin conjugated with fluorescein isothiocyanate (Nordic Immunological Laboratories, Maidenhead, Berks, U.K.). After a further wash in PBS, coverslips were mounted in buffered glycerol and examined with a Leitz Ortholux microscope under u.v. light. The titre of the serum was the highest dilution giving a detectable reaction with the antigen.

Some of the conjugates, particularly the anti-chicken globulin, had high antibody titres against rotavirus. This antibody was removed by absorption of the conjugates with CEL cells
infected with Ch 1 chicken rotavirus and a reaction was no longer detectable when the absorbed conjugate was tested at its working dilution.

**Characterization of virus RNA.** 132 virus and Ch 1 chicken rotavirus were purified from the faeces of experimentally infected birds. Approx. 10% faeces suspensions were extracted with fluorocarbon (Arcton 113, ICI), treated with 0.25% SDS for 5 min at room temperature and pelleted through a 30% sucrose cushion at 110000 g for 1.5 h at 15 °C (Todd et al., 1980). Pellets were resuspended in PBS and layered on to 20 to 40% (w/w) CsCl gradients prepared in PBS. The gradients were centrifuged at 110000 g for 17 h at 10 °C. Sharp opalescent bands containing intact particles of both 132 virus and Ch 1 rotavirus were present at a density of approx. 1.36 g/ml as determined by refractometry. 132 virus and Ch 1 rotavirus were recovered from the gradient bands by centrifugation at 110000 g for 1.5 h at 4 °C. Virus pellets were resuspended in PBS. Cell culture harvests of Ch 1 rotavirus and the Northern Ireland isolate of calf rotavirus were partially purified as described under preparation of antisera above.

RNA was released from purified and partially purified virus preparations by treatment with 6 M-urea, 1% SDS and 1% mercaptoethanol at 60 °C for 10 min and electrophoresed on 3.75% polyacrylamide gels using a discontinuous buffer system (Todd et al., 1980). Alternatively, suspensions of purified 132 virus were diluted with 0.1 M sodium acetate pH 5 (acetate) buffer. SDS was added to a final concentration of 1% and the mixture extracted twice with an equal volume of phenol. Virus RNA was precipitated by the addition of 3 vol. ethanol at -20 °C. Pellets of RNA were collected by centrifugation at 10000 g for 30 min at 0 °C and dissolved in acetate buffer. Gels were stained with ethidium bromide and photographed using an orange filter under u.v. light or with methylene blue. Methylene blue-stained gels were scanned at 590 nm using a Gilford spectrophotometer and the molar ratios of RNA bands calculated as detailed previously (Todd et al., 1980).

RNA prepared by phenol–SDS extraction from 132 virus and Vero cell ribosomal RNA were dissolved in acetate buffer and treated with pancreatic ribonuclease (Sigma, London) at a concentration of 0.1 μg/ml for 30 min at 37 °C. RNA was prepared for electrophoresis by the addition of an equal volume of 0.05 M-tris-glycine buffer pH 8.9 (upper tray electrophoresis buffer) containing 40% sucrose. In addition, phenol–SDS-extracted 132 virus RNA and *Escherichia coli* DNA (Sigma, London) were dissolved in acetate buffer containing 0.0025 M-MgSO₄ and treated with deoxyribonuclease I (Sigma, London) at a concentration of 20 μg/ml for 1 h at 37 °C.

**Infection of chickens.** Batches of SPF chickens between 1 day and 4 weeks of age were infected orally with 1 ml trypsin-treated faeces supernatant or cell culture-grown virus. Chickens were infected and subsequently maintained in isolators. Samples of small intestine were removed under halothane (Fluothane, ICI) anaesthesia, immediately deep frozen in 2-methylbutane using liquid nitrogen and processed for immunofluorescent staining. Additional material was fixed in 4% glutaraldehyde in sodium cacodylate buffer pH 7.2, for 2 h at 4 °C, post-fixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated and prepared for electron microscopic examination (Pearson & McNulty, 1979).

**RESULTS**

**Isolation of 132 virus**

In the course of direct electron microscopic examination of faeces from clinically normal 3-week-old chickens, particles morphologically indistinguishable from rotaviruses were observed. Both intact 67 to 70 nm and 54 to 58 nm particles lacking the outer capsid layer were present (Fig. 1). However, when faecal suspensions containing these particles were trypsin-treated and inoculated into CEL cell cultures, it was not possible to demonstrate
synthesis of rotavirus antigen in any cells by immunofluorescent staining with convalescent Ch 1 chicken rotavirus or hyperimmune lamb rotavirus antisera. Both of these antisera react with all of the avian and mammalian rotaviruses investigated or isolated in this laboratory (McNulty et al., 1979a, 1980; M. S. McNulty, unpublished observations).

In view of this lack of reaction, it was initially thought that the particles were not replicating in cell cultures. However, the faecal suspensions were given a further two blind passages in CEL cultures. Using the above antisera no cells with specific immunofluorescence were detected during these passages, but large numbers of rotavirus-like particles were detected by electron microscopy in a lysate of 3rd passage material. It seemed unlikely that these particles had survived from the original inoculum.

It was possible, therefore, that this virus, designated 132 virus, was a rotavirus which did not possess the group antigen shared by all previously characterized rotaviruses. The other alternative was that 132 virus was not, in fact, a rotavirus. The experiments described below were designed to distinguish between these two possibilities.

Using trypsin treatment, 132 virus has to date undergone 19 serial passages in CEL cultures. The virus did not produce a recognizable c.p.e. and specific immunofluorescence was never obtained using Ch 1 chicken or lamb rotavirus antisera during any of these passages. In contrast, specific cytoplasmic immunofluorescence was observed when cultures at all passage levels were stained with the FITC-conjugated hyperimmune antiserum to 132 virus (Fig. 2 a).

Antigenic relationship of 132 virus and previously characterized rotaviruses

The antigenic relationship between 132 virus, Ch 1 chicken rotavirus and Nebraska calf rotavirus was investigated using indirect immunofluorescence. Hyperimmune and convalescent antisera prepared against a variety of avian and mammalian rotaviruses reacted with both Nebraska calf and Ch 1 chicken rotavirus antigens, but not with 132 virus antigens. Furthermore, no reaction was obtained between hyperimmune antiserum to 132 virus and Nebraska and Ch 1 rotavirus antigens (Table 1).
**Table 1. Antigenic relationship of 132 virus, Ch 1 chicken rotavirus and Nebraska calf rotavirus by indirect immunofluorescence**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>132 virus</th>
<th>Ch 1 chicken rotavirus</th>
<th>Nebraska calf rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>132 virus, hyperimmune</td>
<td>&gt;10,000*</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ch 1 chicken rotavirus, convalescent</td>
<td>&lt;20</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>Ch 1 chicken rotavirus, hyperimmune</td>
<td>&lt;20</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>N. Ireland calf rotavirus, hyperimmune</td>
<td>&lt;20</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Ty 1 turkey rotavirus, hyperimmune</td>
<td>&lt;20</td>
<td>ND†</td>
<td>&gt;2560</td>
</tr>
<tr>
<td>Ty 2 turkey rotavirus, convalescent</td>
<td>&lt;20</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td>Ty 3 turkey rotavirus, convalescent</td>
<td>&lt;20</td>
<td>1280</td>
<td>320</td>
</tr>
<tr>
<td>U.K. calf rotavirus, convalescent</td>
<td>&lt;20</td>
<td>&gt;100</td>
<td>1280</td>
</tr>
<tr>
<td>SW 20/21 pig rotavirus, hyperimmune</td>
<td>&lt;20</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

* Indirect immunofluorescence titre of antiserum.
† ND, Not done.
Fig. 3. Polyacrylamide gel electrophoresis of: (a) 132 virus RNA, note separation of segment 7 from segments 8 and 9; (b) N. Ireland calf rotavirus RNA. Gels stained with methylene blue. RNA segments are numbered.

Table 2. Mol. wt. of 132 virus RNA segments

<table>
<thead>
<tr>
<th>Segment no.</th>
<th>Estimated mol. wt. (x 10^-6)</th>
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<tbody>
<tr>
<td>1</td>
<td>2.07</td>
</tr>
<tr>
<td>2</td>
<td>1.82</td>
</tr>
<tr>
<td>3</td>
<td>1.62</td>
</tr>
<tr>
<td>4</td>
<td>1.43</td>
</tr>
<tr>
<td>5</td>
<td>1.41</td>
</tr>
<tr>
<td>6</td>
<td>0.75</td>
</tr>
<tr>
<td>7</td>
<td>0.63</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>0.45</td>
</tr>
<tr>
<td>10</td>
<td>0.27</td>
</tr>
<tr>
<td>11</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Nucleic acid of 132 virus

The nucleic acid of 132 virus was resistant to digestion with both deoxyribonuclease and pancreatic ribonuclease, under conditions in which E. coli DNA and Vero cell ribosomal RNA were hydrolysed, indicating that it consisted of double-stranded RNA. Following
Fig. 4. (a) Villous epithelial cell from mid small intestine of chicken experimentally infected with 132 virus. Note viroplasm (V) and numerous virus particles (arrows) within dilated cisternae of rough endoplasmic reticulum. Bar marker represents 500 nm. (b) Portion of cytoplasm of chick embryo liver cell infected with 132 virus. Note viroplasm (V) containing virus cores, non-enveloped virus particles (small arrows) and enveloped virus particles (large arrow). Bar marker represents 200 nm.
electrophoresis on 3.75% polyacrylamide gels, 11 RNA bands were obtained (Fig. 3). Determination of molar ratios indicated that each band consisted of only 1 species of RNA. The mol. wt. of the RNA segments of 132 virus (Table 2) were determined by co-electrophoresis with Northern Ireland 75-447 calf rotavirus (Todd et al., 1980). The RNA of 132 virus has been analysed by polyacrylamide gel electrophoresis at various passage levels in CEL cultures and experimentally infected chickens. Results identical to those in Fig. 3 have always been obtained.

**Biological behaviour of 132 virus**

SPF chickens between 1 day and 4 weeks of age were susceptible to infection with 132 virus. The virus was detected by direct electron microscopy in the faeces of orally infected birds from 2 to 8 days after infection. Peak virus excretion occurred 3 to 4 days after infection. Coincident with this, a mild diarrhoea of about 24 h duration was observed. Birds bled 3 weeks after infection had developed antibodies detectable by indirect immunofluorescence against 132 virus, but not against Ch 1 chicken and Nebraska calf rotavirus.

132 virus antigen was detected by immunofluorescent staining in the villous epithelial cells of the small intestines of experimentally infected chickens (Fig. 2b). No fluorescence was obtained when the same material was stained by hyperimmune antiserum to Ch 1 chicken rotavirus (Fig. 2c).

Areas of small intestine containing large numbers of fluorescing cells were examined by electron microscopy. Virus particles were observed in the cytoplasm of villous epithelial cells (Fig. 4a). They were located primarily within dilated cisternae of rough endoplasmic reticulum (RER), and were morphologically indistinguishable from previously characterized rotaviruses (McNulty, 1979). Enveloped virus particles were about 76 nm in diam.; non-enveloped particles were about 57 nm. Masses of granular viral precursor material or viroplasm containing virus cores were frequently observed outside cisternae of RER which contained virus particles. Similar virus particles and associated structures were present in CEL cultures infected with 132 virus (Fig. 4b).

**DISCUSSION**

This paper describes the isolation and characterization of an unusual virus from chickens. This virus, designated 132 virus, is morphologically indistinguishable from rotaviruses. In both cell cultures and intestinal epithelial cells the morphogenesis of 132 virus closely resembles that of rotaviruses (McNulty, 1979). The genome of 132 virus has the characteristics of double-stranded RNA, which was separated into 11 segments by polyacrylamide gel electrophoresis. The mol. wt. of the genome segments of 132 virus are in the correct size range for rotavirus RNA (McNulty, 1979; Todd et al., 1980). Like rotaviruses, 132 virus replicates in vivo in the villous epithelial cells of the small intestine. Therefore, on morphological, biochemical and biological criteria, 132 virus is a rotavirus. However, using immunofluorescence, it was not possible to demonstrate an antigenic relationship between 132 virus and previously characterized avian and mammalian rotaviruses. Thus, 132 virus does not appear to possess the group antigen shared by all other known isolates of rotavirus.

Recently, Saif et al. (1980) have described a virus from pigs which may also be a rotavirus which lacks the group antigen. This virus is morphologically identical to rotaviruses but has not been further characterized. It has not yet been isolated in cell cultures but has been passaged in gnotobiotic pigs. It does not cross-react with pig rotavirus by immune electron microscopy or immunofluorescence. If this pig virus is a rotavirus, as seems likely, it will be interesting to see if it is antigenically related to 132 virus.
Antigenically unusual rotavirus from chickens

If infections with antigenically unconventional rotaviruses like 132 virus are widespread, the diagnostic procedures currently used in many medical and veterinary laboratories will need to be revised. At the moment serological tests such as ELISA, counterimmune-electro-osmophoresis and complement fixation are widely employed to detect rotavirus antigens in faeces. However, these tests are based on the assumption that all rotaviruses are antigenically related through the group antigen.

Polyacrylamide gel electrophoresis revealed some interesting features of 132 virus RNA. Firstly, it is extremely unusual for segment 7 to be so widely separated from segments 8 and 9. Secondly, segments 10 and 11 were well separated from one another. This is unusual for a rotavirus from chickens, in that segments 10 and 11 of all other avian rotavirus RNAs examined so far have identical or very similar electrophoretic mobility (Todd et al., 1980; McNulty et al., 1980). This raises the possibility that 132 virus may be a mammalian virus which has cross-infected chickens.

We are grateful to Mr W. L. Curran for assistance with the electron microscopy.

REFERENCES


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