Purification and Characterization of Ovine Astrovirus

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SUMMARY

Astrovirus, purified in aggregated form from epithelial cells of the small intestinal villi of infected gnotobiotic lambs, was shown to have a single-stranded RNA genome with an S value of 34 and poly(A) tract. Only two major capsid polypeptides were detected with similar mol. wt. of approx. $3.3 \times 10^4$.

INTRODUCTION

Astrovirus was first described by Madeley & Cosgrove (1975) as a small round virus, 28 to 30 nm in diam., commonly observed in the diarrhoeic stools of babies with gastroenteritis. It was recognized as a novel virus species since it showed a distinctive morphology and the name astrovirus was proposed because some of the particles had a five- or six-pointed stellate configuration. Astroviruses have since been described associated with diarrhoea in lambs (Snodgrass & Gray, 1977) and calves (Woode & Bridger, 1978).

Attempts to grow all three astroviruses in cell culture have failed although it has been possible by immunofluorescent techniques to demonstrate limited replication of both human and bovine viruses on the first passage (Lee & Kurtz, 1977; Woode & Bridger, 1978). In addition, the ultrastructure of human astrovirus in cultured cells has been described (Kurtz et al., 1979).

Lamb astrovirus has been shown to replicate in the columnar absorptive epithelial cells of the small intestine and to produce symptoms of mild diarrhoea in gnotobiotic lambs (Snodgrass et al., 1979; Gray et al., 1980). In this communication we report the purification of ovine astrovirus from intestinal cells. Characterization of this virus has shown that the RNA genome resembles that of the picornaviruses and caliciviruses but that the polypeptide composition is unlike that of either of these groups.

METHODS

Growth of virus. Gnotobiotic lambs were infected by the oral route with virus-infected gut contents at 24 h after birth. Animals were killed from 24 to 48 h post-inoculation (p.i.), the gut removed and contents extruded. The small intestine was cut into 25-cm sections which were then opened lengthwise, pinned out and the mucosal surface lightly scraped with a microscope slide. Examination of these intestinal scrapings by optical microscopy showed that they contained large numbers of detached villi. Each lamb yielded from 5 to 10 ml of such material.

Purification of virus

Method 1. Intestinal scrapings were suspended in 20 ml phosphate-buffered saline (PBS, 0.008 M-sodium phosphate buffer pH 7.2, 0.14 M-NaCl) containing 1% (w/v) SDS (PBS/SDS) per ml of scrapings. The mixture was then thoroughly blended with a glass–teflon homogenizer and centrifuged at 560 g for 5 min. The supernatant was removed and centrifuged at 95000 g for 1 h at 20 °C and the pellets resuspended, with the aid of a homogenizer, in 15 ml PBS. Aggregation of the virus occurred and the resulting precipitate of
virus was pelleted and washed twice with PBS by centrifugation at 560 g for 10 min. Such preparations are referred to below as crude virus or crude virus pellets.

**Method 2.** Five to 7 ml of intestinal scrapings were suspended in 100 ml PBS containing 1% (w/v) Triton X-100 (BDH) (PBS/TX-100) and disrupted with seven strokes of a glass–teflon homogenizer. The mixture was cleared at 1250 g for 10 min and the pellets washed with 20 ml PBS/TX-100. Supernatants were pooled and centrifuged at 10000 g for 30 min at room temperature. The resulting large pellets were resuspended in 30 ml PBS/SDS with a homogenizer, recentrifuged at 95000 g for 1 h at 20 °C and finally washed twice in PBS as for the final pellets in method 1.

Most results presented in this paper were obtained with virus preparations from three lambs. These were: B216 killed at 24 h p.i. and processed by method 1, C254 killed 48 h p.i. and processed by method 2 and C259 killed 24 h p.i. and processed by method 2.

**CsCl gradient centrifugation.** Virus pellets from the methods above were resuspended in PBS containing 1% (w/v) Sarcosyl (Ciba-Geigy, N.Y., U.S.A.) and 20 mM-2-mercaptoethanol and incubated at 37 °C for 30 min followed by a 3 min treatment in a bath-type sonicator. This suspension was then loaded on to a 10 to 46% CsCl gradient consisting of five layers with concentrations of 10, 19, 28, 37 and 46% (w/w) CsCl in PBS, Sarcosyl, mercaptoethanol buffer and centrifuged for 17 h at 114000 g in an SW40Ti rotor (Beckman) at 20 °C. In one experiment, a preformed linear 10 to 46% (w/v) CsCl gradient was used and the centrifugation time was 2·5 h. The gradients were fractionated using an ISCO model 185 fractionator and u.v. monitor by upward displacement with Maxidens (Nyegaard & Co., Oslo, Norway).

**Nucleic acid preparation.** Virus pellets were resuspended in 50 mM-tris-HCl pH 8·3 containing 2% (w/v) SDS and 0·1 M-NaCl and extracted twice with 0·6 vol. phenol–cresol mixture (Parish & Kirby, 1966) and 0·4 vol. chloroform. The final aqueous phase was precipitated with 2 vol. ethanol at −20 °C overnight. Ribonuclease A (Sigma) digestion was performed in 0·15 M-NaCl, 0·015 M-sodium citrate pH 7 (1 × SSC).

**Sucrose gradient centrifugation of RNA.** Virus RNA was analysed on 10 to 30% (w/v) sucrose gradients in 50 mM-tris-HCl pH 7 containing 0·1 M-NaCl, 0·001 M-EDTA and 0·5% (w/v) SDS. Gradients of 12·5 ml were centrifuged for 4·5 h at 202000 g and 20 °C in an SW40Ti rotor (Beckman) and fractionated as for the CsCl gradients. Sedimentation coefficients were calculated relative to 18S and 28S ribosomal RNA standards centrifuged in parallel gradients by the method of Martin & Ames (1961).

**Polyacrylamide gel electrophoresis of RNA.** This was performed as described by Loening (1967) using 2·3 and 2·6% gels. Gels were scanned with a Gilford 252 spectrophotometer fitted with a gel transport system and amounts of virus RNA calculated from the peak areas by comparison with known quantities of ribosomal RNA. Gels were stained overnight with 0·001% toluidine blue (Sigma) after prior washing for 5 h with H2O; under these conditions double-stranded nucleic acids stain pink and single-stranded species stain blue (Bevan et al., 1973).

**Reverse transcriptase assay for poly(A) RNA.** To 1 μg of sucrose gradient-purified virus RNA was added 50 μl reaction mixture containing 40 mM-tris-HCl pH 8·3, 50 mM-NaCl, 5 mM-MgCl2, 15 μg/ml bentonite, 1 mM-dATP, dTTP and dCTP, 10 μCi 3H-dGTP (13·2 Ci/mmol). 12·5 units of avian myeloblastosis virus reverse transcriptase and 0·25 μg oligo(dT) (Boehringer, Mannheim). RNA and oligo(dT) were omitted in control incubations and poly(rC).oligo(dG) (P.-L. Biochemicals, Milwaukee, Wis., U.S.A.) was added as a positive control. After 30 min incubation at 37 °C the reaction was stopped by dilution with 200 μl of a 0·02% solution of bovine serum albumin (BSA) and cooling to 4 °C. Two 120 μl amounts were taken and precipitated with 10% TCA, harvested on to Whatman GFC glass fibre filters, washed, dried and counted in toluene-based scintillation fluid.
Characterization of ovine astrovirus

Table 1. Effect of various treatments on astrovirus aggregates in gut contents as judged by observation with the electron microscope

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Treatment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>50% (v/v)</td>
<td>Emulsification</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>Arcton</td>
<td>50% (v/v)</td>
<td>Emulsification</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>NaCl</td>
<td>2 M</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>CsCl</td>
<td>2.6 M</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1% (w/v)</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>SDS</td>
<td>1% (w/v)</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>Sarcosyl</td>
<td>1% (w/v)</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2-5 mg/ml</td>
<td>Incubation at 37 °C for 30 min</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>Incubation at ambient temperature</td>
<td>No disaggregation</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>Ultrasonication for 5 min</td>
<td>Very few particles survived treatment</td>
</tr>
<tr>
<td>Urea</td>
<td>3 M</td>
<td>Incubation at 37 °C for 30 min</td>
<td>Partial disaggregation</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>20 mM</td>
<td>Incubation at 37 °C for 30 min</td>
<td></td>
</tr>
<tr>
<td>plus Sarcosyl</td>
<td>1% (w/v)</td>
<td>with 3 min mild sonication</td>
<td></td>
</tr>
</tbody>
</table>

Hybridization of virus RNA with 3H-poly(U). The method used was that of Bishop et al. (1974).

Polyacrylamide gel analysis of virus polypeptides. This was performed as described by Weber & Osborn (1969) and Laemmli (1970), using phosphorylase A, BSA, catalase, ovalbumin, aldolase and chymotrypsin (Boehringer, Mannheim) as mol. wt. standards. Gels were stained with 0.25% (w/v) Coomassie blue after fixation in 20% (w/v) sulphosalicylic acid.

Electron microscopy of astrovirus. Preparations of purified astrovirus were dried on to formvar/carbon-coated grids, stained with 1% phosphotungstic acid pH 7 for 30 s and examined in a Siemens Elmiskop IA electron microscope.

RESULTS

Purification of virus

All the procedures described were monitored by electron microscopy which was the only method available to detect virus. Initial attempts to purify the virus from infected faeces or gut contents utilized the fluorocarbon extraction and CsCl density-gradient techniques which successfully purified rotavirus (Todd & McNulty, 1976). This approach failed for astrovirus since the majority of the virus particles were aggregated, either with other particles or with cell debris, and were consequently lost in the initial low-speed centrifugations. A variety of treatments were then investigated to attempt to disaggregate the virions in gut contents. The results are summarized in Table 1; none of the treatments led to the production of discrete virions but incubation with mercaptoethanol and Sarcosyl did prevent aggregation with debris. These experiments showed that the particles were stable to a range of reagents including ionic detergents.

The discovery that virus multiplied in the epithelial cells of the small intestine (Gray et al., 1980) led us to investigate this tissue as a source of virus. Virus was isolated from cells scraped from the mucosal surface by exploiting its stability to treatment with the ionic detergent SDS. However, whilst simple disruption of the cells with this detergent was successful, it was found to be preferable to carry out the initial lysis with Triton X-100 as this avoided the high viscosity of the homogenate caused by the release of DNA from chromatin (see methods 1 and 2 above).

Neither method resulted in virus pellets which could be disaggregated in buffer irrespective of the presence of detergent. The crude preparation was thus an aggregate of virus which could be sedimented by low-speed centrifugation. Aggregates of several thousand particles
were seen with the electron microscope as illustrated in Fig. 1. Particle morphology was poorly preserved and very few virions showed the typical stellate surface configuration. Only occasional pieces of contaminating cell debris were observed in such preparations.

CsCl density-gradient centrifugation

The absorbance profile of a crude astrovirus preparation centrifuged overnight on a preformed gradient of CsCl is shown in Fig. 2, together with the amount of virus RNA in the relevant fractions. The virus nucleic acid was identified and estimated by pelleting the individual fractions at high speed followed by phenol extraction and analysis of the nucleic acid on polyacrylamide gels (see below). The absorbance profile shows two peaks at densities of 1.365 and 1.39. The denser peak was due to a very sharp band which was clearly visible and could be seen to consist of aggregated virus. Virus was also present in the fractions containing the upper peak but the particles were observed to be single. The bulk of the virus RNA (see below) was found in the fraction which contained the band of aggregated virus. Similar gradient profiles were obtained with overnight centrifugation on a stepped preformed gradient, or with short centrifugation (2.5 h) on a linear continuous CsCl gradient. The buoyant densities observed with crudely purified intracellular virus were consistent with those obtained in an attempt to purify virus from gut contents using self-generating gradients of CsCl in which virus was mainly present in fractions with densities from 1.38 to 1.40 g/ml.

Analysis of astrovirus nucleic acid

Nucleic acid preparations obtained by phenol extractions of both crude and CsCl-purified virus were analysed by sucrose density-gradient centrifugation and by gel electrophoresis (Fig. 3 and 4). All preparations contained a nucleic acid species with an estimated sedimentation coefficient of 34S (Fig. 3). This species was identified as single-stranded RNA
Characterization of ovine astrovirus

Fig. 2. CsCl density-gradient centrifugation of crude astrovirus from lamb C259. ■—■, Density of individual fractions; ..., absorbance profile at 254 nm; ●--●, virus RNA content of individual fractions.

Fig. 3. Sucrose gradient analysis of nucleic acid from a crude astrovirus preparation from lamb C259 produced by method 2. The positions of 18S and 28S ribosomal RNA standards in a parallel gradient are shown.

by its sensitivity to digestion with 1 μg/ml ribonuclease A in 1 x SSC buffer (Fig. 4) and by the broad band it produced in polyacrylamide gels, which stained blue with toluidine blue. It had an apparent mol. wt. of 2-7 × 10^6, as judged by aqueous gel electrophoresis relative to ribosomal RNA standards. The 34S RNA was the only species detected in nucleic acid prepared from crude virus purified by method 2 and CsCl gradient-purified virus; in a preparation of crude virus produced by method 1 some DNA contamination was evident from the gel profiles. The association of the 34S RNA with the virus particles was confirmed by the CsCl gradient data (Fig. 2).

Results obtained with reverse transcriptase and hybridization with ^3H-poly(U) both indicated that the 34S astrovirus RNA contained a poly(A) tract. The results shown in Table 2 were obtained when 34S RNA was incubated with reverse transcriptase, nucleotide triphosphates and oligo(dT) primer; ^3H-dGTP was used as the label so that only DNA synthesis on a heteropolymeric RNA template was detected. DNA synthesis occurred only in the presence of the oligo(dT) primer. One result obtained by ^3H-poly(U) hybridization is presented in Fig. 5. The method was found to estimate the poly(A) standards quantitatively.
Fig. 4. Polyacrylamide gel electrophoresis of astrovirus RNA from lamb B216. (a) Absorbance profile of a gel loaded with virus RNA; the positions of the peaks of 18S and 28S ribosomal RNA in a parallel gel are indicated. (b) Profile of a gel loaded with virus RNA which had been incubated with 1 µg/ml ribonuclease A.

Table 2. Incorporation of $^3$H-dGTP into DNA by reverse transcriptase using astrovirus RNA as a template as measured by acid-insoluble radioactivity

<table>
<thead>
<tr>
<th>Additions to reaction mixture</th>
<th>Oligo(dT) (0.5 µg)</th>
<th>RNA (0.5 µg)</th>
<th>Poly(rC), oligo(dT) (0.25 µg)</th>
<th>Ct/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>326</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>327</td>
</tr>
<tr>
<td>+</td>
<td>+ (B216)</td>
<td>-</td>
<td>-</td>
<td>92214</td>
</tr>
<tr>
<td>+</td>
<td>+ (C259)</td>
<td>-</td>
<td>-</td>
<td>115707</td>
</tr>
<tr>
<td>-</td>
<td>+ (B216)</td>
<td>-</td>
<td>-</td>
<td>2594</td>
</tr>
<tr>
<td>-</td>
<td>+ (C259)</td>
<td>-</td>
<td>-</td>
<td>1446</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>882235</td>
</tr>
</tbody>
</table>

Fig. 5. Estimation of the poly(A) content of astrovirus RNA by $^3$H-poly(U) hybridization. • • • • •. Ct/min in ribonuclease-resistant $^3$H-poly(U) found with the poly(A) standards. Dotted lines to the ordinate and abscissa indicate the counts found when astrovirus RNAs from two virus preparations (1 µg of each) were hybridized under identical conditions and the equivalent amounts of poly(A).
Characterization of ovine astrovirus

The levels of hybridization with 34S RNA were all positive but were variable; from three such experiments estimates of the size of the poly(A) tract varied from 7 to 30 residues, with a mean of 14.

Polyacrylamide gel electrophoresis of astrovirus polypeptides

Gel analysis of polypeptides from crude and CsCl gradient-purified virus was performed in both continuous and discontinuous buffer systems using a range of gel concentrations. Optimum resolution was achieved with an 11% gel and discontinuous buffer system as shown in Fig. 6. Two polypeptide species, present in roughly equimolar amounts, were seen when gradient-purified virus was analysed. The mol. wt. of these two closely migrating polypeptides was estimated as approx. $3.3 \times 10^4$. A similar value was obtained using the continuous buffer system but the two polypeptides were not resolved. When crude virus preparations were analysed, up to three additional faint bands were seen on the gels.

DISCUSSION

The purification of astrovirus is complicated by aggregation which is a property of both human and ovine astrovirus. Bridges between the particles which appear to be projections from the virus surface can be seen with the electron microscope (Madeley & Cosgrove, 1975; Snodgrass & Gray, 1977) and clearly the formation of the crystalline arrays described by Gray et al. (1980) requires inter-particle bonds. Virus in the gut lumen is found both in aggregates of various size and as single particles, which suggests that the large aggregates and
crystalline arrays seen in the cytoplasm of infected cells are broken down either during or after release from infected cells. The purification method described above isolates these intracellular aggregates directly and the non-dispersible nature of the crude virus pellets and their appearance in the electron microscope suggests that further aggregation takes place during the purification.

It has been suggested that the formation of aggregates has a function in the preservation of infectious virus in the environment outside the cell but the property of aggregation is usually only found under certain well-defined conditions of ionic strength and pH (Galdiero, 1979). Astrovirus aggregates appear to be extremely stable and certainly cannot be dispersed merely by raising the salt concentration; a detailed study of the effects of pH on aggregation has not been carried out but preliminary experiments in which the pH was lowered to 4 did not appear to produce disaggregation (A. J. Herring, unpublished results). Now that preparations of purified aggregated astrovirus can be produced, studies of the nature of the inter-particle bonds should be facilitated.

The interpretation of the data from the CsCl gradient is also complicated by aggregation of the particles. The absorbance profile shows two clear peaks, the denser of which consisted of aggregated particles so that its area in the absorbance profile does not measure the amount of virus present. As RNA analysis showed, the bulk of the virus was clearly in this aggregated peak. Heterogeneity of buoyant density has also been reported for the calicivirus which causes vesicular exanthema of swine, the two peaks of virus occurring at the same densities as those found for astrovirus but with the major peak at 1.36 g/ml. This effect was attributed to differences in solvation and Cs⁺ ion binding (Oglesby et al., 1971). The effect of aggregation on these properties may explain the two peaks of astrovirus.

The nucleic acid analysis showed the genome of astrovirus to be a single-stranded RNA molecule very similar in its sedimentation and electrophoretic behaviour to those found in the picornavirus and calicivirus groups (Newman et al., 1973; Kerr & Martin, 1972). Astrovirus RNA also resembles the genome of these viruses in the possession of a short poly(A) tract, the estimated size of which resembled that reported for encephalomyocarditis virus RNA (Giron et al., 1976; Emtage et al., 1976). The variability of the 3H-poly(U) hybridization results (Table 2) was most probably due to the acid lability of poly(U) described by Williams & Klett (1978).

The results of the polypeptide analysis showed that astrovirus is not a calicivirus since members of this group have a single characteristic major polypeptide with a mol. wt. of about twice that found for astrovirus (Bachrach & Hess, 1973; Burroughs & Brown, 1974). However, the results do not allow the virus to be classified as a picornavirus since members of this group possess four major structural polypeptides (Cooper et al., 1978). It is possible that other structural proteins have been lost due to the harsh treatments used in purification. However, picornaviruses are able to withstand treatment with 1% SDS (Kerr & Martin, 1972; Talbot et al., 1973).

Judged on the present evidence, the astroviruses must be considered a separate group with a polypeptide composition intermediate between that of the picornavirus and calicivirus. A final decision on their classification will have to await further elucidation of their structure.

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REFERENCES


Characterization of ovine astrovirus


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