Cultivation and Partial Characterization of Bovine Astrovirus

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ABSTRACT

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Bovine astrovirus serotype 2 (US2) was adapted to primary neonatal kidney cell (NBK) cultures by the addition of 50 μg ml^{-1} of trypsin in the medium. Infectious virus was released from the cells within 7 days post-infection in early passages and within 3 days in later passages. In the absence of trypsin, neither passage of infected cells nor release of infectious virus occurred. The virus was shown to be similar to the fecal astrovirus by a neutralization test and by ultrastructural studies of infected cells. Primary embryo bovine kidney (EBK) and NBK cell cultures supported infection with both fecal and tissue culture adapted (TCA) astrovirus. The time-related development of infection, as studied by immunofluorescence, was similar for both fecal and TCA astrovirus and for both cell culture types. The first indication of viral infection and expression of viral antigens occurred at 7 h post-infection and was characterized by the appearance of a diffuse faint immunofluorescence (IF) of the cytoplasm. Soon after, two or three brilliant IF granules were observed in the nucleus, which appeared to involve the nucleoli. Subsequently, dense granular IF was seen in the perinuclear region of the cytoplasm, which later extended to involve all the cytoplasmic area. In both EBK and NBK cultures infected with either fecal or tissue culture adapted astrovirus, only a minority of cells became infected, even when the multiplicity of infection exceeded one. Occasionally 10–20% of cells were infected, but in most cultures the proportion did not exceed 2% and in NBK cultures, from 3/9 calves, no infected cells were observed. The virus did not infect bovine cell lines. Infectivity of the virus was not removed by treatment with chloroform, and iododeoxyuridine and actinomycin D when added to the medium, did not block replication. Masses of virions were observed by electron microscopy in discrete areas in the cytoplasm, with similar distributions as the viral antigen foci as seen by IF. The mean diameter of the virions was 34 nm. In conclusion, bovine astrovirus lacks both essential lipids and an envelope, probably has an RNA genome, may have a nuclear phase of replication involving the nucleoli which is not blocked by DNA inhibitors, and has a selective cell tropism.

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INTRODUCTION

Astrovirus was first detected by Madeley and Cosgrove (1975) associated with human diarrhea. The name "astrovirus" was proposed for a particle, 28 nm in diameter, which had a round unbroken edge, a six-pointed star with an electronlucent center, and surface hollows that were triangular (Madeley, 1979). Morphologically similar viruses have been discovered in lambs (Snodgrass and Gray, 1977), calves (Woode and Bridger, 1978), pigs (Bridger, 1980), turkeys (McNulty et al., 1980), puppies (William, 1980), deer (Tzipori et al., 1981), cats (Hoshino et al., 1981), ducks (Gough et al., 1984) and mice (Kjeldsberg and Hem, 1985).

Bovine astrovirus (U.K. isolate) was first identified in England from diarrheic calves as a small round virus by Woode and Bridger (1978). They assumed that the particles possessing six-pointed star configuration and 28 nm in diameter were not pathogenic because transmission of the virus did not induce diarrhea in gnotobiotic calves. Later, two additional isolates of the virus (US1 and US2) were reported in the U.S.A. (Woode et al., 1984, 1985). These three isolates of bovine astrovirus were shown to possess a common antigen detected by immunofluorescence. However, as there was no cross-neutralization among the three isolates, it was concluded that there are at least two distinct serotypes and probably three: US1 serotype 1; US2 serotype 2; UK serotype 3 (Woode et al., 1985).

Low numbers of astrovirus are found in calf feces by electron microscopy, although the infectivity titer may be as high as $10^{5.0}$–$10^{6.0}$ $\log_{10}$ immunofluorescent focus forming units (IFFU) ml^{-1}. The only site of replication in the animal which has been identified is in the dome epithelial cells of the Peyer's patches, usually the M cells. This infection is subclinical in gnotobiotic calves, although it does result in the destruction of the dome cells (Woode et al., 1984).

Successful in vitro cultivation of astrovirus has been limited to human astrovirus, which was passed serially in primary human embryo kidney cells with the aid of trypsin (Lee and Kurtz, 1981). Passages of bovine astrovirus in primary, secondary and tertiary bovine embryo kidney cells showed a decline in the number of infected cells (Woode et al., 1984). It was suggested that this failure might be caused by either a lack of susceptible cells in the secondary cultures or lack of trypsin in the medium, which was required for human astrovirus cultivation in vitro (Lee and Kurtz, 1981).

The properties of astrovirus have been reported for lamb and human astroviruses. Lamb astrovirus was purified from the fecal contents and intestinal epithelium of infected lambs, and two forms of the virus were found: single particles and viral aggregates. Analysis of viral nucleic acid showed the estimated sedimentation coefficient value of 34S. The viral genome was shown to contain a single-stranded positive sense RNA with the molecular weight of 2.7×10^6, and there were two major polypeptides of approximately 3.3×10^4.

The authors concluded that lamb astrovirus seemed to fit between the families Caliciviridae and the Picornaviridae (Herring et al., 1981). In contrast, although the human astrovirus also has a positive strand RNA genome, it apparently possesses four polypeptides (Kurtz and Lee, 1987). Molecular biological studies of astroviruses of other species are needed in order to provide information for the taxonomic classification of these viruses.

In this report the successful propagation of bovine astrovirus 2 in bovine tissue culture is described and preliminary studies made into the properties of the virus. Attempts to subculture bovine astrovirus were performed only in bovine cells because animal astrovirus infections seem to be restricted to host species in vivo and in vitro (Snodgrass and Gray, 1977; William, 1980; Woode et al., 1984).

MATERIALS AND METHODS

Astrovirus isolates

The origin of the bovine astrovirus 2 isolate has been described (Woode et al., 1985). For infection of tissue cultures, virus-containing fecal samples of two experimentally infected gnotobiotic calves (GC54 and GC83) were diluted 1:10 with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at $6000 \times g$ for 30 min. Fecal virus preparations were used at a titer of $10^{3.0}$ IFFU ml^{-1}.

Antisera

Convalescent antisera, GC37 and GC43 to astrovirus 1, and GC39 to astrovirus 2, GC5 to bovine rotavirus 1, GC78 to bovine coronavirus, GC76 and SB219 to Breda virus 1 and 2, and antiserum to UK bovine astrovirus, were obtained from calves bled at 21-days post-infection (Woode et al., 1984, 1985).

Cell cultures

Madin Darby bovine kidney cells (MBDK), GBK bovine kidney cell line and bovine turbinate (BT) cell line were received from Dr. David Reed, Iowa State University. Primary embryo bovine kidney (EBK) cell cultures and primary neonatal bovine kidney (NBK) cell cultures were prepared as previously described (Woode et al., 1984).

Virus culture and assay

For all infectivity studies, cultures were washed once with MEM prior to inoculation with virus. Tissue culture assay for virus and the indirect immunofluorescence (IF) method have been described previously (Woode et al.,

1984, 1985). For the microtiter plate assay the mean IF cell count was determined from four wells and expressed as IFFU ml^{-1}.

To determine the percentage of cells that became infected, NBK cells were grown in eight-chamber tissue-culture slides (Miles Scientific, Naperville, IL) and six chambers were inoculated with fecal astrovirus. After 1–3 days of incubation, four chambers were fixed for IF and the mean IF cell count determined and from the other two chambers the cells were removed with trypsin-versene and the total cells counted in a hemocytometer chamber.

For adaptation of the astrovirus to cell culture, 50 μg ml^{-1} of trypsin (1:250 GIBCO Laboratories, Grand Island, NY) was added to the MEM.

To follow time-related development of IF, two sets of primary NBK cell cultures in tubes containing cover-slips were infected with fecal astrovirus. A pair of cover-slips was fixed for IF every 30 min from 1 to 12 h post-infection and kept at $-25°$ C. The cover-slips were stained and observed next day. The experiments were repeated twice.

Adaptation of astrovirus to replicate in primary cells

Primary EBK and NBK cell cultures in 25-cm^2 flasks were used for subculturing fecal astrovirus at 7-day intervals, with and without 50 μg ml^{-1} of trypsin in MEM. On Day 7 post-infection the cells were scraped off the flask with a "plastic policeman" or if they had stripped, as usually happened with the passages containing trypsin, the medium and cell debris were collected. Serial passages, including cells or cell debris plus medium, were inoculated into 25-cm^2 flasks and also into cover-slip cultures. The cover-slips were tested by IF at 24–48 h post-infection.

Neutralization test (NT)

Approximately 10^3 IFFU ml^{-1} of tissue culture adapted (TCA) astrovirus were mixed with an equal volume of each antiserum dilution. The mixture was incubated at 37°C for 1 h; 0.1 ml was then inoculated into each of four microtiter plate wells containing NBK cell monolayers. After 24 h the plates were fixed and read by IF (Woode et al., 1985).

Ultrastructural studies of infected cell cultures

NBK primary cells grown in 25-cm^2 flasks were infected at 6 days with 10^6 IFFU of TCA astrovirus passage 23 with 50 μg ml^{-1} of trypsin in MEM. At 24 and 48 h post-infection, the medium was removed and 1.5 ml of 2.5% glutaraldehyde in 0.1 M cacodylate added. The cells from infected and uninfected cultures were scraped off with a rubber policeman and pelleted in the glutaraldehyde solution at 100 × g for 10 min. The glutaraldehyde was removed and

replaced with fresh glutaraldehyde to 10 vol. of the pellet, and post-fixed in 1% buffered osmium tetroxide (Fagerland et al., 1986). Cells were dehydrated in ethanol, embedded in EMbed 812 (Electron Microscopy Sciences, Ft. Washington, PA 19034) and sectioned on a diamond knife. Sections of 60–90 nm were collected on uncoated copper grids and stained for 15 min each in 2% methanolic uranyl acetate and Reynolds' lead citrate. Thin sections were studied by transmission electron microscopy. Cover-slip cultures were infected at the same time, in order to determine the proportion of infected cells by IF at 24 h post-infection.

Effect of chloroform, 5-iodo-2'-deoxyuridine (IDU) and actinomycin D (AMD) on astrovirus replication

For control of the tests, bovid herpes virus 1 (BHV1) obtained from Dr. R.A. Crandell, Texas Veterinary Medical Diagnostic Laboratory and rotavirus Strain B223 (Woode et al., 1983) were included. BHV1, B223 and fecal and TCA astrovirus were titrated in secondary or tertiary NBK cell cultures grown in microtiter plates using eight wells per dilution. Virus infectivities for rotavirus and astrovirus were determined from IF at 24 h, whereas BHV1 infectivity was determined from cytopathic effect of the infected monolayers at 72 h. The viruses were subjected to the following treatments.

Chloroform sensitivity test

The method of Rovozzo and Burke (1973) was followed. To one of each pair of tubes containing 1 ml of each of the virus samples was added 0.05 ml chloroform (Chloroform anhydrous, Fisher Scientific Company, Fair Lawn, NJ). The mixtures were shaken for 10 min and the aqueous layer titrated for viral infectivity.

Effect of iododeoxyuridine and actinomycin D on astroviral replication

Astrovirus, BHV1 and B223 replication growth curves in primary NBK cultures were compared with and without the addition to the medium of $100 \mu g$ ml^{-1} iododeoxyuridine (Aldrich Chemical Co., Milwaukee, WI) or $0.05 \mu g ml^{-1}$ actinomycin D (Sigma Chemical Co., St. Louis, MO) following the method of Horzinek et al., 1984. Two flasks ($25 cm^2$) of NBK cells were infected with one of the following: 10–100 IFFU of TCA astrovirus or 10–100 TCD_{50} of BHV1 or B223. At intervals over 3 days samples were taken and viral titers determined. Titration end points were calculated using the method of Reed and Muench (1938).

RESULTS

Studies on the IF pattern and distribution in infected cells

The appearance of immunofluorescent cells in primary or secondary cultures of EBK or NBK cells infected with the fecal astrovirus was similar when the fixation and staining was performed at 24, 48, and 72 h post-infection. The highest number of positive cells was detected at 48 h post-infection, double those counted at 24 h. However, only a minority of cells apparently became infected as the ratio of infected cells to total cells did not exceed 1:60.

Infected IF positive cells were first observed at 7–8 h post-infection (Fig. 1a). The earliest sign was faint IF in the cytoplasmic area, followed shortly thereafter by the appearance of large, spherical, strongly positive regions in the nucleus (Fig. 1b), with the same distribution of nucleoli as determined by Giemsa staining. This was followed by the appearance of densely stained cytoplasmic granules in the perinuclear region (Fig. 1c). At this time and later, some brilliant positive cells were seen with dense IF occupying most of the cytoplasmic and nuclear areas (Fig. 1d).

Adaptation of astrovirus to replicate in cell culture

Following inoculation of fecal astrovirus into cultures of the continuous cell lines MDBK, GBK and BT, no IF positive cells were observed at 24, 48 and 72 h post-infection. Similarly, by the third passage of EBK cells, the cultures would no longer support viral infection. In contrast, no apparent reduction of fecal astrovirus titer, approximately $10^{5.0}$ IFFU ml^{-1}, occurred up to Passage 5 of NBK cells, and positive immunofluorescent cells were detected when NBK passage 19 cells were infected with fecal virus. However, the proportion of astrovirus infectable cells in a culture decreased slowly as the NBK cells were subcultured from primary cultures, with a rapid decline in these cells after Passage 19. In primary and secondary EBK and up to Passage 5 of NBK the proportion of infected cells varied between 1–20% of the cells of the cultures, with a minority of NBK primary cultures (prepared from 3/9 calves) in which no infected cells were observed.

Subculturing of astrovirus in primary EBK or NBK at 24-, 48- or 72-h incubation periods, resulted in loss of infectivity by the third or fourth passage of virus.

When the bovine astrovirus was passaged every 7 days in NBK cell cultures, with 50 µg ml^{-1} of trypsin in the MEM, the virus was subcultured successfully. In contrast, passage of the virus at intervals of 1–3 days resulted in loss of infectivity. Because the 50 µg ml^{-1} trypsin caused detachment of cells from the plastic of both infected and uninfected cell cultures within 48 h, infectivity of each passage was determined by infecting cultures without trypsin and fix-
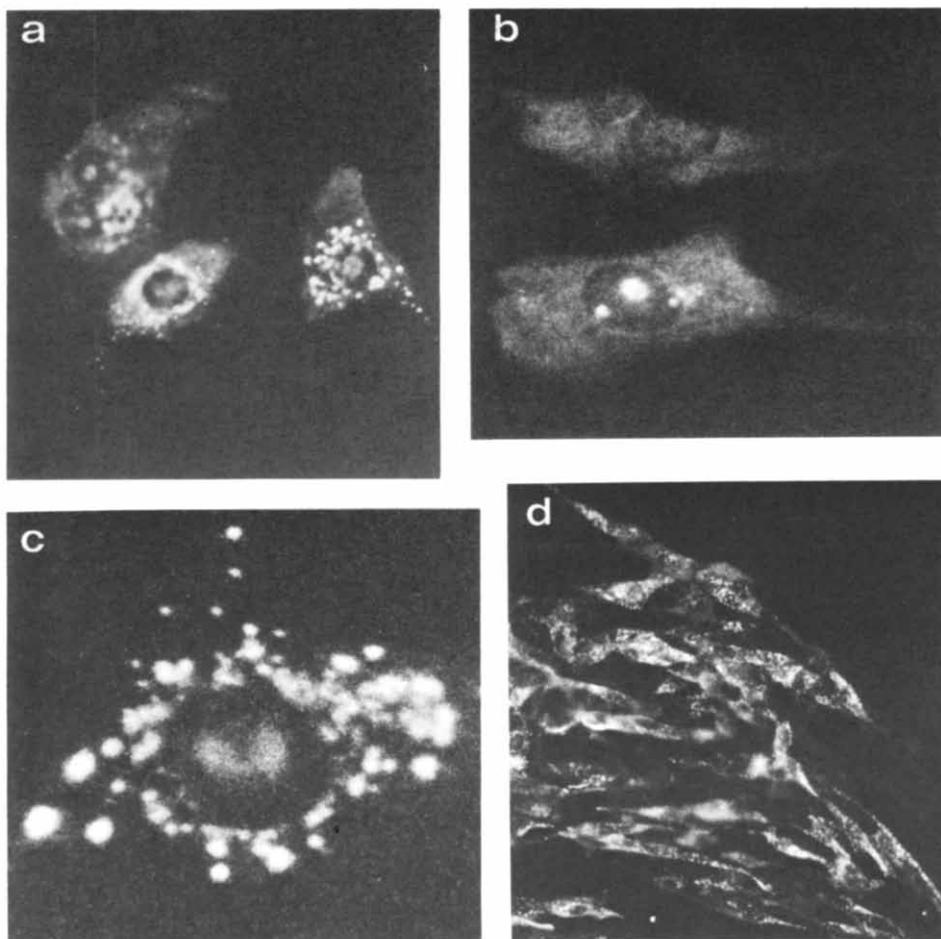
Fig. 1. IF staining of NBK cells infected with bovine astrovirus: (a) fecal astrovirus (GC54) fixed at 24 h post-infection to show cells at different stages of infection in the same culture; (b) TCA astrovirus Passage 8 infected in NBK primary cells fixed 48 h post-infection to show fine granules in the cytoplasm and densely stained granules in the nucleus; (c) TCA astrovirus Passage 12 fixed at 24 h post-infection to show large, strongly positive cytoplasmic granules and two round granules in the nuclear region; (d) TCA astrovirus Passage 22.

ing at 24 h for IF. From virus-passage 15–29, virus replicated and was released from cells within 72 h, and after Passage 30, within 48 h. To confirm that cell-free virus was produced during passaging, the cells and cell debris were removed by centrifugation at $2000 \times g$. The infectivity titer of Passage 19 cell-free virus was approximately 10^6 IFFU ml^{-1}. This result provided clear evidence of viral replication.

Even when the multiplicity of infection of TCA astrovirus was one or greater,

80–99% of cells of infected cultures were uninfectable, as judged by IF. However, in discrete areas most of the cells became infected, particularly in older cultures (Fig. 1d).

The pattern of IF and its distribution in the cells infected with TCA astrovirus at all passages, was similar to that observed in cells infected with fecal astrovirus.

Identification of the virus adapted to cell culture

Serology

There was no cross-reaction by IF between TCA astrovirus and antisera to Breda virus, bovine coronavirus, or bovine rotavirus B223. The TCA astrovirus virus reacted with antisera to bovine astroviruses US 1 and 2, and UK astrovirus (possible serotype 3) which have cross-immunofluorescent relationships (Woode et al., 1985).

By NT the titers of GC39 antiserum (to US2) and GC43 antiserum (to US1) with TCA astrovirus were 320 and <10, respectively. These titers were approximately the same as those reported for fecal US2 astrovirus (Woode et al., 1985) and confirmed that the TCA astrovirus was serotype 2 (US2).

Ultrastructural studies of infected cell cultures

The NBK primary cultures were homogeneous with most of the cells apparently of epithelial origin, as evidenced by the presence of desmosomes. Seven of 462 cells (1.5%) of the infected cultures at 24 h post-infection contained virions. Virions were not observed in an equal number of control cells or in the 48-h infected cultures. In one case, 3 contiguous cells contained virions (Fig. 2). Within infected cells, virions were found in numerous discrete cytoplasmic foci which were not enclosed within any type of membrane-bound structure. Nuclei and nucleoli appeared to be normal and lacked virions or unusual accumulations of virus-associated material. Measurements of 20 virions gave an average diameter of 34 nm with a range of 30–37 nm. At high magnification the virions were round in cross-section with spiky outer projections. The proportion of infected cells as determined by IF of cover-slip cultures was similar at 1–2%.

Preliminary studies on the properties of bovine astrovirus

The results of chloroform treatment and the effect of actinomycin D and iododeoxyuridine on the infectivity or replication of astrovirus, rotavirus and BHV1 virus are given in Tables 1 and 2. BHV1 infectivity was removed by the chloroform and its replication significantly reduced by both actinomycin D and iododeoxyuridine. In contrast, infectivity and replication of astrovirus and rotavirus were unaffected by these treatments.
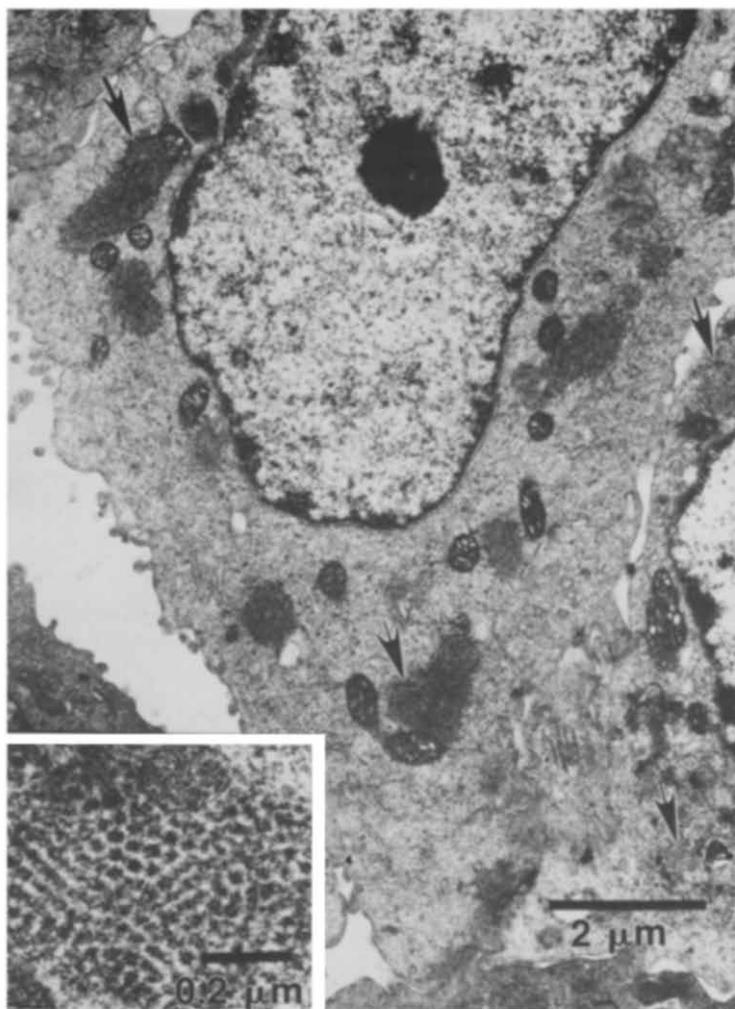
Fig. 2. Portion of two contiguous astrovirus-infected cells. Arrows indicate several focal accumulations of virions. Inset shows virions at a higher magnification.

TABLE 1

Effect of chloroform on astrovirus infectivity

Virus	Virus titer ($\log_{10}$ ml^{-1})	
	Chloroform (5%)	Control
TCA astrovirus	$10^{4.0}$	$10^{4.0}$
Fecal astrovirus	$10^{6.2}$	$10^{6.3}$
BHV1	$<10^{1.0}$	$10^{6.0}$
Rotavirus	$10^{6.2}$	$10^{6.4}$

TABLE 2

Effect of actinomycin D and iododeoxyuridine on astrovirus replication

Virus	Virus titer ($\log_{10}$ ml^{-1})			
	AMD[1] (0.05 μg ml^{-1})	Control[1]	IDU[2] (100 μg ml^{-1})	Control[2]
TCA astrovirus	$10^{3.5}$	$10^{3.5}$	$10^{4.3}$	$10^{4.1}$
BHV1	$<10^{1.0}$	$10^{7.7}$	$10^{2.4}$	$10^{7.5}$
Rotavirus	$10^{5.12}$	$10^{5.5}$	$10^{6.4}$	$10^{6.1}$

[1]Cultures sampled 48 h post-infection and viruses titrated.
[2]Cultures sampled 72 h post-infection and viruses titrated.

DISCUSSION

Infection and replication in tissue culture of bovine astrovirus (US2) was studied with both fecal virus and tissue-culture adapted virus. Infection and at least partial replication was assumed to be associated with the appearance of immunofluorescent positive cells, a condition which was an essential prerequisite for the release of infectious virus under the influence of trypsin. The optimal concentration of trypsin needed for astrovirus replication, release of infectious virus and virus passage has not been determined, nor have other proteases been tested for their effect. At a concentration of 50 μg ml^{-1} of trypsin, cell-free infectious astrovirus was produced from infected cultures, eventually reaching a titer of approximately 10^6 IFFU ml^{-1}. The mechanism of the effect of trypsin on the replication of bovine astrovirus was not determined in these studies, nor was it determined for human astrovirus (Lee and Kurtz, 1981). Trypsin, a proteolytic enzyme, has been used with rotavirus and influenza virus in order to increase viral infectivities. Cleavage of the hemagglutinin glycoprotein by proteolytic enzymes is necessary for influenza virus infection (Klenk et al., 1975; Lasorowitz and Choppin, 1975). Trypsin has a similar effect on rotavirus by specific cleavage of one of the outer-capsid proteins that facilitates the uncoating stage of viral replication (Theil et al., 1977; Clark et al., 1981; Espejo et al., 1981).

Astrovirus infection was not inhibited by the treatment of virus with chloroform, indicating a lack of essential lipids. The addition of iododeoxyuridine (a thymidine analogue), or actinomycin D (an inhibitor of DNA transcription) to the medium did not inhibit infection. All these treatments blocked the infectivity or replication of BHV1 but they had no effect on bovine rotavirus. Thus, one can conclude that bovine astrovirus lacks a lipid-containing envelope and is not a DNA virus nor a DNA-dependent RNA virus. The lack of an envelope confirms the electron-microscopic description of the virus (Madeley

and Cosgrove, 1975; Woode and Bridger, 1978) that it has an unbroken circular border with a five- or six-pointed surface star and no envelope is observed.

Astrovirus infection in primary bovine cells (EBK, NBK), and in passages of these cells, was similar as judged from immunofluorescent studies. The course of development of the virus infection was construed to be as follows: the earliest events in replication are cytoplasmic, followed shortly by involvement of nuclear structures which are possibly nucleoli. Areas of intense viral antigen concentration then develop in the cytoplasm, initially perinuclear and then spreading to involve much of the cytoplasmic space. Ultrastructural studies showed masses of virions with a similar cytoplasmic distribution as the IF.

The role of the cell nucleus (or nucleolus) in astrovirus replication was partially tested by the use of actinomycin D. Horzinek et al. (1984) have reported that the Berne equine virus, a member of the proposed family Toroviridae, a ssRNA virus, requires host-cell nuclear activity to support viral replication because actinomycin D and alpha-amanitin, a specific inhibitor of nucleoplasmic DNA-dependent RNA polymerase II, can reduce viral multiplication when the reagents are present during the first 8 h after infection. The lack of an inhibitory effect of actinomycin D on astrovirus replication suggests that the nucleolus is either uninfluenced by the actinomycin D treatment or that it is not essential for replication.

The size and appearance of the virions in the cytoplasm of infected cell cultures was similar to those observed in thin sections of infected cells of Peyer's patches (Woode et al., 1984). In the infected cell cultures the infected cells appeared similar morphologically to the uninfected cells, both apparently of epithelial cell origin and thus no morphological evidence was obtained to explain the restricted cell tropism of the virus. No ultrastructural evidence was obtained for the involvement of the nucleoli in replication which supported the data obtained by the lack of an inhibitory effect of actinomycin D. Similarly, ultrastructural observations of human astrovirus infection in primary human embryo kidney cells (Kurtz et al., 1979) and lamb astrovirus in gnotobiotic lambs (Gray et al., 1980), showed that virions were found in the cytoplasm of infected cells and there were no reports of the involvement of the nucleus in the replication and assembly of virions.

NBK cell passages produced monolayers containing predominantly cuboidal-shaped cells, in contrast to the spindle-shaped cells, possibly indicating fibroblasts, which were the majority of the cells seen in secondary and tertiary cultures of EBK cells. The cell passages of EBK cells may have resulted in an overgrowth of fibroblasts and stromal cells, as these cells generally have shorter generation times in culture than do epithelial cells (Douglas and Kaighn, 1974). These differences in the cell populations may be the reason for the loss of virus-infectable cells in subpassages of EBK cultures.

The difficulties experienced in attempting to cultivate astrovirus are not surprising, when one considers the restricted cell tropism of the virus in vivo.

The only cells known to be infected are the dome cells, usually the M cells, of the Peyer's patches; even if such cells exist in the kidneys, it is unlikely that they would be cultured selectively from trypsinized kidneys. The tubular kidney cell, probably the dominant epithelial cell of kidney tissue culture, supports rotavirus replication well. Rotavirus also replicates in the villus epithelial cells of the small intestine, which contain the Ia antigens, but not in the M cells. In rats, the epithelium lining the proximal tubules in the kidney cortex, similar to the villous epithelium in the gut, express Ia antigens in the basal part of the cells. In contrast, no Ia antigen expression is detected in M cells (Wiman et al., 1978; Hart and Fabre, 1981; Mayrhofer et al., 1983). It is possible that astrovirus can only infect or replicate in epithelial cells lacking Ia antigens.

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