Cultivation and characterization of bovine astrovirus

by

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INTRODUCTION

Astrovirus was first discovered associated with human gastroenteritis by Madeley and Cosgrove in 1975. The virus particle in negatively stained preparations was approximately 28 nm in diameter had a smooth surface and 5 to 6 pointed star shape of the internal configuration (Madeley and Cosgrove, 1979). Viruses resembling this astrovirus morphology have been detected in a variety of animals including calves (Woode and Bridger, 1978). The bovine astrovirus did not cause diarrhea in experimental calf infection, but serological surveys and isolation studies showed that the virus was a common infection in cattle (Woode and Bridger, 1978, Woode et al., 1985).

In lambs infected with astrovirus, the virus has been shown to destroy absorptive epithelial enterocytes lining the small intestine (Snodgrass et al., 1979), whereas bovine astrovirus is restricted to infection and destruction of the M cells covering the Peyer's patch follicles (Woode et al., 1984). M cells have been shown to be important in the local immune response of the gut, by transporting antigens, bacteria and viruses, to the underlying lymphoid tissue (Bockman and Cooper, 1973, Wolf et al., 1981). It has not been determined whether this new virus plays an important role in the reduction of the local immune responses which would predipose to secondary infection.

Only lamb astrovirus has been characterized for its viral properties, because large quantities of virus can be produced in the intestine of gnotobiotic lambs (Herring et al., 1981). The nucleic acid

is single-stranded RNA with a molecular weight of 2.7×10^6 daltons and 34S sedimentation coefficient. In their judgement, it could not be classified in the family Caliciviridae, which contains a single major polypeptide (Burroughs and Brown, 1974) nor in the family Picornaviridae, which has four major structural polypeptides (Cooper et al., 1978), as lamb astrovirus possesses two major polypeptides with similar molecular weight of approximately 3.3×10^4.

As bovine astrovirus is released in small quantities in the feces, full biochemical characterization of the virus and its antigenic properties have not been determined. Successful in vitro cultivation must be achieved in order for the virus properties to be determined for taxonomic purposes.

Cultivation of astroviruses in tissue culture has not been successful (Snodgrass and Gray, 1977, Gough et al., 1985, Tzipori et al., 1981, William, 1980, Woode et al., 1984) except for human astrovirus which was passed serially in primary human embryo kidney, primary baboon kidney and a continuous line of rhesus monkey kidney cells with the aid of trypsin (Lee and Kurtz, 1981).

This study was concerned with determining the conditions required for in vitro cultivation of bovine astrovirus. Successful cultivation permitted the use of chloroform, and the replication inhibition of DNA virus synthesis by iododeoxyuridine and actinomycin D, to determine the presence or absence of essential lipids and the probable nature of the genome.

LITERATURE REVIEW

Neonatal Calf Diarrhea

Neonatal diarrhea is very common in calves aged two days to three weeks, and is less frequent in older calves. The economic loss varies among herds and may depend on the management. An annual cost based on retardation of growth and mortality of calves was estimated at 95.5 million dollars (House, 1978). The etiology of diarrhea is complex. Three major groups of microorganisms are considered important as causes of diarrhea in young animals: bacteria, protozoa, and viruses (Tzipori, 1981).

The most common bacterium causing diarrhea in vertebrate animals is Escherichia coli. The estimated average annual loss from E. coli was between $48.6 and $71.2 million, whereas that from coronavirus was between $16.7 and 28.4 million and from rotavirus was between $3.1 and $8.7 million, in two studies; from Cryptosporidia was $6.2 million in one study (House, 1978).

Pathogenic mechanisms of each enteric microorganism are different. Pathogenic E. coli strains adhere to the intestinal epithelium and produce an enterotoxin causing intestinal secretion and watery diarrhea. Invasive strains of E. coli do not produce toxins but invade and destroy intestinal epithelial cells, causing enteritis and dysentery (Moon, 1974).

Cryptosporidium is common in neonatal diarrhea of calves. Certain stages of its life cycle attach to the microvillous border of epithelial

cells at villous tips, as extracellular parasites (Panciera et al., 1971, Pohlenz et al., 1978, Pearson and Logan, 1978). The parasites were detectable as spherical bodies of various sizes distributed over the surface of the atrophic villi, of the upper part of the small intestine.

Since the early 1970s, the use of the electron microscope in the investigation of diarrheic fecal samples has provided new discoveries of previously undetectable viruses such as rotavirus, coronavirus, astrovirus, calicivirus, Breda virus, etc. (Mebus et al., 1969, Stair et al., 1972, Madeley and Cosgrove, 1975, 1976, Woode and Bridger, 1978, Woode et al., 1982).

Viruses are found commonly in association with neonatal calf diarrhea: rotavirus and coronavirus are being associated with 20-80% of epizootic or enzootic infections (Woode, 1982). Other viruses such as parvovirus, calicivirus, astrovirus, and Breda virus have been shown to be experimentally pathogenic but their roles in natural disease have not been fully determined.

Enteric viruses cause damage of the intestine leading to diarrhea in newborn or young animals. Rotavirus infects absorptive cells of the anterior part of the small intestine (Mebus et al., 1971). Bovine coronavirus infects the absorptive epithelium of the small intestine, cecum, and colon, resulting in small intestinal villous and colonic ridge atrophy (Mebus et al., 1975, Patel et al., 1982). Bovine parvovirus has a preference for actively dividing cells of the crypts at the base of villi of the small intestine (Storz and Bates, 1973, Durham et al., 1985). The virus causes villous atrophy and fusion, due to crypt cell

damage.

The newly identified virus, bovine Breda virus, causes cytopathic changes of the enterocytes of posterior or lower small intestine, large intestine and dome epithelial cells (Woode et al., 1982, Pohlenz et al., 1984). In contrast, bovine calicivirus was restricted the anterior half of the small intestine causing degeneration and exfoliation of the enterocytes (Hall et al., 1984). Bovine astrovirus had a restricted site of infection, the M cells lining the lymphoid follicles of the small intestine, and did not cause diarrhea or clinical signs of disease (Woode et al., 1984, 1985).

The severity of intestinal disorders is influenced by other factors also. Animal housing conditions, individual animal variations, virulence of pathogens, and hygiene management all are believed to play significant roles in the development, severity and frequency of animal diarrhea.

Astrovirus

Astrovirus was described in humans associated with diarrhea, in 1975, by Madeley and Cosgrove. The name "astrovirus" is proposed for a particle, 28 nm in diameter, which has a round unbroken edge, a six-pointed star with a white center, and surface hollows that are triangular (Madeley, 1979). The virus was observed in the fecal samples of more than fifty babies. Subsequently, more viruses from a variety of animal species, resembling the morphology of human astrovirus have been discovered in lambs, calves, pigs, chickens, puppies, deer, cat, ducks,

and mice (Snodgrass and Gray, 1977, Woode and Bridger, 1978, Bridger, 1980, McNulty et al., 1980, Saif et al., 1985, William, 1980, Tzipori et al., 1981, Hoshino et al., 1981, Gough et al., 1984, Kjeldsberg and Hem, 1985).

Human astrovirus associated with gastroenteritis was found in both adults and children (Kurtz et al., 1977). Astrovirus particles were detected in feces of 17 of 27 symptomatic children and 4 of 14 ward staff members who reported an episode of diarrhea during that time. Transmissible infection of human astrovirus by experimental oral ingestion of a filtrate (Kurtz et al., 1979), prepared from feces of a child with mild gastroenteris shown by electron microscope to contain numerous astrovirus particles, to eight volunteers resulted in the development of diarrhea with large amounts of astrovirus shedding in the feces of one. Another individual had mild symptoms with a lower level of virus shedding and two of nine volunteers receiving fecal filtrate from the diarrheic volunteer, had astrovirus shedding but did not develope diarrhea.

Most astroviruses in animals were found associated with gastroenteritis disorders in the young. In piglets, the virus particles were detected together with calicivirus and rotavirus-like particles in the feces of weaning piglets showing diarrhea (Bridger, 1980). In three to four-week-old deer suffering from diarrhea associated with Cryptosporidium, 5 of 56 samples were found containing a few aggregates of particles resembling astrovirus (Tzipori et al., 1981). And, canine astrovirus-like particles were observed associated with coronavirus-like

and parvovirus-like particles in the diarrhea stools of beagle pups, of a 3-month-old litter (William, 1980).

Although astrovirus usually has been reported to infect young animals, there is a report of astrovirus in the feces of a 4-month-old cat who had diarrhea 5 days after receiving vaccination for feline panleukopenia, feline viral rhinotracheitis and feline calicivirus. The cat had poor body condition with a green, watery diarrhea. It is possible that vaccination altered the susceptibility of the cat to astrovirus infection.

In addition, astrovirus was isolated from the gut contents of a colony of nude mice from both diarrheic and non diarrheic animals (Kjeldsberg and Hem, 1985). There were 94% and 62% of diarrheic mice and non-diarrheic mice, respectively, 4 weeks to 9 months old, had astrovirus infection.

Avian astrovirus was detected in fecal samples of diarrheic turkeys in two cases (McNulty et al., 1980, Saif et al., 1985). The first detection of astrovirus in turkey fecal contents was obtained from 6- and 11-day-old poults. In both cases, history of diarrhea and increased mortality was reported. Recently, astrovirus is claimed to be the second most frequently detected virus with adenovirus and/or enterovirus from diarrheic poults; rotavirus was the most frequent (Saif et al., 1985). In this observation, 33 intestinal samples from 10-to-21-day-old diarrheic turkeys were examined.

In contrast to enteric astrovirus infection, astrovirus-like particles were detected from ducks with hepatitis (Gough et al., 1984,

1985). The agents were observed and isolated from liver suspension of 6-to-14-day-old ducks with 50% mortality and 4-to-6-week-old ducks with 25% mortality. Inoculation into chicken embryos showed growth stunting with green necrotic livers. Experimental infection of 20 ducklings with a liver-suspension preparation of the virus caused 5 deaths at 2 to 4 days postinoculation (pi.). The survivers developed a serological response positive for the related virus. Astroviruses were detected from both infected chicken embryos and from ducklings showing lesions (Gough et al., 1985).

More experimental studies have been performed with lamb astrovirus which was isolated from diarrheic feces by Snodgrass and Gray (1977). The 5-to-6-pointed star particles, 29.7 nm in diameter, were detected in fecal sample from 4-to-6-week-old lambs. The virus was shown to be pathogenic to 6 gnotobiotic lambs by causing diarrhea after oral infection with fecal filtrates (Snodgrass and Gray, 1977, Snodgrass et al., 1979). The infected lambs developed diarrhea with loose yellow stools, 44 to 48 hours after infection. The viral particles could be found in the intestinal content of every lamb killed after 23 hours pi. (Snodgrass and Gray, 1977, Snodgrass et al., 1979).

Immunofluorescent positive cells were present in the mucosal epithelium and subepithelial tissues between 14-70 hours after infection. Infected absorptive enterocytes were found scattered through the apical half of the villi. The most affected sites of astrovirus infection were found in the midgut and posterior ileum. Astrovirus infected cells were limited to mature villous epithelial cells (absorptive enterocytes) and

subepithelial macrophages. The destruction of infected epithelium caused partial villous atophy, and all the lesions gradually disappeared by 5 days after inoculation (Snodgrass et al., 1979).

Following the early experiment, Gray and his associates (1980) reported an ultrastructural study of the astrovirus infected intestinal cells. The viral particles were observed as aggregated form in cytoplasm of mature columnar epithelial cells covering the apical two-thirds of villi during 14 to 38 hours pi. Viral release followed desquamation and disintegration of the mucosal epithelium. The recovery of the intestinal villi occurred by 120 hours post infection. Viral particles were observed in apical pits, tubules and lysosomes.

Bovine astrovirus (UK 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 6-pointed star configuration and 28 nm in diameter, were not pathogenic because transmission of the virus could not induce diarrhea in gnotobiotic calves. Later, two more isolates of the virus were reported in the USA (Woode et al., 1984, 1985), they were known as astrovirus US1 and US2. The bovine astrovirus US1 was detected from fecal samples of a calf from a herd with diarrheic history in Florida (Woode et al., 1984). The bovine astrovirus US2 was detected from a colostrum deprived calf prepared for an experiment in Woode's laboratory, associated with bovine rotavirus (Woode et al., 1985). These three isolates of bovine astrovirus: UK, US1, US2 possessed common antigen detected by immunofluorescence (Woode et al., 1985). However, there was no cross neutralization among the three isolates.

Therefore, there are at least two distinct serotypes and probably three: US1 serotype 1, US2 serotype 2, UK serotype 3.

Thirty percent of serum samples collected from calves and cows were positive for antibody to astrovirus (Woode et al., 1984). Twenty-eight of 1,060 field samples of diarrhea in calves aged 3 days to 3 weeks were positive for astrovirus (Woode et al., 1985). Sixteen samples were tested for their serotypes: five belonged to serotype 1, none belonged to serotype 2, six belonged to serotype 3. The rest were not neutralized by any of the three antisera.

Pathogenesis of astrovirus infection of US1 and US2 was studied by experimental infection of the viruses in gnotobiotic calves (Woode et al., 1984, 1985). Astrovirus alone did not cause diarrhea or clinical illness, although the viruses were shed in feces. When astrovirus was mixed with rotavirus or Breda virus 2, the calves developed severe greenish-yellow watery diarrhea which persisted for 3 to 5 days. The incubation time between astrovirus infection and the first detectable excretion of virus in feces, 24-96 hours, varied independently of the age of calves, and dose of virus inoculated, implying individual animal variation. Identification of astrovirus, US1 and US2, was made by infected tissue cultures (Woode et al., 1984, 1985). In contrast to lamb astrovirus, bovine astrovirus did not infect mature villous enterocytes but only the dome epithelial cells (M cells) of the jejunal and ileal Peyer's Patches. There was fusion of dome epithelium with adjacent absorptive enterocytes near the dome base. The astrovirus infected cells appeared to be necrotic and sloughing. They were either flattened or

rounded and extruded from the tip, and some were free in the intestinal lumen. Scanning electron microscopy confirmed that M cells exclusively were sloughing; they were irregular in shape and devoid of microfolds. Histopathological studies showed that cell debris covering domes consisted of sloughing epithelial cells (M cells), mononuclear cells, and eosinophilic cells.

Two forms of the virus were found in virus infected M cells: large masses of virions in an electron-dense matrix clustered around the nucleus and small aggregates of virions packed between apical tubules. One M cell from a mixed infection was found to have both astrovirus 1 and Breda virus 2.

Cytopathic infections of M cells of domes by astroviruses, US1 and US2, may have an important pathogenic effect on the host as M cells appear to play a very important role in the local immune response of the gut (Woode et al., 1984, Wolf and Bye, 1984). Degeneration of M cells may exacerbate infection of calves with other pathogens since astrovirus infection from field studies has been followed by, or has been coincidental with, infection of other pathogens such as rotavirus, coronavirus, or Cryptosporidium (Woode et al., 1984).

Like most enteric viruses, obtaining astrovirus replication in tissue culture has not been easy. No successful propagation of astrovirus has been reported with the exception of the human astrovirus which was passed serially in primary human embryo kidney cells with the aid of trypsin (Lee and Kurtz, 1981). Foci of single infected cells could be detected in the first passage in primary cell cultures with the

following viruses: lamb astrovirus in fetal lamb kidney cells (Snodgrass and Gray, 1977), bovine astrovirus in primary bovine embryo kidney cells (Woode and Bridger, 1978), canine astrovirus in canine kidney and human embryo kidney cells (William, 1980), deer astrovirus in bovine embryonic kidney cells (Tzipori et al., 1981), feline astrovirus in feline kidney and fetal rhesus monkey kidney cells (Hoshino et al., 1981), and duck hepatitis virus in chicken embryo liver cells and duck kidney tissue culture (Gough et al., 1985).

Passages of bovine astrovirus in primary, secondary and tertiary bovine embryo kidney cells showed a decline in the number of positive infected cells (Woode et al., 1984). It was suggested that this failure might be due to a combination of relative lack of susceptible cells in the secondary cells and the lack of trypsin in the medium which had been proved necessary on human astrovirus culture (Lee and Kurtz, 1981).

The immunofluorescent cells could be observed in cells fixed 24 to 72 hours pi. (Woode et al., 1984). No cytopathic effect was detected during the next 7 to 10 day period. Meanwhile, bovine astrovirus was detected in the feces of gnotobiotic calves 24 to 96 hours after infection, and it was shed for as long as 7 to 9 days.

Interestingly, although the morphology of the star shaped viruses in different species are similar, there is no serological antigenic relationship among human, bovine, ovine, and porcine astroviruses (Bridger, 1980, Tzipori et al., 1981, Snodgrass et al., 1979, Woode et al., 1985).

The properties of astrovirus have been reported only in lambs

(Herring et al., 1981) because of the lack of viral propagation in culture and because the bovine astrovirus is produced in small quantities. Lamb astrovirus was obtained 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 determined to contain a single stranded (ss) RNA with the molecular weight of 2.7×10^6 daltons, and there were two major polypeptides of approximately 3.3×10^4.

From the classification and nomenclature of viruses (Matthews, 1982), viruses in the family Caliciviridae possess positive-sense ssRNA, with one major polypeptide, a MW of $10\text{-}15 \times 10^3$ and no essential lipid. The Picornaviridae family has positive ssRNA, no envelope and four major polypeptides: three of MW $24\text{-}41 \times 10^3$, and one of MW $5.5\text{-}13.5 \times 10^3$.

Astrovirus seems to fit between the Caliciviridae and the Picornaviridae groups because lamb astrovirus was demonstrated to have two major polypeptides (Herring et al., 1981). However, more studies of astroviruses of other species are needed in order to provide information for the taxonomic classification of the virus.

M Cell

Cell tropism of astroviruses is different among species. In duck, the virus can cause disease due to damage of the liver (Gough et al., 1984, 1985). In contrast, lamb astrovirus destroys the majority of the

absorptive epithelium lining the intestinal wall (Snodgrass et al., 1979). On the other hand, bovine astrovirus selectively infects and multiplies in M cells only (Woode et al., 1984, 1985). The restriction of bovine astrovirus to the M cells with the resulting destruction of the infected cells, is very interesting and might be an important pathogenic effect of the virus. However, as there is no precedent for an agent that only infects M cells, and thus no evidence available concerning the loss of M cells on the local or general immune responses, the effect of astrovirus pathogenesis has not been elucidated.

M cells or membraneous cells are defined as "specialized epithelial cells overlying the gut associated and bronchial associated lymphoid tissues (GALT and BALT) that transport antigens from the lumen to the extracellular space, allowing access to lymphocytes, macrophages, and plasma cells" (Wolf and Bye, 1984). They are also called lymphoepithelial cells or follicular-associated epithelial cells in the literature.

The cells were first described in the late 1960s and have been found overlying the lymphoid follicle in various species including mice, rabbits, chickens (Bockman and Cooper, 1973), human (Owen and Jones, 1974), hamster, monkeys, dogs (Owen and Nemaric, 1978), pigs, calves (Torres-Medina, 1981, Landsverk, 1981a). Along the intestinal tract of human and mice, M cells are found as cuboidal cells (Bockman and Cooper, 1973, Owen and Jones, 1974). However, calf M cells are described as columnar cells rather than cuboidal cells (Torres-Medina, 1981, Landsverk, 1981a).

Studies of the ultrastructure of M cells with the transmission electron microscope and the scanning electron microscope was necessary for their identification (Owen and Jones, 1974, Bye et al., 1984). In mice, the follicular epithelial cells that cover the lymphoid follicles of ileal Peyer's patches are composed of goblet cells, absorptive epithelium, M cells and enteroendocrine cells (Bye et al., 1984). M cells can be distinguished from others by their microfolds or microvilli which are shorter, wider and fewer compared to the microvilli of adjacent absorptive cells. The microfolds appear on the apical luminal surface and the nucleus is usually seen in the basal cytoplasm beneath the central invaginated space, generally containing intraepithelial lymphocytes (Bye et al., 1984, Owen and Jones, 1974, Egberts et al., 1985).

In mice, Bye and his colleagues (1984) have studied and classified the M cell from its appearance into two categories: mature and immature M cell. Immature M cells lack the central cytoplasmic hollow, which in immature cells contain lymphoid cells. The morphology of the immature M cells is intermediate between the mature M cell and the absorptive epithelial cell because the immature cell have the microvilli which were more numerous and regular appearing than those of mature cells.

The origin of M cells is not clear. Bye et al. (1984) believed that many if not all M cells derived from undifferentiated crypt cells for many reasons. Firstly, they thought that immature M cells appeared to be the intermediate between the mature M cells and the absorptive mucosal cells. Secondly, they observed both mature and immature-appearing M

cells at the base of the follicular domes near the mouth of surrounding crypts. Thirdly, using (^{3}H) thymidine labeling, the nuclei of most crypt epithelial cells, but not M cells, were labeled at 6 and 12 hours after (^{3}H) thymidine injection. On the other hand, some immature M cells were labeled at 24 hours, and both labeled mature and immature M cells were seen on domes harvested at 48 hours after initial exposure of the labeling. This result supported the observation of Bhalla and Owen, 1982, who demonstrated nuclear labeling of the dome epithelium 72 hours after the labeling initiation.

In young milk-fed calves, dome epithelial cells were described as short, conical or tongue-shaped columnar cells (Landsverk, 1979). Correlated to this, Torres-Medina (1981) demonstrated the dome epithelium of Peyer's patches of gnotobiotic calves had a uniform population of cells which were columnar, in shape, in contrast to the cuboidal cells usually found in other species. The surface of bovine domes bulge toward the intestinal lumen and form deep intercellular crevices. The apical surface of the lymphoepithelial cells were covered by densely packed, blunt microvilli which were irregular, short and thick. Bovine M cells contain basal nuclei with a distinctive nucleolus and cytoplasm which is rich in mitochondria, microtubules and vacuoles (Torres-Medina, 1981).

The specialized maturation of M cells probably plays a significant role in the local immune response of the gut. In addition to the morphological differences, there are differences in the biochemical composition of M cells and absorptive enterocytes. Higher cholesterol and a low protein to lipid ratio of the M cell were recognized (Madara et

al., 1984). There was more elastase activity over luminal surface of M cells than on absorptive cells but more alkaline phosphatase was detected over absorptive cells than M cells (Owen and Bhalla, 1982, 1983). It was suggested that the absence of alkaline phosphatase may reflect rapid turnover of surface membrane due to vesicle formation during transportation. And, esterase activity may result from higher metabolic activity of M cells. Enzyme histochemistry of bovine M cells (Pospischil et al., 1986) showed the presence of acid phosphatase, succinic dehydrogenase, and B-galactocidase in both control animals and the animals infected with rotavirus and/or pathogenic E. coli. These properties may be involved in the antigen uptake and transportation of M cells (Owen and Bhalla, 1982, 1983).

Peyer's patches are identified as circumscribed areas of aggregation of lymphatic nodules appear at intervals in the antimesenteric site (Doughri et al., 1972). Total number of patches of bovine fetal gut per individual varies from 3 to 76 at the 18th and 40th week of fetal life, respectively. The greatest number and surface area of Peyer's patches throughout fetal development occurred in the jejunum, approximately 1:10, followed by the duodenum, the ileum, the ileocecal valve, and the proximal loop of the ascending colon.

Peyer's patches have a significant role in the local immune responses of the gut. From studies in rabbits, Peyer's patches are a highly enriched source of lymphoid cells which have high potency to proliferate and differentiate into IgA-producing cells (Graig and Cebra, 1971). Stimulation of lymphoid cells in Peyers' patches followed when M

cells allowed pinocytosis and transportation of antigens to the lamina propia (Bockman and Cooper, 1973). Ingestion of horseradish peroxidase showed the adherence of the substance to mice M cells and it was also found in the vesicles of M cells (Owen, 1977).

The local immune responses of the gut is a very complex process. The sensitized IgA-producing lymphoblasts leave Peyer's patches and enter the lymphatic system to travel to the mesenteric lymph nodes, and there via the thoracic duct to the blood circulation (Husband and Gowans, 1978). Mature lymphocytes will return to their original organ, the intestine, and finally reach the lamina propria, where they produce IgA dimers which bind specifically to the secretory component (SC) found on the epithelial cell basal membrane (Brandtzaeg and Baklien, 1977). The complex is transfered to the glycocalyx coat overlying the luminal surface of the epithelium by reverse pinocytosis. IgA presumably play an important role in the local immune response of the mucosa (Husband, 1985).

According to Woode et al., 1984, bovine astrovirus was observed in the dome epithelium of the ileal Peyer's patches. The virus caused focal necrosis and loss of columnar epithelium at the tip of domes. There was replacement of columnar M cell by flat or cuboidal cells. Degeneration effects of dome epithelium occurred whether the cells were infected with astrovirus alone, both astrovirus and Breda virus 2 or astrovirus and rotavirus. Large masses of astrovirus virions were arranged in an electron-dense matrix clustered around the nucleus and smaller aggregates of packed virion were found between numerous apical tubules and vesicles

(Woode et al., 1984).

Besides bovine astrovirus, reovirus type 1 and 3, Escherichia coli, and chlamydia have been reported to adhere to M cells (Wolf et al., 1983, Wolf et al., 1981, Inman and Cantey, 1983, Landsverk, 1981b, Bye et al., 1984). Reovirus type 1 inoculated into closed ileal loops of live suckling mice were detected adhering to all the M cells of the samples removed 30 minutes after infection (Wolf et al., 1981). Apparently, the reovirus type 1 initially adheres to the surface of the M cell, is endocytosed into the cell, transverses in vesicles, and then, passes through the extracellular space between M cells and lymphoid cells.

Comparison between reovirus type 1 and 3 in mice was studied again by Wolf and her colleagues (1983). Reovirus type 1 adhered to a minority of absorptive cells, beside M cells, in adult mice, but no viral particles were detected in the cytoplasm of the absorptive cells. In contrast, in suckling mice reovirus type 1 adhered to and was endocytosed by M cells selectively, while type 3 virus adhered and was endocytosed by both M cells and absorptive cells.

In addition, adherence of bacteria, E. coli, was found to be specific to M cells rather than absorptive epithelial cells of the lymphoid follicle epithelium (Inman and Cantey, 1983). However, experimental infections of calves with rotavirus and enterotoxigenic E. coli showed no viral or bacterial propagation in M cells (Torres-Medina, 1984, Pospischil et al., 1986). E. coli was not seen adhering to M cell but the microorganism was detected either free in the intercellular space or intracellular region of lymphoid cells beneath M cells (Torres-Medina,

1984). No immunofluorescence of rotavirus was detected at the dome region.

Recently, Bass et al. (in press) reported M cell infection by reovirus 1 in mice. Virus replication consisting of virions and dense viroplasms were found in dome absorptive epithelial, crypt and M cells, with a cytopathic effect on M cells occurring 72 hours post infection.

All of the evidence available shows that M cells are active for antigen transportation which results in the local immune responses. However, there are no studies on the effect of M cell destruction on the normal function of the Peyer's patches. It is reasonable to conclude that loss of this specialized cell might adversely affect the gut immune function.

MATERIALS AND METHODS

Astrovirus Isolates

The astrovirus isolate studied in this work is bovine astrovirus (US2) supplied by G. N. Woode (Veterinary Microbiology and Parasitology, Texas A & M University, TX). This isolate was reported in 1984 by Woode et al., and two sources of the virus were used, both were from gnotobiotic calves, GC54 and GC83.

Fecal samples of the gnotobiotic calves (GC54 and GC83) were diluted 1:10 with phosphate-buffered saline (PBS; pH 7.2), centrifuged at 6000 x g for 30 minutes. This viral suspension was used as the inoculum for the experiments.

Antisera

Convalescent gnotobiotic calf 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, obtained from G. N. Woode, which had been prepared in gnotobiotic calves bled at 21 days post oral infection.

Cell Culture

Three kinds of cell culture were used: primary bovine embryo kidney (PBEK) cells, primary newborn calf kidney (PNCK) cells, and bovine kidney (BK) cells.

Primary bovine embryo kidney (PBEK) cells

Bovine embryo kidneys were obtained from the National Animal Disease Center (NADC, Ames, IA) and prepared aseptically as followed:

a. The fat around the kidney and its capsule were removed.

b. The outer layer (cortex) of the kidney nodules was sliced off and collected for cell preparation. This was repeated for each kidney, the preparations pooled. Kidneys from 2 or more animals were used on each occasion.

c. The collected tissue was cut into small pieces of 2-4 mm.

d. After the tissue was washed once with PBS, it was trypsinized using 50 ml 0.25% trypsin with stirring in an incubator at 37 C. The trypsin was replaced every 10 minutes and the trypsinized cell suspension were added to centrifuge tubes containing 2 ml fetal bovine serum (GIBCO Laboratories, Grand Island, NY) and held at 4 C.

e. When the tissue trypsinization was complete, the cell suspensions were spun at 200 x g for 10 minutes. The packed cells were used for the fresh preparation of cell cultures, or they were frozen and kept in a liquid nitrogen tank. In order to freeze the cells, 1 ml of 40% fetal bovine serum in Eagle minimum essential medium (MEM, Flow Laboratories, Inc., Mclean, VA) and 1 ml of 15% dimethyl sulfoxide (DMSO, GIBCO Laboratories, Grand Island, NY) were added to 2 ml of packed cells. The cell suspension was dispensed in 2 ml aliquots, and frozen slowly in a polystyrene container at -80 C overnight and then stored in liquid nitrogen.

f. One ml of packed cells or 1 vial of frozen cells were resuspended in 200 ml growth medium containing 10% fetal bovine serum in MEM supplemented with 0.25% lactalbumin hydrolysate (Difco Laboratories, Detroit, Mich.), penicillin (100 iu/ml), streptomycin (100 ug/ml), amphotericin B (5 ug/ml; Fungizone, E. R. Squibb & Sons, Princeton, NJ). The resuspended cells were grown in 25 cm^2 flasks (Corning Glass Works, Corning, NY), or 96-well-microtiterplates (Costar, Cambridge, MA) or glass tubes containing cover-slips. Medium changes were needed for the culture after 2-3 day incubation, and the monolayers were confluent at 5-7 days. Before inoculation of the virus onto the cell monolayer, the culture was washed once with serum-free medium.

Primary newborn calf kidney (PNCK) cells

Newborn calf kidneys were obtained by aseptic removal of kidneys from newborn calves supplied by the Dairy Cattle Center (Texas A & M University, Collage Station, TX). Tissue preparations were processed by the same method as for PBEK, except for the freezing process. For the modified freezing process (Paul, 1975), DMSO (10%) and growth medium (80%) were mixed with 1 ml of packed cells (10%). The cell suspension was then aliquoted in 2 ml volumes, left overnight at -80 C and stored in a liquid nitrogen tank. One vial was resuspended in 20-25 ml serum free MEM, centrifuged at 200 x g for 10 minutes and the cells resuspended in growth medium for the establishment of primary cell cultures. The MEM used was from GIBCO Laboratories (Grand Island, NY), and some experiments were carried out using the cultures grown in Lab-Tek tissue culture 8-chamber slides (Miles Scientific, Naperville, Ill.).

Bovine kidney (BK) cells

The PNCK cell culture was trypsinized off the flasks using a trypsin-versene solution, and diluted 1:2 to 1:3 in growth medium. BK cells up to the tenth passage were used. The cells were grown in the same growth medium as for primary cells.

The formula of the solutions used in cell culture are as described below:

1. 0.25% Trypsin for primary cell

10X Dulbecco's Phosphate Buffer Solutions I		
NaCl	320	gm
KCl	8	gm
Na_2HPO_4	44.8	gm
KH_2PO_4	8	gm
q.s. Distilled Water	3200	ml

10X Dulbecco's Phosphate Buffer Solutions II		
$MgCl_2.6H_2O$	5	gm
q.s. Distilled Water	500	ml

Working Solutions

10X Dulbecco's PO_4 Buffer Solutions I	480 ml
10X Dulbecco's PO_4 Buffer Solutions II	60 ml
Distilled Water	5400 ml
Trypsin (Difco 1:250)	15 gm

Adjust pH to 6.8

2. Trypsin-Versene Solution

EDTA (diNa)	1	gm
Trypsin	2	gm
NaCl	8	gm
KH_2PO_4	0.2	gm
Na_2HPO_4	1.15	gm
q.s. Distilled Water	1000	ml

Adjust pH 7.2

Immunofluorescence Test (IF)

For all studies on astrovirus infection the cell cultures were washed once with serum free MEM, followed by application of the relevant viral preparation. The inoculated cover slip monolayers were fixed with acetone at 24 to 48 hours after infection. The microtiter plate cultures were fixed for 10 minutes with 80% acetone in PBS at -25 C, the cultures were dried and, then, rehydrated with PBS. The relevant antisera were diluted 1:20 in PBS and applied to the cultures for 1 hour at room temperature, at which time they were washed with PBS. Next, rabbit anti-bovine IgG fluorescein conjugates (RAB, Cappel, Cooper Biomedical, Inc., Malvern, PA) diluted at 1:60, was applied to the cultures for one more hour. Finally, after the last wash, the stained culture was mounted with 90% glycerol in PBS and observed under the fluorescent microscope, Leitzfluovert (E. Leitz, Inc., Rockleigh, NJ).

Quantitation of Astrovirus Infection

For the microtiter plates, the immunofluorescent cells in an area at the center of the well viewed with the 10x objective were counted; 4 wells per dilution. For the cover slips or chamber slides, all the immunofluorescent cells of the monolayer were counted.

To determine the percentage of cells that became infected, a total count of cells was obtained. Cells were grown in chamber-slides, 6 wells were inoculated with the virus, and after 1-3 days of incubation, 4 well cells were fixed for IF and from 2 wells the cells were stripped off by adding 0.2 ml of trypsin-versene to each well. The cells were counted in a hemocytometer chamber, and the total count per well obtained from the formula n x 0.2 x 1000 (n=number of cells/cu.mm., mean count of two wells).

Time Related Development of Immunofluorescence

Two sets of PNCK cell cultures in cover-slip tubes were infected with astrovirus fecal sample (GC83 1:100 dilution) and tissue culture adapted virus (A9, subcultured 5 times in PBEK and 4 times in PNCK). A pair of cover-slips were fixed with acetone every 30 minutes from 1 to 12 hours after infection and kept at -25 C. The cover-slips were stained next day with a 1:20 dilution of GC37 followed by a 1:60 dilution of RAB. The experiments were repeated twice.

Adaptation of Astrovirus to Cell Culture

Bovine continuous cell line

Two kinds of continuous cell line were used: GBK (Bovine kidney cell line, passage 170) and BT (Bovine turbinate cell line, passage 20) were received from Dr. David Reed (Nationional Animal Disease Center, Ames, IA). The cell monolayer on cover slips were infected with a 1:100 dilution of fecal astrovirus (GC54), at different ages of the cell monolayer: 2, 4, 5, and 10 days. The cultures were fixed at 24, 48, and 72 hours postinfection, and observed for immunofluorescent cells.

Bovine primary cell

Primary spleen cells A spleen was aseptically removed from a newborn calf and placed in serum-free MEM until used. The spleen was cut and cells were squeezed out of the capsule. The spleen cells were washed twice and diluted in 10% FBS MEM as 1:100 dilution, and incubate 37 C. The monolayer was confluent 10 days later and infected with a 1:100 dilution of fecal astrovirus (GC83). Infected and control cultures were fixed every 24 hours, for 7 days.

Peritoneal wash cells The peritoneal cavity of a newborn calf was washed with phosphate buffer saline (PBS), 500 ml, by intraperitoneal injection and the fluid was collected through the surgical opening of the abdomen. The fluid was centrifuged at 200 x g for 10 minutes, and resuspended to the final concentration of approximately 1-2 x 10^6 cells/ml. in 10% FBS MEM. The monolayers of 2 weeks old were infected with fecal astrovirus (GC83) and fixed for IF every 24 hours, for 7 days.

Effect of fetal bovine serum on astrovirus infection of cell culture

Different concentrations of fetal bovine serum (FBS) at final concentrations of 0, 5, 10 and 15 %, were added to the astrovirus (1:100 dilution of GC83) inocula when BK cells at passages 5 to 7 were infected. The number of immunofluorescent cells and total cells of the monolayers, infected with astrovirus with or without FBS at various concentrations were recorded at 24 hours.

Exposure of cell culture with ultraviolet irradiation prior to infection

PBEK cells layer growing in microtiterplates were exposed to ultraviolet (uv) irradiation (Philips, 15W, Germicidal Lamp, with a radiation intensity of 5 J/m^2 s.), with a different exposure time for each row of 4 wells. The exposure time varied by two seconds steps from 0-20 seconds. The monolayers were then inoculated with a 1:100 dilution of fecal astrovirus (GC54) and incubated at 37 C for 24 hours. Immunofluorescence cells were counted in one area viewed with 10x objective, four wells for each of the uv exposure time.

Effect of centrifugation of cell culture on astrovirus infection

Microtiter plates of PBEK cell monolayer were spun at 2500 rpm, for 30 minutes intermediately post inoculation (pi.) with a 1:100 dilution fecal astrovirus (GC54). The uncentrifuged plate, acted as a control plate. The infected monolayer were incubated 37 C and fixed for IF 24 hours later. An immunofluorescence cell count was made as before.

Effect of trypsin on astrovirus infection

Fecal astrovirus (GC83) was diluted and inoculated into PNCK cell cultures grown on cover slips. The following trypsin concentrations in

serum free MEM were prepared: 0, 10, 25, 50 and 100 ug/ml. The cultures were fixed for IF after 24 hours incubation at 37 C. The total number of immunofluorescent cells per cover slip was compared between infected cultures with or without the various concentrations of trypsin.

Passage of PBEK and PNCK cells and astrovirus infection

Both PBEK and PNCK cell cultures were trypsinized with trypsin-versene and resuspended in 1:2 dilution of 10% FBS MEM. The passages of PBEK cells were grown in microtiter plates; the passages of PNCK cells were grown in chambered slides. The secondary and tertiary cell cultures, confluent within 24-48 hours were infected with a 1:100 dilution fecal astrovirus (GC54). The IF titer of astrovirus in each culture was determined as before at 24 hours pi.

Adaptation of Astrovirus to Replicate in Primary Cells

Set of tubes containing PBEK cells growing on cover-slips were inoculated with astrovirus GC54 in serum free MEM. At 24, 48, and 72 hours post infection, the cover-slips were fixed and stained for immunofluorescence with GC37 antiserum (1:20) and RAB (1:60). Meanwhile, fluid and cells in the tubes were subcultured to new culture tubes; the incubation period was consistent for each line. Each line with different incubation period had at least four tubes: two had virus in serum-free medium, the other two had virus with medium containing 25 ug of trypsin (0.25% Trypsin used to trypsinize kidneys for primary cell preparation).

PBEK and PNCK cell culture in 25-cm^2-flasks was used for 7-day

subcultures. Three lines of the viral passages were set up: One was in serum-free medium, the other two in medium containing 25 and 50 ug of trypsin per ml, respectively. No media change was done over 7 days. On day 7, the cells were scraped off the flask with a "plastic policeman" or if they had stripped, as usually happened with the passages containing trypsin in the medium, the media containing cell debris, was collected. Serial passages, including cells or cell debris plus the medium, were inoculated in 25 cm^2 flask and cover-slips washed once with serum free MEM. The cover-slips were tested for its infectivity of that early passages by IF test at 48 hours incubation. Sometimes the harvest was frozen to -80 C and stored before the next passage.

Lipid Sensitivity Test

A modification of the chloroform sensitivity test, of Rovozzo and Burke, 1973, was performed in order to determined whether astrovirions contained essential lipids. An amount of 0.05 ml chloroform (Chloroform anhydrous, Fisher Scientific Company, Fair Lawn, NJ) was added to different tubes containing 1 ml of one of the following four samples: a 1:10 fecal suspension of astrovirus (GC54), astrovirus passage 10 in BK cells, MEM (negative control), and bovid herpesvirus 1 (BHV1 or Infectious bovine rhinotracheitis virus, as positive control). The BHV1 was obtained from R. A. Crandell (Texas Veterinary Medical Diagnostic Laboratory, Texas A & M University, TX). The mixtures, were then shaken for 10 minutes, and centrifuged at 800 rpm for 10 minutes. The upper

layer of the fluid was removed and titrated by ten fold serial dilution in serum free medium. The cultures were examined for virus 48 hours later; by immunofluorescence for astrovirus and by cytopathic effect (CPE) for BHV1. The titer of chloroform treated virus was compared with the titer of untreated virus (Reed and Muench, 1938).

Effect of Nucleic Acid Analogue on Viral Replication

The effect of 5-iodo-2'-deoxyuridine (IDUR, Sigma Chemical, St. Louis, MO) on astrovirus multiplication (Rovozzo and Burke, 1973) in PNCK cells was studied. The optimal concentration of IDUR to be used was first determined by titration of BHV1 using BK passage 5 cells, 8 wells per dilution. In four different concentrations of IDUR, 0, 40, 100, and 200 ug/ml in serum-free medium, BHV1 was titrated and after 3 day, the titer was recorded, calculated by the Reed and Meunch method. The effect of IDUR treatment on the titer of astrovirus was then determined by using the optimal concentration of BHV1 incorporating into the test; BHV1 as positive (sensitive) control and bovine rotavirus (B223; supplied by G. N. Woode as a negative (insensitive) control. Cell infection of fecal astrovirus (GC83), tissue adapted astrovirus passage 22, and rotavirus viruses was determined by IF staining at 48 hours after infection and of BHV1 by CPE at 72 hours.

Effect of Host Cell DNA Inhibition on Astrovirus Replication

DNA inhibition by actinomycin D (AMD, Sigma Chemical, St. Louis, MO) was determining by a modification of the methods of Horzinek et al. (1984). Different concentrations varying between 1 and 0.001 ug/ml of serum free MEM were first tested with BHV1 in BK cells passage 6. Viral dilutions of 10^{-1} to 10^{-8}, 0.1 ml was adsorbed to the monolayer for an hour at 37 C. Then, the culture was washed three times with serum free medium. New medium containing AMD at the various concentrations was then added. Results were recorded 3 days pi; viral infectivity titer was assayed.

For the fecal astrovirus test, AMD medium was added at 1 or at 12 hours after infection and the cultures were incubated for 24 hours. The relevant virus titer of the AMD treated cultures were compared with the titer of untreated cultures. Later, the effect of AMD on the replication of tissue culture adapted astrovirus (passage 20 in BK cells) was studied, but without washing the monolayer prior to the addition of AMD. The virus titer of the AMD treated (0.05 ug/ml) and untreated cultures were compared.

RESULTS

Studies on the Immunofluorescence Pattern and Distribution in Infected Cells

The appearance of immunofluorescent cells in infected cultures of PBEK or PNCK or BK cells was similar when the staining was performed at 24, 48, and 72 hours pi. The proportion of positive cells to number of total cells of the monolayer showed that only a minority of cells were infected (Table 1). The highest number of positive cells was detected at 48 hours pi and was double those counted at 24 hours. No positive cells were detected at day 7, which confirmed the experience of Woode et al., 1984.

The appearance of positive cells could be observed as single cells with both fine and dense immunofluorescent granules in their cytoplasm. Occasionally, some thick granules were seen apparently in the nucleus with the same distribution as nucleoli, as determined by Giemsa staining. In most cells, the large dense granules had a perinuclear area distribution (Figs. 1 to 6).

Time Related Development of Immunofluorescence

Earlier studies had suggested that there was a nuclear phase of replication, possibly involving the nucleolus. To determine the sequence of events in the viral replication in cells, infected cultures were fixed

Table 1. Proportion of cells infected with astrovirus

Incubation time(hour)	Fecal dilution	Number of cells/well (x1000)	Number of pos.cells per well	Proportion of pos.cells to total no.
24	$1:10^2$	107.4	782	1:137.34
	$1:10^3$	147.4	63	1:2339.68
	$1:10^4$	158.4	0	0
	0	170.0	0	0
48	$1:10^2$	93.0	1544	1:60.23
	$1:10^3$	150.0	127	1:1181.10
	$1:10^4$	145.0	21	1:6904.76
	0	135.8	0	0
72	$1:10^2$	184.4	247	1:746.55
	$1:10^3$	148.6	73	1:2035.62
	$1:10^4$	130.4	2	1:65200.0
	0	144.2	0	0

at 30 minute intervals and examined by IF. The earliest immunofluorescent cells appeared at 7-8 hours pi. Faint staining was seen first in the cytoplasmic area, and followed by large spherical strongly positive regions in the nucleus. Then, this was followed by the appearance of thick densely stained cytoplasmic granules around the nuclear membrane (Figs. 1, 2, 3). At this time and later, some brilliant positive cells were seen with thick immunofluorescent occupying most of the cytoplasmic and nuclear areas (Fig. 4). Some cells had clearly defined large nuclear granules, with a distribution similar to that of the nucleoli of this cultures, again as determined by Giemsa staining (Figs. 2, 3, 6).

When fecal bovine astrovirus was cultured in BK cells, there was a slower development of the immunofluorescence and this was weaker (Fig. 5) than that observed in primary cells (Fig. 6). Both fecal astrovirus (GC83) and tissue culture virus (Passage 9) showed similar characteristics of cell IF.

Adaptation of Cell Culture to Astrovirus

Bovine continuous cell line

When GBK and BT cells were infected with fecal astrovirus, no immunofluorescence was observed in cultures fixed at 24, 48, and 72 hours.

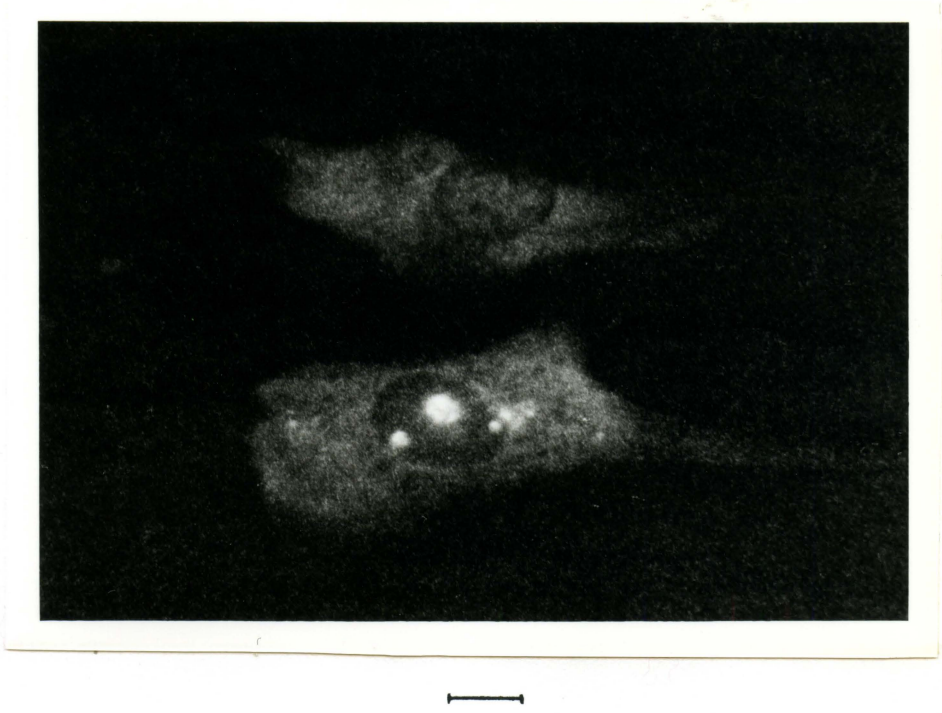

Figure 1. Culture adapted bovine astrovirus passage 8 infected in PNCK cells, fixed at 48 hours pi., showed fine granules in the cytoplasm and densely round granule in nuclear region (Bar represents 2 um)

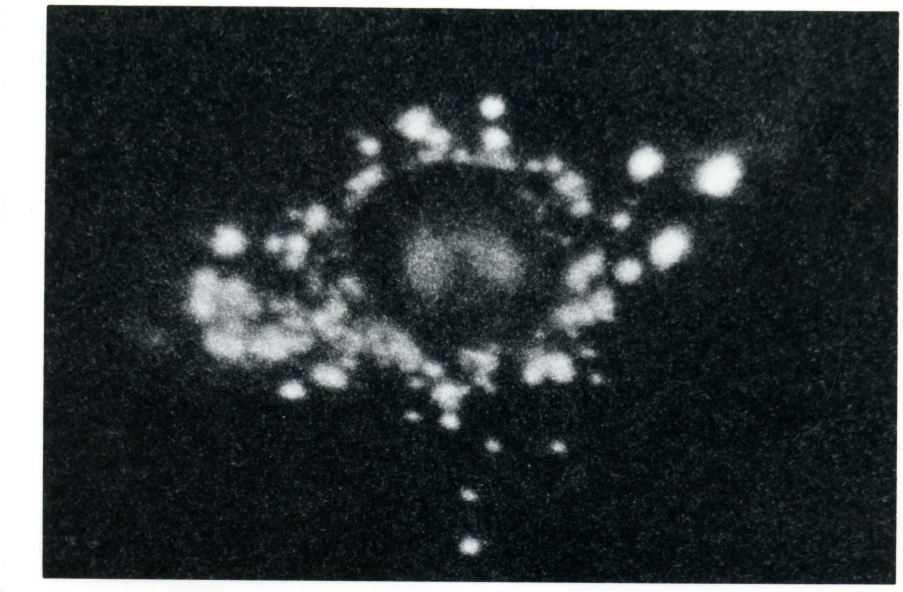

Figure 2. Culture adapted bovine astrovirus passage 12 inoculated on BK cells passage 15, fixed at 24 hours pi., showed large densely thick cytoplasmic granules and two round granules in nuclear region (Bar represents 2 um)

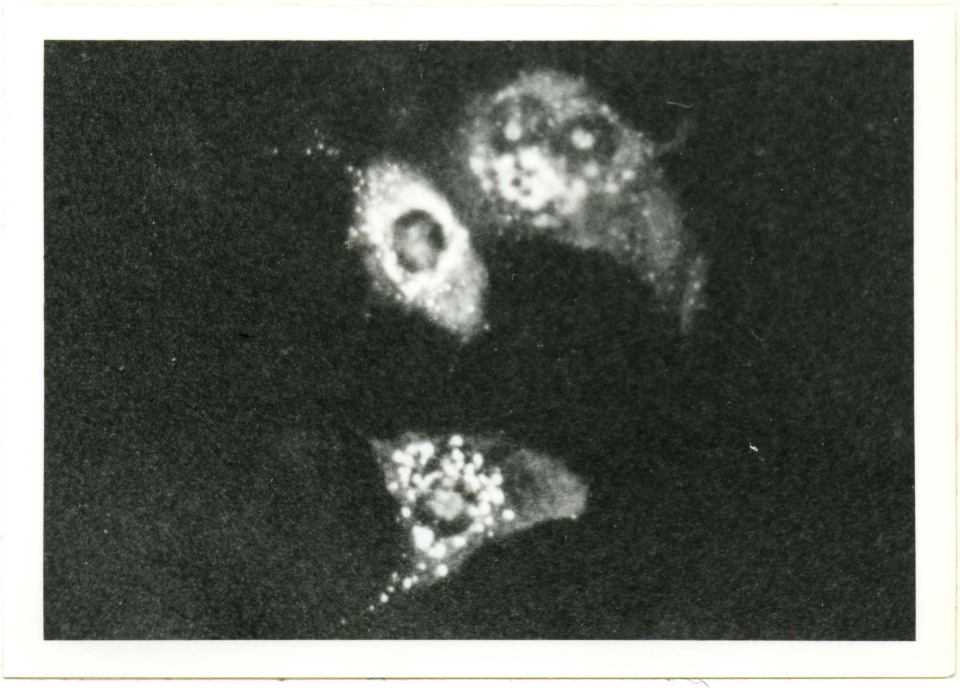

Figure 3. Fecal astrovirus (GC54) inoculated in BK cells passage 2, fixed at 24 hours pi., showed cells at different stages in the same culture (Bar represents 5 um)

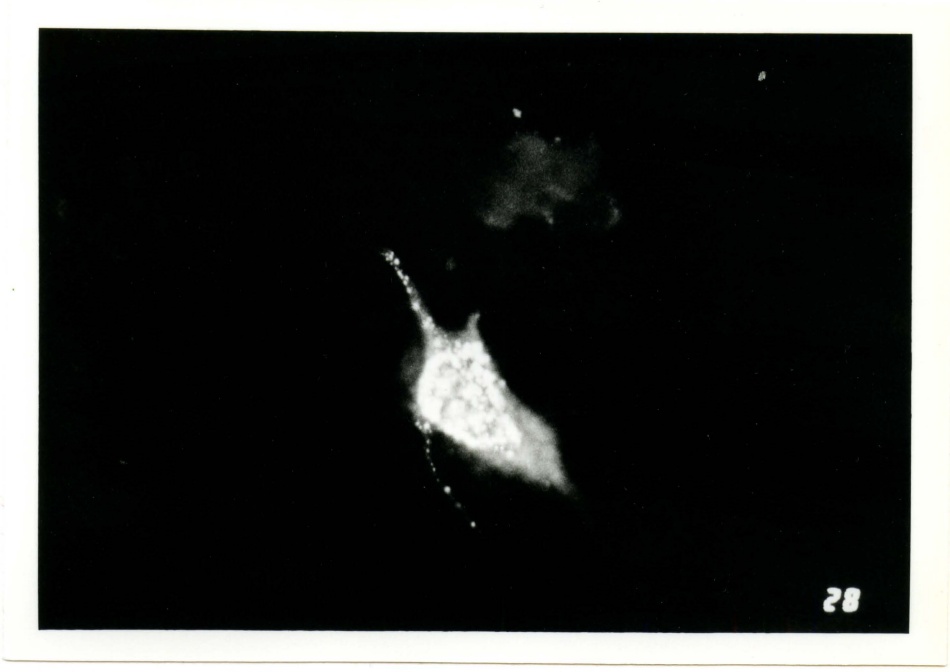

Figure 4. Culture adapted bovine astrovirus passage 8 inoculated in primary cell, fixed 5 days pi., showed thick immunofluorescent granules covering the nuclear and perinuclear regions (Bar represents 5 um)

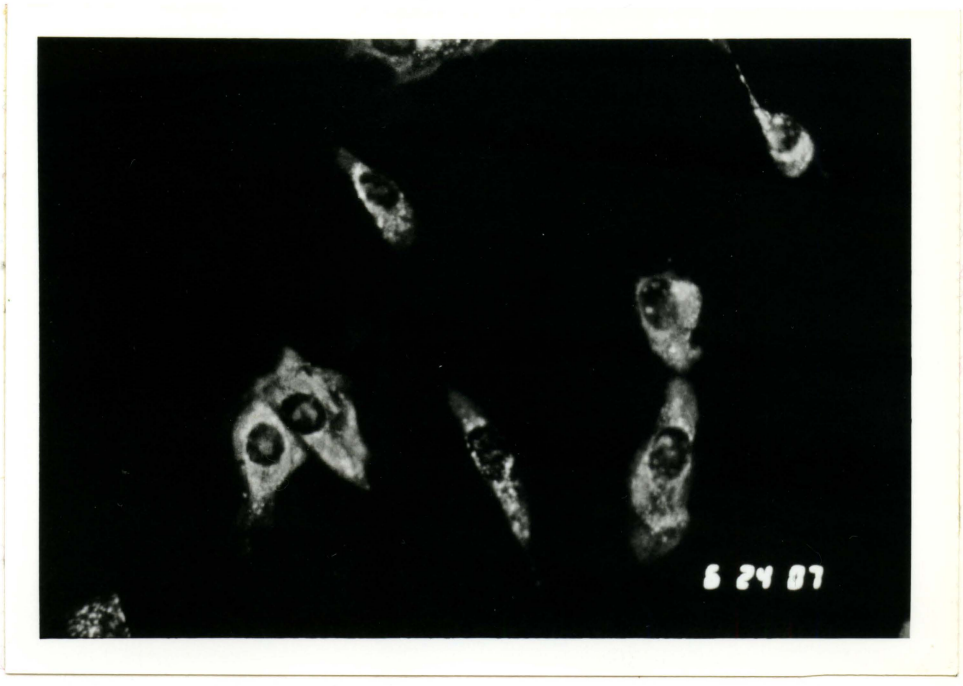

Figure 5. Fecal bovine astrovirus (GC54) inoculated in BK cells passage 2, fixed at 24 hours pi. (Bar represents 5 um)

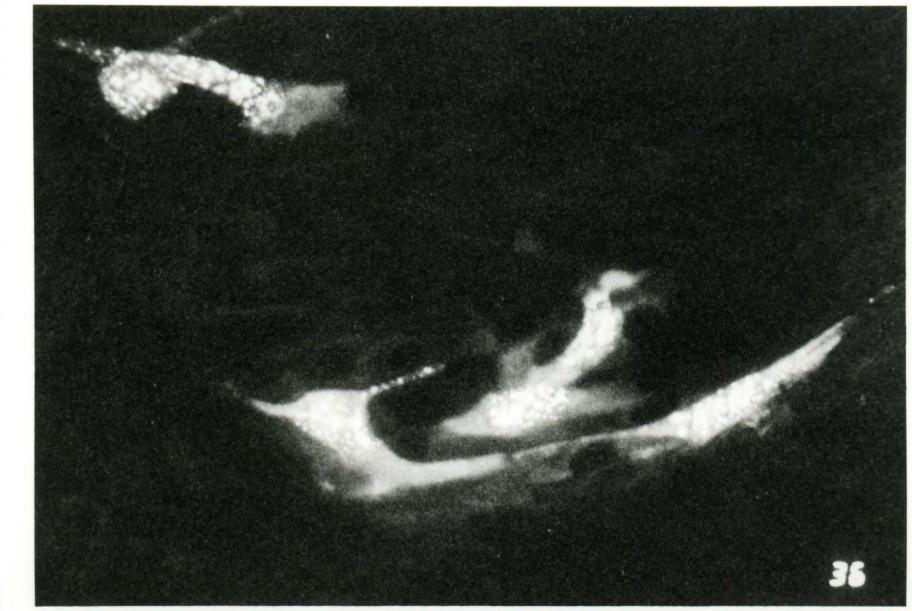

Figure 6. Culture adapted astrovirus passage 8, infected in PNCK cells, fixed 48 hours pi. (Bar represents 5 um)

Bovine primary cells

Besides primary bovine kidney cells, early studies by Woode and Bridger (1978) showed the ability of bovine astrovirus (UK) to infect primary bovine testicular cells. Bovine astrovirus infection in other primary cells have not been reported. The primary spleen cell and peritoneal wash cell cultures, were infected with fecal astrovirus (GC83), but no specific IF was detected when the cultures were fixed every 24 hours, for 7 days.

Effect of fetal bovine serum on astrovirus infection of cell culture

From the previous studies, there appeared to be an involvement of the nucleus in an early phase of astrovirus replication. As 10% FBS does not neutralize astrovirus infection (Woode et al., 1985), different concentrations of FBS (0, 5, 10, 15%) in MEM were included in the viral culture medium to determine whether FBS would effect viral replication, as a consequence of an increase in cell nuclear activity. The number of astrovirus infected cells increased over a 24 hour period as the percentage concentrations of FBS in the medium increased (Table 2). This increase exceeded the increase in total number of cells (Table 3).

Exposure of cell culture with ultraviolet irradiation prior to infection

Exposure of cells to uv irradiation has been shown to increase the susceptibility of cells to virus infection (Horzinek et al., 1984). To investigate whether one can achieve a higher percentage of infected cells, monolayers of PBEK cells were exposed to UV light at different interval before viral inoculation. Short exposure times did not increase the number of infected cells, but longer exposure time appeared to damage

the cells. The number of the positive infected cells per microscopic area was slightly reduced when the exposure time was longer than 12 seconds (Table 4).

Table 2. Positive cell count of astrovirus infection with different concentrations of FBS

Serum conc. (%)	No. of pos.cells/well				Mean[1]
0	23	41	30	23	29.25±9[a]
5	141	109	82	109	110.25±29.5[b]
10	126	164	183	152	156.25±28.5[b]
15	148	137	112	89	121.50±29.5[b]

[1]Significantly different (P<0.001, Tukey test, Zar, 1984).

Table 3. Total count of cells of monolayers infected with astrovirus with 10% FBS

Treatment	Total cell count/well (x1000)				Mean[1]
control	273.6	293.6	229.2	206.0	250.6±43.8
astrovirus	339.6	282.4	350.4	314.4	321.7±28.6
astro+10%FBS	535.6	429.6	437.6	403.2	451.5±66.2

[1]Statistically nonsignificant ($F_{df\ 1,6}$ P>0.25, analysis of variance, Zar, 1984).

Table 4. Effect of UV light on astrovirus infection

Exposure Time(sec.)	No. of IF pos. cells/area				Mean[1]
0	24	26	35	36	30.25±6[a]
2	12	17	38	25	23.00±8
4	32	26	21	19	24.50±6.5
6	23	15	27	32	24.25±8.5
8	21	27	28	25	25.25±3.5
10	15	14	26	38	23.25±12
12	35	31	25	55	36.50±15[b]
14	11	32	31	34	27.00±11.5[c]
16	16	16	13	19	16.00±3[c]
18	6	13	14	7	10.00±4[c]
20	10	14	18	8	12.50±5[d]

[1]Statistical difference between a and d, b and c, b and d. (P>0.05, Tukey Test, Zar, 1984).

Effect of centrifugation of cell culture on astrovirus infection

Centrifugation of a monolayer has been shown to increase the rate of infection of cells with rota virus (Banatvala et al., 1975). When the monolayers were spun during adsorption period of astrovirus to cell culture, there was one log decline of its infectivity compared to its infection onto normal monolayers (Table 5). From subjective examination, the monolayers appeared to suffer as a consequence of the centrifugation and this probably contributed to the decline in the number of infected cells.

Table 5. Effect of Centrifugation on viral infection

Virus dilution		No. of pos. cells/area				Mean
control	$1:10^3$	62	64	59	50	58.75±5
culture	$1:10^4$	11	8	14	12	11.25±3
	$1:10^5$	3	0	1	0	1.00±1
centrifuged	$1:10^2$	16	15	12	20	15.75±4
culture	$1:10^3$	0	3	2	1	1.50±1
	$1:10^4$	0	0	0	0	0

Effect of trypsin on astrovirus infection

It has been shown that trypsin is essential for primary isolation of rotavirus and enhances the titer of culture adapted rotavirus (Babiuk et al., 1977, Theil et al., 1977, Almeida et al., 1978). A concentration of 100 ug/ml of trypsin in the medium caused detachment of the cells within 24 hours. At trypsin concentrations of 25 and 50 ug/ml, the immunofluorescence of infected cells was more brilliant, than at 10 ug/ml. The titer of fecal astrovirus (GC83), with and without trypsin (25 ug/ml) in the medium, were compared. No significant difference was observed (Table 6).

Table 6. Number of positive cells of monolayers infected with astrovirus with and without trypsin cooperation

Virus dilution	No. of pos. cells/cover-slip			
	no trypsin		with trypsin 25 ug/ml	
$1:10^2$	471	738	859	609
$1:10^3$	48	85	40	32
$1:10^4$	0	0	0	0

Passage of PBEK and PNCK cells and astrovirus infection

Passages of PBEK cells to secondary and tertiary cell cultures showed a reduction in the infectivity titer of GC54 fecal astrovirus (Table 7). In contrast, passages of PNCK cells (BK cells) produce an increase rate of astrovirus infection. No reduction of astrovirus titer occurred upto passage 5 of BK cells, and, positive immunofluorescent cells were detected when BK cells passage 19 were infected with GC54 fecal virus. The number of astrovirus supported cells decreased as the BK cells were subcultured further. BK cell passage 39 infected with a 1:10 dilution of tissue culture adapted astrovirus passage 15 could support and express a few number of infected cells (<10 cells/well of 8 well-chamber-slide), when the monolayers were fixed at 48 hours pi.

Table 7. Comparison of astrovirus infectivity in cell passages of PBEK and PNCK cells

Cell culture and subculture		Viral Dilutions $1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$
PBEK	1	+	+	+	-
	2	+	+	-	-
	3	-	-	-	-
PNCK	1	+	+	+	-
	2	+	+	+	-
	3	+	+	+	-
	4	+	+	+[a]	-
	5	+	+	+	-

[a]33 and 35 pos.cells/well of chamber slide.

Differences were observed in the ability of kidney cells from different calves to support astrovirus infection. All primary cell cultures prepared from approximately 40 embryonic kidneys, supported astrovirus infection. In contrast, PNCK culture from 3 out of 8 calves could not support the virus, The ability of the PNCK cells to support astrovirus did not appear to depend on breed, age, or colostral feeding of the calf (Table 8).

Table 8. Differences between calves used for PNCK cell preparations

Batch no. of cells	Age of calf(hr)	Breed	Colostrum[1] fed	IF test[2]
1 (1-7-86)	24	Holstein	Y	+
2 (1-21-86)	24	Jersey	Y	+
3 (2-4-86)	24	Holstein	Y	+
4 (2-20-86)	48	Holstein	Y	-
5 (2-5-86)	24	Holstein	Y	-
6 (3-26-86)	24	Holstein	Y	+
7 (4-4-86)	2	Jersey	N	-
8 (4-10-86)	1	Jersey	N	+

[1]Y = colostrum fed; N = colostrum deprived.

[2]Results detected when cells were infected with astrovirus GC54 and stained at 48 hours pi.

Adaptation of Astrovirus to Replicate in Primary Cells

In an attenpt to adapt astrovirus to replicate in cell culture and release infectious virus, fecal astrovirus (GC54) was subcultured in PBEK cells after 24, 48, or 72 hour incubation period. There was a reduction in the number of infected cells with each passage, and all infectivity was lost by the third to fourth passage (Table 9). However, passing the virus in primary cells with the presence of trypsin appeared to provide more positive immunofluorescent cells with stronger fluorescein staining.

When the bovine astrovirus was subcultured every 7 days, with two different concentrations of trypsin in the medium, 25 and 50 ug/ml, the virus was subcultured successfully 4 and 18 times with 50 and 25 mg/ml trypsin respectively (Tables 10, 11). Each passage further diluted the virus approximately 10 fold, although some surviving infected cells may have been long lived. This result provide probably evidence of viral replication. To confirm that cell free virus was produced during passing, the cells and cell debris were removed by centrifugation at 2000 x g. The infectivity titer of passage 19 cell free virus with 50 ug/ml of trypsin was approximate 10^5. Tissue culture adapted virus remained infectious after storage at -80 C.

Table 9. Immunofluorescence result of astrovirus (GC54) subcultured every 24, 48 and 72 hours in PBEK cells

Incubation time (hr)	Trypsin presence (25 ug/ml)	Times subcultured 1	2	3	4
24	+	+	+	-	-
24	-	+	+	-	-
48	+	+	+	-	-
48	-	+	+	-	-
72	+	+	+	+	-
72	-	+	+	-	-

Table 10. Immunofluorescence result of astrovirus (GC54) subcultured every 7 days with different concentrations of trypsin in PBEK cells

Trypsin conc.(ug/ml)	Times virus was subcultured[1] 1	2	3	4	5	6...12		18
0	+	+	-	-	ND	ND	ND	ND
25	+	+	+	-	+	+	+	+
50	+	+	+	+	-	ND	ND	ND

[1]Cells using for the 6th to 18th passage were PNCK cells with trypsin 25 and 50 ug/ml and both were positive.

Table 11. Immunofluorescence result of astrovirus (GC54) subcultured every 7 days with different concentrations of trypsin in PNCK cells

Trypsin conc (ug/ml)	Times virus was subcultured							
	1	2	3	4	5	6	14	15
25	+	+	+	-	-	+	ND	ND
50	+	+	+	+	-	+	+	+

Serological Identification of Tissue Culture Astrovirus Passage 9

Tissue culture astrovirus passage 9 (A9) was tested for its antigenic relationship to bovine astrovirus and other enteric viruses. The result showed that there was no cross immunofluorescence between astrovirus and Breda virus, bovine coronavirus, or bovine rotavirus serotype 2 (B223) (Table 12). The tissue culture adapted virus reacted with antisera to both bovine astrovirus 1 and 2, and UK astrovirus (possible serotype 3) which have cross immunofluorescent relationships (Woode et al., 1985). Both fecal astrovirus (GC83) and tissue culture adapted astrovirus produced the same results when cultured and tested in either PNCK cells or BK cells passage 6.

Table 12. IF test of tissue culture astrovirus

Antiserum to	In PNCK cells		In BK6	
	GC83	A9	GC83	A6
SB219 Breda virus 1	-	-	-	-
GC76 Breda virus 2	-	-	-	-
GC78 coronavirus	-	-	-	-
GC 5 rotavirus 2	-	-	-	-
GC43 astrovirus 1	+	+	+	+
GC39 astrovirus 2	+	+	+	+
UK astrovirus 3	+	+	+	+

Lipid Sensitivity Test

Determination of the lipid sensitivity of bovine astrovirus to chloroform treatment showed no effect of the chemical on viral infectivity of both fecal astrovirus and culture adapted astrovirus (passage 10 in BK cells) (Table 13). The titer of astrovirus passage 10 with and without chloroform treatment was $29x10^3$ and $20x10^3$ respectively. On the other hand, chloroform destroyed the infectivity of bovid herpesvirus 1 in the positive control system.

Table 13. Chloroform test of BHV1 and fecal astrovirus

Virus Dil.	BHV1 (CPE) Control	BHV1 (CPE) $CHCl_3$ treated	Fecal astrovirus (IF) Control	Fecal astrovirus (IF) $CHCl_3$ treated
1:10	+	-	ND	ND
$1:10^2$	+	-	+	+
$1:10^3$	+	-	+	+
$1:10^4$	+	-	-	-
$1:10^5$	+	-	-	-
$1:10^6$	-	-	-	-

Nucleic Acid Determination

In order to determine whether bovine astrovirus is a DNA dependent virus, the effect of the nucleic acid analogue, 5'-iodo-2'-deoxyuridine (IDUR) was determined. Different concentrations of IDUR were tested with the BHV1 in order to select an optimal concentration for the test (Table 14). The 100 ug/ml concentration of IDUR was selected. The same test repeated with bovine rotavirus and astrovirus using 100 ug/ml IDUR, showed no effect on the replication of both viruses (Tables 15, 16, 17). Consequently, astrovirus is not a DNA dependent virus and must be concluded to contain RNA.

Table 14. BHV1 with different concentrations of IDUR

IDUR conc. (ug/ml)	Virus titer (TCID50/0.1 ml)
0	10^{7}
40	$10^{4.57}$
100	$10^{4.5}$
200	$10^{4.5}$

Table 15. IDUR test with rotavirus

Virus dilution	Control[1]	IDUR treated (100ug/ml)[1]
$1:10^2$	8/0	8/0
$1:10^3$	8/0	8/0
$1:10^4$	5/3	6/2
$1:10^5$	0/8	0/8
Virus Titer (TCID50/0.1ml)	log 4.57	log 4.80

[1]Number of positive wells/negative wells (IF).

Table 16. IDUR test with fecal astrovirus (GC83)

	Virus dil.	Immunofluorescence result	
		a[1]	b[2]
Control	$1:10^2$	4/4	1099, 939, 1172, 1099
	$1:10^3$	4/4	
	$1:10^4$	0/4	
IDUR treated (100 ug/ml)	$1:10^2$	4/4	1025, 1298, 980, 1076
	$1:10^3$	4/4	
	$1:10^4$	0/4	

[1]Number of positive/total wells.

[2]Number of positive cells/well.

Table 17. IDUR test with tissue culture adapted astrovirus passage 22

	Viral Dilution	Immunofluorescence Result	
		a[1]	b[2]
control	1:10[3]	8/8	
	1:10[4]	8/8	21.00±16
	1:10[5]	7/8	
IDUR treated	1:10[3]	8/8	
(100 ug/ml)	1:10[4]	8/8	15.38±22
	1:10[5]	7/8	

[1]Number of positive/total wells.

[2]Number of positive cells/well.

Effect of Actinomycin D on Astrovirus Replication

Presence of Actinomycin D, a DNA replicaton inhibitor, at a concentration of 0.05 ug/ml at 1 hour postinfection showed no effect on rotavirus replication, but had the ability to inhibit IBRV infectivity (Table 18). The titer of fecal and tissue culture adapted astrovirus in PNCK cells, showed no significant difference between the presence or absence of AMD (0.05 ug/ml) at 1 hour after inoculation (Tables 19, 20). Similarly, there was statistically no difference between the presence or absence of AMD (0.05 ug/ml) when added to fecal astrovirus infected cultures at 12 hours pi. The difference in titer of astrovirus at 1 hour

and 12 hours reflects the fact that virus was removed by washing immediately prior to AMD treatment.

Table 18. Actinomycin D test with BHV1 and rotavirus

Virus	Virus titer (log, TCID50/0.1ml)	
	control	AMD treated (0.05 ug/ml at 1 hr pi)
BHV1	4.67	1.57
Rotavirus	3.57	3.80

Table 19. Actinomycin D test with fecal astrovirus (GC83)

Time of media replacement	AMD (0.05 ug/ml)	No.of pos. cells/well	Mean
1 hr.	-	9, 11, 14, 10	11.0
	+	10, 12, 11, 9	10.5
12 hr	-	23, 22, 26, 25	24.0
	+	28, 32, 40, 39	34.75

Table 20. Actinomycin D test with tissue culture adapted astrovirus passage 20

Viral Dilution	Immunofluorescence Result control	AMD treated
$1:10^2$	8/8	8/8
$1:10^3$	7/8	5/8
$1:10^4$	1/8	0/8
$1:10^5$	0/8	0/8
Virus titer (TCID50/0.1ml)	log 3.5	log 3.2

DISCUSSION

Replication of Bovine astrovirus (US2) was studied with both fecal virus and tissue culture adapted virus, in tissue culture. 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. Astrovirus replication was not inhibited by the treatment of virus with chloroform, indicating a lack of essential lipids. The addition of IDUR (a nucleic acid analogue), or actinomycin D (an inhibitor of DNA transcription) to the medium did not inhibit replication. All these treatments blocked the infectivity or replication of bovine infectious bronchitis virus or bovid herpes virus 1 (BHV1). Thus, bovine astrovirus lacks a lipid-containing envelope and is not a DNA virus or DNA dependent RNA virus. The lack of an envelope confirms the electron-microscopic description of the virus (Madeley, 1979, Woode and Bridger, 1978) that the virus has an unbroken circular border with a five-or six pointed surface star and no envelope is observed.

Astrovirus infection in primary bovine cells (PBEK, PNCK) and BK cells was similar as judged from immunofluorescent studies. The earliest IF was observed at 7 to 8 hours pi. Fine green granules appeared in the cytoplasm, occasionally associated with round granules in nuclear regions, which had the same distribution as nucleoli as seen by Giemsa staining. Dense thick cytoplasmic particles were seen later in the cytoplasmic area especially at the perinuclear region. The timing of each stage is not accurate since more than one stage of immunofluorescent

cells were seen on one cover-slip, for example: a cell with dense cytoplasmic immunofluorescence was seen together with cells which had fine cytoplasmic granules when fixed at 7 hours pi. The course of development of the virus infection was constructed to be as followed: The earliest events in replication are cytoplasmic, with possibly nucleolus association. Areas of intense viral antigen concentration then develop in the cytoplasm, initially perinuclear and then spreading to involve much of the cytoplasmic space.

The highest number of positive immunofluorescent cells occurred 48 hours pi. at 37 C. More recent observations with the tissue culture adapted virus (author, unpublished data), suggests that the greatest number of infected cells occur at 24 hours pi. Studies of bovine astrovirus (Woode et al., 1984) and human astrovirus (Lee and Kurtz, 1981) on immunofluorescence of infected primary cells with fecal virus showed similar results.

No cytopathic effect (CPE) was observed in infected cultures over at least a 7 day period. The reasons for this may be as follows: First, the positive immunofluorescence may represent only partial replication and partial expression of viral antigens, without the development of complete virions. In earlier studies, no virus particles were seen in ultrastructural studies of infected cultures (Woode and Fagerland, Department of Veterinary Pathology, Iowa State University, personal communication). Secondly, the proportion of infected cells to total cells of the monolayer, approximately 1:60 to 1:2000, reflected the fact that a minority of cells are infected. Thus, the virus could effect and

destroy the cells, but no CPE may be seen because most cells are still unaffected and healthy.

A steadily declining titer of astrovirus after subculturing in the primary cells, supported the idea that few complete infectious virions are released from these infected cultures. When trypsin was added to the culture medium, adaptation of the virus to replicate in cell culture occurred, and the virus at passage 19 had a titer of approximately 10^5. No CPE was observed in these cultures, because the trypsin caused rounding of the cells and detachment from the plastic, at the same time in both infected and control cultures.

Virus at 10^5 titer infected only a minority of cells, approximately 10% or less, when cells were fixed at 24, and only 1-5% when fixed at 48 hours pi. The cell population was approximately 10^5, indicating that most cells, even in a susceptible culture, cannot be infected. Using fecal virus, when fetal bovine serum was added to the culture, the number of cells per well increased approximately 1.4 folds, but the number of infected cells increased 4 to 5 fold.

The infected cells of primary cultures aged 6-7 days showed immunofluorescent cells randomly scattered throughout the monolayer. In contrast, older cultures (10-14 days), when examined 24 hours pi. showed an increased number of IF positive cells but these had a plaque-like distribution, and included all the cells in discrete areas (20-50 cells), but still the majority of cells of the culture were uninfected. It is unlikely that these plaques of infected cells resulted from virus spread from cell to cell within 24 hours, as when attempts were made to

subculture the virus every 24 and 48 hours, infectivity rapidly declined to zero. This indicates that the virus requires more than 24 hours for full replication and release from the cell.

In summary, for an optimal infectious rate and release of virus, one must select a susceptible cell type of 7-10 days of age or older, and incubate the cells with fetal bovine serum during viral replication. However, trypsin (inhibited by the presence of serum) is required for the production of infectious virus. The study did not determine the mechanism of action of serum factors or trypsin on the replication.

Human astrovirus has been subcultured in primary human embryo kidney cells with the addition of 10 ug/ml of crystalline trypsin to the medium, and an attempt to adapt human astrovirus to cells in a trypsin-free medium was unsuccessful. Higher concentration of trypsin, 50 ug/ml, resulted in a more rapid yield of virus although not a higher titer of the virus. The mechanism of the effect of trypsin on human astrovirus has not been reported (Lee and Kurtz, 1981).

Only the addition of trypsin to the culture medium permitted the successful adaptation of bovine astrovirus to replicate in vitro. The optimal concentration of trypsin needed for astrovirus replication has not been determined. At a trypsin concentration of 25 and 50 ug/ml of trypsin, cell free infectious astrovirus was produced from infected cultures, reaching a titer of approximately 10^5. Trypsin, a proteolytic enzyme, has been used with rotavirus and influenza virus in order to increase viral infectivities (Babiuk et al., 1977, Theil et al., 1977, Almeida et al., 1978, Klenk et al., 1975, Lazarowitz and Choppin, 1975).

Cleavage of hemagglutinin glycoprotein (HA) by proteolytic enzymes is necessary for influenza virus infection (Klenk et al., 1975, Lazarowitz 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 (Espejo et al., 1981, Clark et al., 1981).

In order to determine whether astrovirus used a nuclear phase for its replication, actinomycin D, an inhibitor of DNA transcription, was tested on astrovirus infectivity. In contrast to HBV1 which is blocked by AMD (0.05 ug/ml), when present from 1 hour pi., there was no effect of the chemical on astrovirus replication. Horzinek et al. (1984) have reported that the Berne equine virus, a member of the proposed family Toroviridae, an ssRNA virus, requires host cell nuclear activity to support viral replication because AMD 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 hours after infection. Thus, despite the suggestive evidence of nuclear (possibly nucleoli) involvement in viral replication, these inhibitors were not effective.

The nucleolus is described as a cellular location for the formation and accumulation of ribosomal precursors (Thorpe, 1984). Nucleoli are situated in the nucleus at specific sites of certain chromosomes, and the number of nucleoli is related to the number of these chromosomes. The evidence that nucleolar elements contain large amounts of protein in addition to RNA and the detection of ribonucleoprotein particles in the

nucleolus (Birnstiel et al., 1963) indicate the ability of the nucleolus to perform protein synthesis.

Actinomycin D acts as a DNA transcription inhibitor and blocks the formation of mRNA, the manufacture of transfer RNA and ribosomal RNA (Sobell, 1974). Its mechanism is to bind tightly to double-strand DNA, not ssDNA or ssRNA, not dsRNA or RNA-DNA hybrids (Stryer, 1981), thus, astrovirus, as a probable RNA virus, would not be inhibited by AMD. However, it may use the protein machinery located in the nucleolar area to synthesis viral protein, resulting with the development of the IF of the nucleoli. There is evidence that H-1 parvovirus effect host cell nucleoli at the early stage of its replication (Singer and Toolan, 1975, Singer, 1976). At 10-12 hours pi., empty virions of virus capsid rings were found associated with the damaged nucleoli. The binding of unassembled H-1 proteins to specific regions of chromatin was believed to cause early nucleolar destruction and nuclear damage during infection. Later studies of the same researcher as reviewed by Johnson, 1984, indicates that the thermostable chromatin-associated antigens present in proteins have not formed capsids and are associated with nucleolar chromatin. In addition, the beginning of DNA synthesis occurs at localized euchromatic or nucleolar sites and then spread outward. Thus, the H-1 parvovirus synthesis is assumed to be regulated by the H-1 proteins associated with the nucleolus and euchromatin.

Ultrastructural and biochemical studies of cells infected by astroviruses are likely to show that most viral replication and assembly occur in the cytoplasm rather than nucleus. Ultrastructural observations

of human astrovirus infection in primary human embryo kidney cells (Kurtz et al., 1979), lamb astrovirus in gnotobiotic lambs (Gray et al., 1980), and bovine astrovirus in gnotobiotic calves (Woode et al., 1984), all showed results that aggregated virions were found in the cytoplasm of infected cells and there were no reports of the involvement of the nucleus in the assembly of virions.

Attempts to subculture bovine astrovirus have been performed in bovine cells only because animal astrovirus infection seems to be restricted to host species (Snodgrass and Gray, 1977, Woode and Bridger, 1978, William, 1980, Tzipori et al., 1981, Gough et al., 1985). No IF was detected in two continuous bovine cell lines, GBK and BT cells, infected with fecal bovine astrovirus. The results did not change when the virus was inoculated into cultures of different ages, 2, 4, 5, and 10 days, or when fixed at different incubation periods, 24, 48, and 72 hours at 37 C. Only the primary kidney cells of most calves or early passages of PNCK cells (BK cells passage 5) with the exception in one calf to passage BK39, were able to express IF cells of the infected cultures.

In contrast to PNCK cells cultures which did not always process astrovirus infectable cells, all of approximately 40 PBEK cultures supported virus infection. However, by the 3rd passage of these cells, all susceptible cells were lost from the cultures. In contrast to PBEK cells, all PNCK cells subcultured to become BK cells up to the 5th passage could support bovine astrovirus, close to the level supported by PNCK cells. The cell passages of PBEK 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). Spindle-shaped cells, possibly indicating fibroblasts, are the majority of the cells seen in secondary and tertiary cultures of PBEK cells. On the other hand, BK cells or PNCK cell passages produced monolayers containing predominantly cuboidal-shaped cells rather than spindle-shaped cells.

No attempt has been made to determine why the PNCK cells derived from different animals varied so much in their ability to support astrovirus infection. Within these limited studies, no influence was discernible associated with breed, age or the feeding of colostrum. Serum samples from the calves were not taken to determine whether they possessed astrovirus antibodies. In an unrelated study, 3 of 5 calves from this herd were shown to shed astroviruses in the feces between 1 to 2 weeks of age. Thus the virus is probably enzootically present in the herd. However, it is unlikely that kidney cell cultures prepared from calves with astrovirus antibody would be refractory to astrovirus infection, as this has not been observed with such common viruses as rotavirus and parainfluenza 3 virus (Woode, Veterinary Microbiology and Parasitology, Texas A & M University, TX, personal communication).

The difficulties experience in attempting to cultivate astrovirus is not surprising, when one considers the restricted cell tropism of the virus in vivo. The only cell known to be infected is the M cell of the Peyer's patches, and it is unlikely that this cell is cultured readily from trypsinized kidneys. The tubular kidney cell, probably the dominant epithelial cell of kidney tissue culture, supports rotavirus well.

Rotavirus replicates in the villous 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 antigen intracellularly 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). Ia antigens are polymorphic membrane glycoproteins coded for by genes within the major histocompatibility complex (MHC) and play an important role in controlling the level of antibody response to specific antigens (Klein and Hauptfeld, 1976, Shevach, 1976). It is possible that astrovirus can only replicate in epithelial cells lacking Ia antigens.

The successful adaptation of bovine astrovirus to cell culture has been important in our understanding of this possibly unique virus. Using this adapted virus, the biochemical properties of astrovirus will be studied primarily for taxonomic purposes, to determine whether the prediction by Herring et al., 1981, with lamb astrovirus was correct: that these viruses lie between the Picornaviridae and the Caliciviridae, possibly requiring the establishment of a new virus family. The tropism of the virus for the M cell, apparently without replicating or damaging other cells, provides a tool for investigating further the function of M cells by their selective removal, temporarily, by astrovirus infection. Previously, fecal virus had to be used for such in vivo studies, with the attendant problem of possible contamination by other viruses. Plaque

purification of astrovirus from tissue culture, should reduce the chance of contamination with other, at present undiscovered, enteric virus.

SUMMARY

Infection and replication of bovine astrovirus (US2) was studied in primary bovine embryo kidney (PBEK) cell cultures, in primary newborn calf kidney (PNCK) cell cultures, and in serial passages of these cultures, using both fecal virus and cell culture adapted virus. The astrovirus had a restricted cell tropism for a minority of tissue culture cells. Whereas all PBEK cell cultures from approximately 40 kidneys supported astrovirus infection, 3 out of 8 PNCK cultures would not support infection. Passages of one PNCK, up to passage 39, contained a minority of cells which became infected with astrovirus, whereas PBEK cell passages lost all susceptible cells by the third to fourth passage. In all cultures, there were less than one cell in 50 which became infected even when the multiplicity of infection was 1.

The first indication of viral replication occurred at 7 hours postinfection 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 densely granular IF was seen in the perinuclear region of the cytoplasm, which later extended to involve all the cytoplasmic area. Infectious virus was only produced by infected cultures when trypsin was added to the medium, and by this means the virus was adapted to cell culture. Infectivity of the virus was not removed by treatment with chloroform, and iododeoxyuridine or actinomycin D when added to the medium, did not block replication.

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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