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Isolation and Characterization of a Canine Rotavirus

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With 4 Figures

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Summary

Canine rotavirus particles were visualized by direct electron microscopy in the feces from a clinically normal dog. The virus was subsequently propagated in cell cultures; it was chracterized and compared with rotaviruses from other species. Replication of the virus in cell culture was found to be less dependent upon trypsin than that of human, bovine and porcine rotaviruses. Reproducible, sharp-edged plaques of various sizes were produced by the canine rotavirus in an established cell line of fetal rhesus monkey kidney, MA104, under overlays of carboxymethyl cellulose or agarose. Intracytoplasmic inclusion bodies of different sizes and shapes were produced in infected MA104 cells. By plaque reduction neutralization assay, a two-way antigenic relationship was found between the canine (CU-1) and simian (rhesus MMU18006 and SA-11) rotaviruses. The canine rotavirus had a one-way antigenic relationship with feline (Taka), bovine (NCDV), and porcine (OSU) rotaviruses.

Introduction

Rotaviruses are among the most important causative agents of acute neonatal gastroenteritis in various mammalian and avian species. Rotaviruses have been detected in the feces of man, monkey, horse, cattle, bison, antelope, sheep, goat, deer, pig, cat, rabbit, guinea pig, mouse, chicken, turkey, duck and parrot (4, 10, 11, 13, 18, 20, 28, 29, 31).

Recently the worldwide appearance of parvovirus enteritis in dogs has caused an intensified search for infectious agents in dog feces. Subsequently, reports of visualization of rotavirus particles in canine feces, and in two cases cultivation

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of a canine rotavirus (9, 15) have appeared (9, 11, 15, 32, 37, 39, 42). However, a comparison of canine rotaviruses with rotaviruses of other animal species has not been performed. The purpose of the present report is to describe the isolation, propagation, and partial characterization of a canine rotavirus, as well as a serological comparison with human, feline, bovine, porcine, and simian rotaviruses.

Materials and Methods

Preparation of Fecal Inoculum

Fecal samples were collected from a clinically normal one-year-old beagle. The preparation of fecal inoculum has been previously described (19, 20). Approximately 20 percent fecal suspension was prepared in Eagle's minimum essential medium (MEM) with antibiotics, centrifuged at $1200 \times g$ for 10 minutes (crude fecal suspension) and the supernatant was ultracentrifuged at $10{,}000 \times g$ for 10 minutes (clarified fecal suspension). The supernatant was used as virus inoculum.

Electron Microscopy

Examination of fecal samples by electron microscopy (EM) was performed as previously described (19, 20). One to 4 drops of crude fecal suspension were mixed with 20 drops of distilled water, one drop of 0.1 percent bovine serum albumin, and 3 to 4 drops of 4 percent phosphotungstic acid adjusted to pH 7.0. This mixture was applied to a carbon-parlodion-coated 200 mesh copper grid with an all-glass nebulizer and examined in a Philips 201 electron microscope at 80 kV. Infected cell culture suspensions, after 3 cycles of quick freezing and quick thawing, were examined as described above.

Cell Culture

An established cell line of fetal rhesus monkey kidney, MA 104 (M. A. Bioproducts, Walkersville, Maryland) was used for rotavirus isolation and for further experiments. Growth medium for MA 104 cell cultures was Eagle's MEM supplemented with 5 percent newborn calf serum, 0.01 percent lactalbumin hydrolysate (LH), 0.075 percent sodium bicarbonate and antibiotics. Maintenance medium (MM) was Eagle's MEM supplemented with 0.01 percent LH, 0.075 percent sodium bicarbonate, antibiotics and no serum.

Isolation and Propagation of Canine Rotavirus in Cultured Cells

Confluent monolayer cultures (16×125 mm culture tubes) were washed once with phosphate buffered saline (PBS), pH 7.2, treated for 10 minutes at 37° C with 1 ml PBS supplemented with 2.5 μg of $2\times$ crystalline trypsin/ml (Nutritional Biochemical Corporation, Cleveland, Ohio), and then were inoculated with 0.1 ml of clarified fecal suspension. After one hour of adsorption at 37° C, cell cultures were washed once with PBS,fed with 1.0 ml of MM and incubated in stationary racks at 35° C. When the cells showed 50—80 percent cytophatic change, the culture tubes were frozen and thawed quickly three times, centrifuged at $1200 \times g$ for 10 minutes and supernatants were inoculated into fresh cell cultures as described above. This isolate was labelled Cornell University 1 (CU-1) and was characterized and compared with rotaviruses from other species.

Examination for Inclusion Bodies

Leighton tube cultures of MA 104 cells were prepared and inoculated with canine rotavirus (CU-1) at MOI 1. When approximately 50 percent of cells showed cytopathic change, coverslips were removed, washed with PBS, fixed with cold methanol, stained with May-Grünwald-Giemsa strain and examined for inclusion bodies.

Viruses

Rotaviruses of different animal species employed in this study are summarized in Table 1. Canine (CU-1) and feline (Taka) rotaviruses were isolated at the Cornell Feline Health Center. Bovine rotavirus (NCDV) was supplied by Dr. C. A. Mebus,

porcine rotavirus (OSU) by Dr. E. H. Bohl, simian rotavirus (SA-11) by Dr. H. Malherbe, and simian rotavirus (rhesus MMU 18006) by Dr. N. J. Schmidt. The DS-1 strain of human rotavirus is a reassortant virus between a temperature-sensitive mutant of a cultivatable bovine rotavirus (UK strain) and a noncultivatable human rotavirus (DS-1 strain) (17). All rotaviruses were plaque-purified prior to use.

Table 1. *Rotaviruses used in this study*

Strain designation	Original isolation			Present study	
	Species	Country	Year	Source	Titer[a]
CU-1	Dog	U.S.A.	1980	MA 104	2×10^6
Taka	Cat	U.S.A.	1980	MA 104	2×10^7
NCDV	Cattle	U.S.A.	1967	AGMK	2×10^8
OSU	Pig	U.S.A.	1976	AGMK	9×10^7
SA-11	Vervet Monkey	S. Africa	1958	AGMK	3×10^8
Rhesus MMU 18006	Rhesus Monkey	U.S.A.	1979	AGMK	6×10^6
Wa	Human	U.S.A.	1980	AGMK	1×10^6
DS-1[b]	Human	U.S.A.	1980	AGMK	2×10^6

[a] PFU/ml

[b] Reassortant virus between temperature-sensitive mutant of cultivatable bovine rotavirus (UK strain) and noncultivatable human rotavirus (DS-1 strain)

Preparation of Hyperimmune Sera

Hyperimmune rabbit sera to canine and feline rotaviruses were prepared by intramuscular (i.m.) inoculation of 0.5 ml of purified virus mixed 0.5 ml Freund's incomplete adjuvant followed by two i.m. re-inoculations of 1.0 ml purified virus without adjuvant on days 21 and 28. Rabbits were bled one week after the last injection. All other hyperimmune antisera used were prepared in guinea pigs (17).

Plaque Assay

Plastic six-well plates (Costar, Cambridge, Mass.) with confluent MA 104 cell monolayers were washed three times with Leibovitz L-15 medium (M. A. Bioproducts, Walkersville, Md.) supplemented with 0.5 percent gelatin-glutamine and antibiotics (L-15/gel) and inoculated with 0.5 ml of virus samples (pretreated for 60 minutes at 37° C with trypsin at a final concentration of 10 μg/ml). The virus inoculum was allowed to adsorb for 60 minutes at 37° C. Excess inoculum was then removed, plates were washed once with L-15/gel and 5 ml of the overlay medium was applied to each well. The overlay medium was either: a) Eagle's basal medium, 0.75 percent carboxymethyl cellulose (CMC, Hercules, Inc., Wilmington, Del.), antibiotics, 100 μg DEAE-dextran/ml (Pharmacia Fine Chemicals, Piscataway, N. J.) and 0.5 μg 2× crystalline trypsin/ml, or b) Eagle's minimum essential medium (MEM) containing 0.9 percent agarose, glutamine, antibiotics, 100 μg DEAE-dextran/ml, and 0.5 μg 2× crystalline trypsin/ml. With the use of CMC overlay, plates were stained with formalin/crystal violet solution after 5 days of incubation in a humidified CO_2 incubator. With the use of agarose overlay, plates were applied a second overlay containing neutral red (0.067 mg/ml) after 3 to 4 days of incubation. Plaques were then counted 1—3 days later.

Plaque Reduction Neutralization Test

Plaque reduction neutralization tests were performed by mixing equal volumes of fourfold dilutions of sera (previously heat-inactivated at 56° C for 30 minutes) and the virus suspension diluted to contain approximately 30 plaque-forming units (PFU)

per well. After an incubation of 60 minutes at 37° C, 1 ml aliquots of the virus-serum mixtures were inoculated onto the cell monolayers of 6-well plates. Two wells were used for each dilution. After 60 minutes of adsportion at 37° C, the inoculum was removed and the cell monolayers were washed once with L-15/gel, an overlay medium was applied, the cultures were incubated, stained and read as described above. The neutralizing titer was expressed as the reciprocal of the calculated serum dilution causing a 60 percent reduction in plaque counts (57).

Criteria for Significance of Neutralization

Neutralization test criteria of significance were arbitrarily established on the basis of enterovirus standard system (7, 23, 35). If the difference between homologous and heterologous neutralizing titers was less than 20-fold, the serological relationship was considered to be significant; if the difference between titers was 20-fold or greater, viruses were considered to be significantly different.

Immunofluorescent Test

Monolayers of MA 104 cells grown either on glass slides (Lab-Tek chamber slides, Lab-Tek Products, Napperville, Ill.) Or on coverslips in Leighton tubes infected with viruses, washed with PBS, fixed with acetone at —20° C for 15 minutes, air-dried at room temperature, and then stored at —20° C until used. The fixed cell cultures were stained by the indirect immunofluorescent methods using rabbit or canine sera and the corresponding fluorescein isothiocyanate (FITC)-conjugated anti-gammaglobulin sera or protein A-FITC conjugate (Pharmacia Fine Chemicals, Piscataway, N. J.). The slides were then counterstained with a 1:500 dilution of 1 percent Evans Blue in PBS for 10 minutes and washed twice with PBS. After air -drying, slides were mounted with 50 percent glycerol in distilled water, and examined for specific flourescence with an epifluorescence UV microscope (Ernst Leitz, Ltd., Midland, Ontario, Canada).

Results

Detection of Rotaviruses in Feces by Electron Microscopy

In negatively stained preparations of the fecal sample, both double-shelled and single-shelled particles were observed (Fig. 1). The canine rotavirus particles were indistinguishable in morphology from those of known rotaviruses of other species. Double-shelled particles had sharply defined margins forming a continuous covering over the main capsid, which is characteristic for rotaviruses (26, 38). Smooth particles were approximately 70 nm in diameter and rough particles were approximately 57 nm in diameter. Complete particles showed a characteristic "spoke-like" arrangement of inner capsomeres surrounded by an outer layer, which gave a honeycomb-like appearance on the surface of the virion.

Isolation and Propagation of Rotavirus in Cultured Cells

The first cythopathic effect (CPE) was observed 1 day after inoculation (DPI). Many cells adhered to the glass wall by only a single process ("flagging" CPE), which is a characteristic cytopathic effect produced *in vitro* by rotaviruses (33) and reoviruses (43). As incubation progressed, these cells detached from the glass wall and floated free in the medium. The rotavirus isolate (CU-1) could be passaged in MA 104 cells without the aid of trypsin. Viruses were serially passaged more than ten times in MA 104 cells. Virus particles were demonstrated by EM in cell culture lysates in all passages.

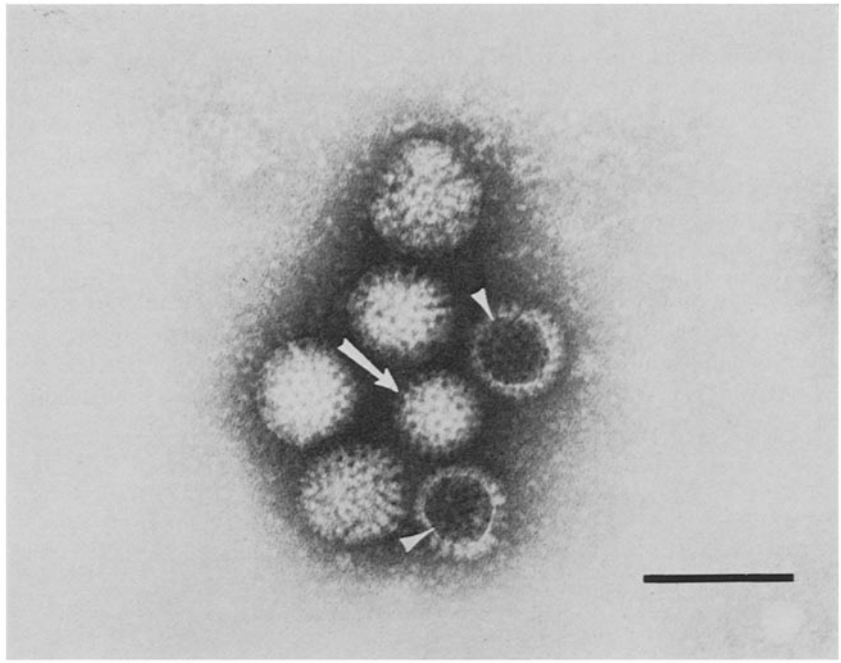

Fig. 1. Electron micrograph of negatively stained canine rotavirus particle in canine feces. Both single-shelled (arrow) and double-shelled particles are seen. Penetration of rotavirus particles by negative stain reveals an electron dense center (arrowheads), approximately 40 nm in diameter and hexagonal in outline, surrounded by an electron lucent layer. Bar represents 100 nm

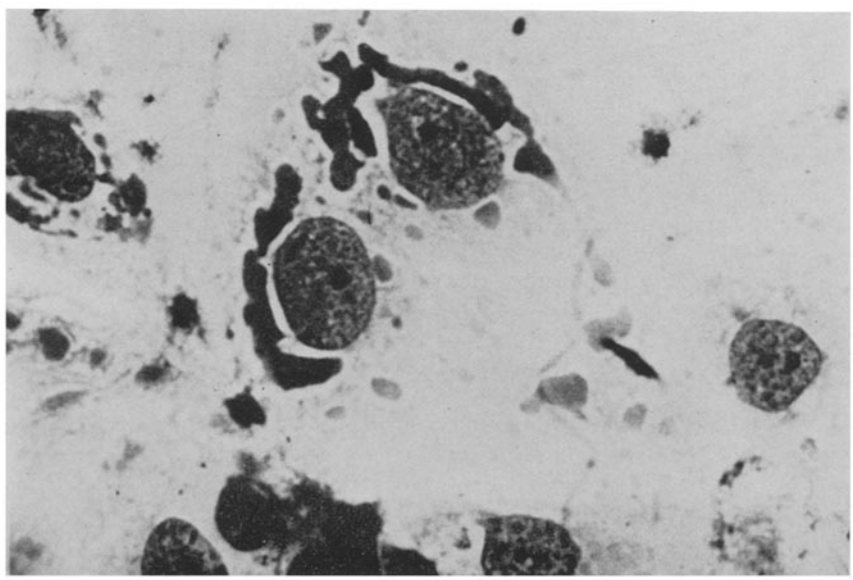

Fig. 2. Intra-cytoplasmic inclusion bodies in MA 104 cells infected with canine rotavirus (CU-1). Both small and coalesced large bodies are seen. May-Grünwald-Giemsa stain, 400×

Examination for Inclusion Bodies

Canine rotavirus (CU-1) induced intracytoplasmic inclusion bodies in MA104 cells. Inclusions were first noticed 24 hours post-infection. As incubation progressed, infected cells detached from the glass wall as noted above, however, inclusions were detectable in cells attached to the glass wall 5 DPI. Inclusions were of different sizes and shapes (Fig. 2).

Plaque Assay

Reproducible and clear-cut plaques were formed 5 DPI under the overlays of CMC or agarose in the presence of trypsin (Fig. 3). Although canine rotavirus readily replicated and produced CPE in monolayer cultures of MA104 cells in the absence of trypsin, detectable plaques were not formed even 8 DPI under the overlays of CMC or agarose without trypsin.

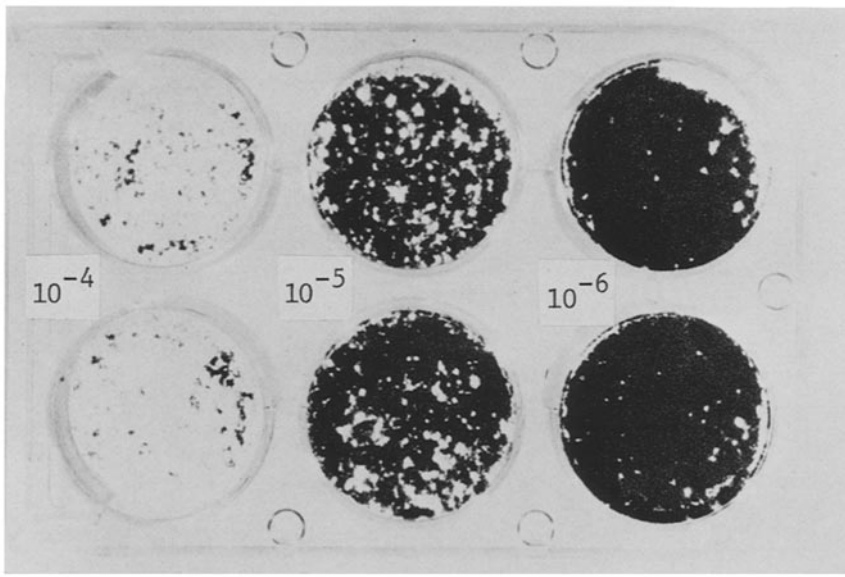

Fig. 3. Plaques produced by canine rotavirus (CU-1) in MA104 cells under agarose overlay after five days of incubation at 37° C

Antigenic Relationship Between Canine and Other Mammalian Rotaviruses

The antigenic relationship between canine and other mammalian rotaviruses was studied by plaque reduction neutralzation assay (Table 2). A two-way relationship between canine and simian (rhesus MMU 18006 and SA-11) rotaviruses was found, indicating that these viruses were similar, if not identical, by this assay. In contrast, canine rotavirus (CU-1) was distinct from two human rotavirus strains (Wa and DS-1). There was a one-way antigenic relationship between the canine and the feline, bovine and porcine rotaviruses; in each instance, serum to the non-canine strain demonstrated the broader reactivity.

Table 2. *Antigenic relationship between canine rotavirus and simian, bovine, porcine, feline and human rotaviruses by plaque reduction neutralization tests*

Rotavirus strain	Reciprocal of 60% plaque reduction neutralizing titer of hyperimmune antisera to indicated antigens (species of origin/strain)							
	Canine/ CU-1	Simian/ rhesus MMU 18006	Simian/ SA-11	Bovine/ NCDV	Porcine/ OSU	Feline/ Taka	Human/ Wa	Human/ DS-1
Canine/ CU-1	*67,350*[a]	67,350[a]	3,296[a]	3,900[a]	15,449[a]	21,104[a]	<80[a]	<80[a]
	65,788[b]	—	—	—	—	—	—	—
	81,441[c]	63,026[c]	4,826[c]	—	—	—	—	—
	68,365[d]	—	—	3,750[d]	16,350[d]	—	—	—
	71,972[e]	—	—	—	—	33,246[e]	<80[e]	—
	65,952[f]	—	—	—	—	—	—	<80[f]
Simian/ rhesus MMU 18006	25,547[b]	—	—	—	—	—	—	—
	27,179[c]	*62,013*[c]	3,658[c]	—	—	—	—	—
Simian/ SA-11	6,975[b]	—	—	—	—	—	—	—
	7,476[c]	74,725[c]	*44,635*[c]	—	—	—	—	—
Bovine/ NCDV	480[b]	—	—	—	—	—	—	—
	632[d]	—	—	*54,689*[d]	—	—	—	—
Porcine/ OSU	777[b]	—	—	—	—	—	—	—
	832[d]	—	—	—	*63,478*[d]	—	—	—
Feline/ Taka	3,125[b]	—	—	—	—	—	—	—
	3,254[e]	—	—	—	—	*48,482*[e]	—	—
Human/ Wa	135[b]	—	—	—	—	—	—	—
	314[e]	—	—	—	—	—	*63,365*[e]	—
Human/ DS-1[g]	164[b]	—	—	—	—	—	—	—
	330[f]	—	—	—	—	—	—	*28,365*[f]

[a–f] This is a composite table of values derived from 6 different tests, a-f. Homologous values italicized.

[g] Human-bovine reassortant rotavirus

Immunofluorescence

Virus-specific immunofluorescence was demonstrated with rabbit immune serum but not with preimmunization serum in the cytoplasm of MA104 cells infected with canine rotavirus (CU-1). In some infected cells, the areas of immunofluorescence were small and scattered in the cytoplasm (Fig. 4A), while in other cases, the areas coalesced to from large diffuse fluorescent foci (Fig. 4B). A cross-reactive antigen(s) was demonstrated in the cells infected with canine rotavirus by indirect immunofluorescence test (IFT) using hyperimmune anti-bovine rotavirus serum. Canine rotavirus antigen was not stained by hyperimmune sera against reovirus type I, II, and III by IFT; neither did hyperimmune serum to rotavirus stain reovirus antigens. Fluorescence was not detected in the uninfected control MA104 cells.

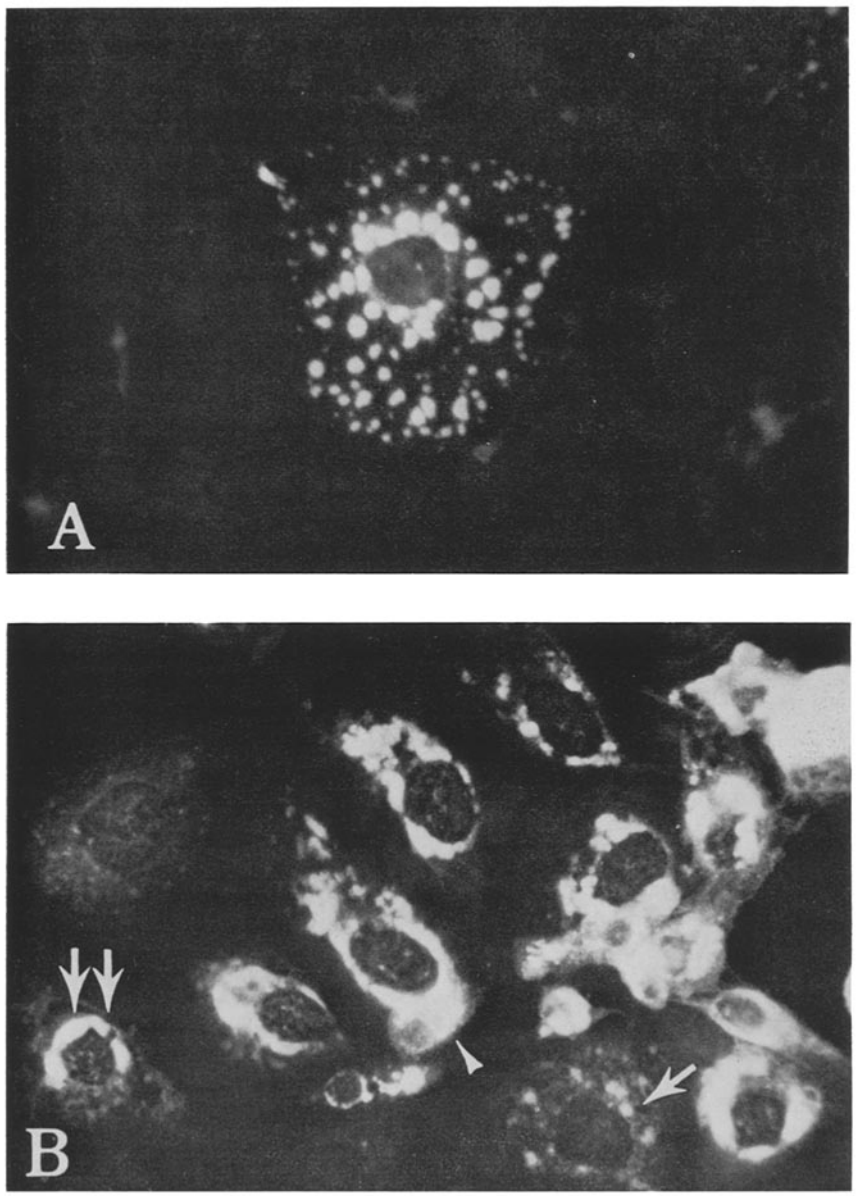

Fig. 4. Indirect immunofluorescent staining of MA 104 cells infected with canine rotavirus (CU-1), 400×. *A* single MA 104 cell with small and scattered immunofluorescent foci in the cytoplasm. Protein A-FITC. *B* small granular (arrow), large perinuclear (double arrows) and diffuse cytoplasmic immunofluorecence (arrowhead) are seen. Rabbit anti-dog IgG-FITC

Enhancement of Virus Infectivity by Trypsin Treatment of Virus Preparation

Infected cell lysates were treated with trypsin (final concentration of trypsin at 10 μg/ml), incubated for 1 hour at 37° C and then directly assayed by plaquing. The PFU infectivity was enhanced approximately 100-fold.

Discussion

In 1978 McNULTY *et al.* (30) reported that antibody to rotavirus was detected by indirect immunofluorescent test (IFT) in 49 of 62 dogs (79 percent) from the Belfast area in Northern Ireland. TAKAHASHI *et al.* (48) in 1979 reported that 10 of 18 dogs (55.6 percent) in Aomori prefecture of Japan had antibody against calf rotavirus as determined by complement fixation test (CFT). Since both IFT and CFT detect only group-specific antibodies, and rotaviruses from one species can infect another species (5, 8, 34, 36, 46, 50, 51, 52, 55), it was not clear from these studies whether the antibodies in the dogs resulted from infection with distinctly canine rotavirus or with human or some other rotavirus.

EUGSTER and SIDWA (11) reported in 1979 that rotavirus was visualized by EM in feces from a 12-week-old puppy with diarrhea. Virus isolation and characterization were not reported. In 1980, ENGLAND and POSTON (9) reported visualization by EM and subsequent isolation in MDCK cells of a rotavirus from a dog with fatal neonatal diarrhea. They also reported that experimental inoculation of two 6-month-old beagles with purified intestinal contents did not result in clinical signs of illness or virus shedding. In 1981, FULTON *et al.* (15) isolated a rotavirus from a newborn dog that died after having clinical signs of diarrhea. They reported that their rotavirus isolate, "designated LSU 79—36, may be a specific canine rotavirus or a rotavirus from another species".

The results of the present study confirm the existence of a canine rotavirus. This canine rotavirus (isolated in New York State) was found to be similar, if not identical, serologically by plaque reduction neutralization assay to simian rotaviruses isolated in California (47) and South Africa (Table 2). However, they are different in RNA electropherotype, in hemagglutination pattern and in plaque size (HOSHINO, Y., KALICA, A. R. *et al.*, unpublished results). In addition, the relatedness of two rotaviruses based on serotype does not always correlate with the relatedness based on genotype (14, HOSHINO, Y. *et al.*, unpublished results). Further biological and biochemical studies, including hybridization analysis, on both canine and rhesus rotaviruses, are in progress. Whether these findings are unique to this specific isolate (CU-1 strain) of canine rotavirus or this is a common characteristic of any canine rotavirus isolate is being investigated in our laboratory. Recently we isolated by cloning two small variants of feline rotavirus which had a two-way antigenic relationship with the CU-1 strain of canine rotavirus. Further studies on plaque variation of rotaviruses are underway. No antigenic relationship was found between this canine rotavirus and two human rotaviruses by plaque reduction neutralization assay (Table 2), although we have observed that there is a one-way antigenic relationship between this isolate of canine rotavirus and another strain of human rotavirus (HOSHINO, Y. *et al.*, unpublished results). Since dogs, as pets, have close contact with humans, it will be interesting to examine

further the serological relationships between canine and human rotaviruses, and to look for evidence of interspecies transmission.

The negatively stained canine rotavirus particles were indistinguishable in morphology from those of known rotaviruses of other species. The spoke-like arrangement of inner capsomeres of double-shelled particles, with a sharply defined outer capsid margin, differs from reovirus which has a featureless layer located either over or among capsomeres of the main capsid (21), and from orbivirus which has a fuzzy indistinct layer covering the main capsid (53).

Rotaviruses are, in general, difficult to isolate and to grow in cell cultures using conventional techniques. However, certain strains may be serially propagated in cell cultures if the virus inuculum is treated with proteolytic enzymes or if proteolytic enzymes are incorporated into the medium after infection (1, 2, 3, 6, 16, 40, 41, 44, 45, 49, 56). England and Poston (9) reported that rotavirus observed in feces by EM from a diarrheal dog grew in cell cultures with the aid of trypsin, but did not grow in cell cultures without trypsin treatment. Our isolate, canine rotavirus CU-1, readily replicated in cell cultures without trypsin treatment. Both double-shelled and single-shelled virus particles were demonstrated by EM in cell culture lysates in all passages.

It has been reported that both simian and bovine rotaviruses induced intracytoplasmic inclusoin bodies which were small with clearly defined round or oval outlines, and they did not coalesce to from large bodies such as those seen in cells infected with reovirus (27, 54). Canine rotavirus, CU-1, produced intracytoplasmic inclusion bodies in different sizes and shapes. Some were small and multiple as have been reported for those induced by bovine and simian rotaviruses and some were large and coalesced like those induced by reoviruses. The size and shape of inclusion bodies may depend upon each virus-cell culture system. We observed large inclusions in bovine rotavirus/MA104 cell and simian rotavirus/MA104 cell systems (unpublished observation).

Further serological studies will determine antibody prevalence to canine rotavirus and establish whether or not infection with this strain is associated with disease. If canine rotavirus (CU-1) is found to be of low virulence, it may be evaluated as a potential vaccine strain for use in homologous or even heterologous hosts. The potential use of a heterologous bovine rotavirus for possible use as a vaccine for humans has been suggested previously (25, 55).

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