

Isolation of Human Rotaviruses in Primary Cultures of Monkey Kidney Cells

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We succeeded in isolating human rotaviruses from the feces of gastroenteritis patients by using roller cultures of primary cynomolgus monkey kidney cells with trypsin in the maintenance medium but without concentration and trypsin treatment of the inocula at each passage level. These cells were found to be more sensitive than MA-104 cells (derived from fetal rhesus monkey kidney) for the propagation of human rotaviruses. Polyacrylamide gel electrophoresis of the genome RNA revealed that there were small differences in the migration pattern of the segments among all the strains isolated from 1976 to 1981. The cultivation of human rotaviruses in primary cell cultures might aid in developing a live rotavirus vaccine.

Human rotavirus (HRV) is now recognized as a major causative agent of infantile gastroenteritis (3, 5). However, many attempts to propagate HRV *in vitro* have failed (1, 2, 4, 10, 11, 18). Recently, HRV (type 2, strain Wa) was cultivated by Wyatt et al. in primary cultures of African green monkey kidney cells after 11 passages in newborn gnotobiotic piglets (19). More recently, Sato et al. (14) and Urasawa et al. (17) succeeded in the direct cultivation of human rotaviruses from fecal specimens by using roller cultures of MA-104 cells together with pretreatment of the specimens with trypsin and incorporation of a small amount of trypsin into the maintenance medium. In this report we describe the successful cultivation of HRV in primary cultures of monkey kidney cells, which were found to be more sensitive than MA-104 cells.

Fecal specimens were collected at the same hospital from infants and children with diarrhea during the winters of 1976 to 1981. All the specimens were stored at -20°C . Primary cultures of cynomolgus monkey kidney (PMK) cells were grown in lactalbumin-Earle medium plus 2% heat-inactivated bovine serum, and MA-104 cells derived from fetal rhesus monkey kidney were grown in Eagle minimum essential medium plus 10% newborn calf serum as described previously (8). Both types of cells were maintained for at least 1 day in Eagle minimum essential medium without serum before they were used for virus isolation. A 10 to 20% fecal suspension was prepared with distilled water or phosphate-buffered saline and then treated with fluorocarbon; the aqueous phase was collected

as a source of virus. Rotavirus particles or antigens in the samples were detected by electron microscopy (EM) or by immune adherence hemagglutination (IAHA) (9). Rotavirus-positive samples were then filtered through a 0.45- μm -pore membrane (Acrodisk; Gelman Sciences, Inc., Australia), mixed with an equal volume of acetyl trypsin (10 $\mu\text{g}/\text{ml}$) (Sigma Chemical Co., St. Louis, Mo.), and incubated at 37°C for 20 min. After the trypsin treatment, the samples were inoculated into two culture tubes each of PMK or MA-104 cells (0.2 ml per tube), which were incubated at 37°C for 1 h. After adsorption of virus to cells, maintenance medium containing 1 μg of acetyl trypsin per ml was added to the tubes without washing off the inocula. Cultures were then rolled at 37°C . After an incubation period of 7 days, the cultures were frozen and thawed three times, 0.2 ml of the fluid was inoculated to two new culture tubes each of PMK or MA-104 cells, and the trypsin-containing maintenance medium was added. The inocula for further passages were not treated with trypsin. At the time of each passage, culture fluids were tested by IAHA for rotavirus antigen. At passage 7, they were examined by EM for rotavirus or any other virus particles. The EM test was performed by negative staining with 2% potassium phosphotungstate (pH 7.2). Cultivated viruses were concentrated with polyethylene glycol 6000 about 20 times (8), layered onto 40% (wt/wt) sucrose, and centrifuged at 35,000 rpm for 90 min in a Beckman SW50.1 rotor. The pellets were suspended in 0.01 M Tris-hydrochloride (pH 7.2), mixed with sodium

TABLE 1. Propagation of HRV in PMK and MA-104 cells

Specimen no.	Cells used	IAHA antigen titer ^a determined with anti-human rotavirus serum ^b							
		Original sample	Passage no.						
			1	2	3	4	5	6	7
76-T4 ^c	PMK	NT ^d	NT	64	64	64	64	32	64
76-T5	PMK	NT	NT	64	32	64	64	32	64
76-T6	PMK	NT	NT	64	128	64	32	16	64
76-T7	PMK	NT	NT	128	64	64	32	32	64
76-T8	PMK	NT	NT	<2	<2	<2	<2	<2	<2
77-21	PMK	≥256	32	64	64	64	32	64	32
77-24	PMK	≥256	<2	<2	<2	<2	<2	<2	<2
77-49	PMK	≥256	32	64	64	128	32	64	32
77-67	PMK	256	8	2	<2	<2	<2	<2	<2
78-9 ^e	PMK	<2	32	16	64	64	32	64	32
78-20	PMK	<2	8	32	32	64	64	32	64
79-20	PMK	256	≥32	16	64	64	32	64	64
79-30	PMK	≥256	≥32	16	64	64	32	64	64
79-33	PMK	≥256	32	64	64	64	32	64	64
79-33	PMK	≥256	32	16	64	64	32	64	64
80-3	PMK	64	64	128	64	64	64	128	128
	MA-104		NT	<2	<2	<2	<2	<2	<2
80-17	PMK	64	128	128	32	64	16	128	128
	MA-104		NT	<2	<2	<2	<2	<2	<2
81-9	PMK	256	256	32	16	8	16	128	128
	MA-104		NT	8	<2	<2	<2	<2	<2
81-18	PMK	256	64	32	64	64	64	128	128
	MA-104		NT	<2	<2	<2	<2	<2	<2
81-27	PMK	128	64	64	16	32	32	64	64
	MA-104		NT	<2	<2	<2	<2	<2	<2
81-31	PMK	256	64	16	<2	<2	<2	<2	<2
	MA-104		NT	<2	<2	<2	<2	<2	<2

^a The IAHA test was performed according to the method of Inouye et al. (6).

^b The preparation of antiserum was as described previously (8).

^c Rotavirus particles were found by EM in the 10% fecal suspension of five specimens collected in 1976.

^d NT, Not tested.

^e Rotavirus antigens in two specimens collected in 1978 were negative (<2) by IAHA but positive (≥32) by the reverse passive hemagglutination test, which was performed according to the method of Sanekata et al. (13).

dodecyl sulfate (0.1%), and treated with chloroform-phenol (1:1). The extracted RNA was analyzed by polyacrylamide gel electrophoresis according to the method of Rodger et al. (12). Gels were stained with 0.5 µg of ethidium bromide per ml in 0.01 M Tris buffer (pH 8.0) and examined on a shortwave UV transilluminator.

We isolated rotaviruses from 17 of 21 fecal specimens in PMK cells; Table 1 summarizes the IAHA antigen titers of culture fluids at each passage level. When PMK cells were used, IAHA antigen was detected at passage 1, and the titer did not vary much with passage levels. In MA-104 cells, however, the titer decreased and remained at undetectable levels (<2) up to passage 7. Cytopathic effects (CPE) appeared in PMK cells at passage 2 to 7 (Fig. 1). Typical rotavirus particles were observed by EM in the

culture fluids which were IAHA antigen positive.

RNA genomes of the 17 isolated strains were subjected to polyacrylamide gel electrophoresis. Figure 2 shows typical migration patterns of rotavirus RNA segments. Small differences in the relative mobility were observed among strains and even among viruses from the same year. This suggests that more than one electropherotype could coexist during one winter season in the same hospital area.

By using PMK cells, 17 strains were isolated easily. We feel that these isolated viruses were of human origin and not bovine or simian rotavirus contaminants. We used purified trypsin and heat-inactivated serum for cell cultures. We could detect rotavirus antigens only in cells inoculated with rotavirus-positive fecal speci-

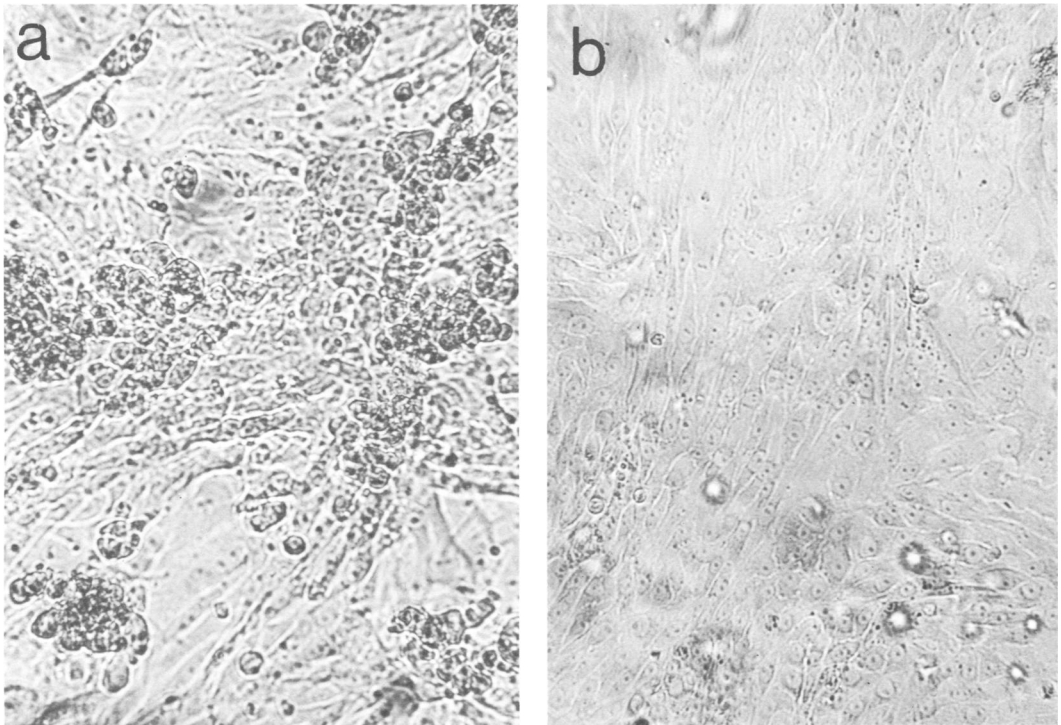


FIG. 1. CPE produced by human rotavirus (strain 79-33) in PMK cells. (a) Cells 3 days after inoculation on passage 4. At the early stage of CPE, fibroblast-like cells took on a round shape, and minute granules appeared in the cytoplasm. (b) Uninfected control.

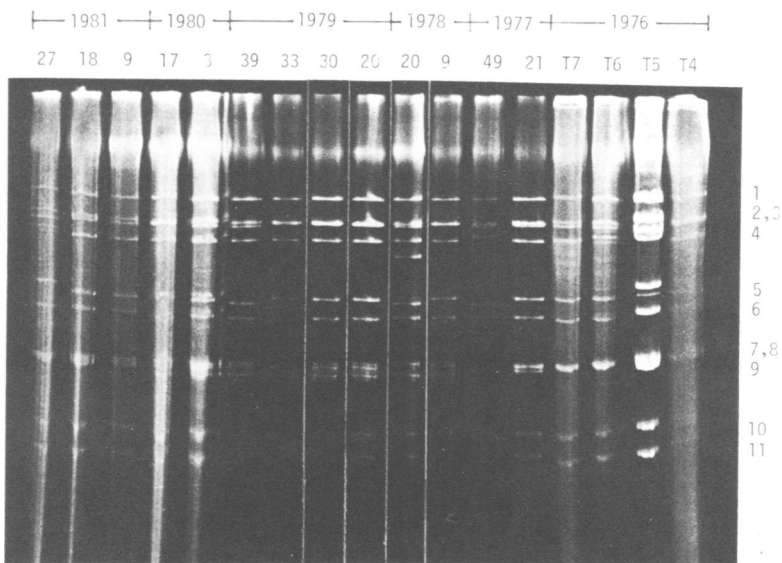


FIG. 2. Electrophoretic patterns of genome RNA of human rotaviruses isolated from 1976 to 1981. Electrophoresis of RNA was performed in 10% polyacrylamide gels with the discontinuous buffer system described by Laemmli (7). Electrophoresis was conducted for 4 h at room temperature at 30 mA. Migration was from top to bottom, and genome segments are numbered in descending order of size.

mens, not in uninfected cell controls. Furthermore, the possibility of contamination by laboratory strains of animal rotaviruses could be ruled out by the fact that there were variations of RNA migration patterns among the isolated strains (Fig. 2); if they were derived from such viruses, the RNA migration patterns would be identical.

In MA-104 cells we could neither observe clear CPE nor detect IAHA antigen up to passage 7. However, Sato et al. reported that clear CPE by human rotavirus appear in MA-104 cells at low passage levels (14), and Urasawa et al. also reported observation of CPE and detection of fluorescent foci in human rotavirus in MA-104 cells (17). The reason for the difference may be that we did not perform concentration and trypsin treatment of the inocula at every passage level. At any rate, we found that PMK cells were more sensitive than MA-104 cells for the propagation of HRV. Stuker et al. also used PMK cells for the isolation of rotaviruses from infant rhesus monkeys with diarrhea (16). PMK cells may be useful for the propagation of human and simian rotaviruses.

In tropical areas infantile gastroenteritis occurs throughout the year, and about 40% of these cases are associated with rotaviruses (15); rotavirus vaccines are needed for the reduction of infant mortality. Successful cultivation of human rotavirus in PMK cells may provide candidate strains for developing an effective live virus vaccine.

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