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Simian Rotavirus SA11 Replication in Cell Cultures

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Understanding the basic virology of rotavirus infections has been hampered by the fastidiousness of most isolates and by the lack of a rapid quantitative assay method. The growth characteristics of the simian rotavirus SA11 were studied because it grows to high titers in tissue culture and infectivity can be quantitated by plaque assay. SA11 replication was analyzed in a variety of primary cell cultures or continuous cell lines derived from both homologous and heterologous hosts. Viral replication was observed in each of the cell cultures examined. The individual cell cultures demonstrated marked variability in their susceptibility to rotavirus infection. The highest titers were obtained with MA104, BSC-1, CV-1, and BGM cells. Observable cytopathic effect was found to correlate with the percentage of infected cells in the culture. This study presents growth curves of the simian rotavirus in a variety of cell cultures.

Rotaviruses are a worldwide cause of neonatal diarrhea and have been isolated from many mammalian species (5, 8). Only limited information is available describing the basic virology of this newly recognized genus in the Reoviridae family, due in part to the fact that most rotaviruses are difficult to propagate in vitro. Previous studies with tissue culture-adapted calf, porcine, or simian rotavirus strains have provided preliminary information on rotavirus replication in selected cell lines, but have suffered from the lack of systematic analyses, or quantitation of viral infectivity, or both (2, 4, 9-11, 13). We have examined rotavirus-cell interactions using the simian rotavirus SA11 as a model system because it grows readily in tissue culture and can be quantitated by plaque assay. These studies sought to identify the most susceptible cell line for growth of SA11 as well as to analyze the basic biological characteristics of the virus. Studies were performed analyzing infectivity and growth characteristics of SA11 in a variety of primary cell cultures and continuous cell lines. A marked difference in cell susceptibility to viral infection was observed, suggesting that a range of virus-cell interactions may occur in cells derived from either homologous or heterologous hosts.

MATERIALS AND METHODS

Virus and cell lines. Simian rotavirus SA11 was kindly supplied by H. H. Malherbe. Virus stocks were prepared by low-multiplicity infection of fetal rhesus monkey kidney MA104 cells (Microbiological Associates, Bethesda, Md.) in media without fetal calf serum as previously described (3a). The titer of virus stocks used in these experiments was 1.2×10^8 PFU/ml. The following cells were used: primary African green monkey kidney (AGMK), primary rhesus monkey kidney (RhMK), primary human embryonic kidney (HEK), human embryonic lung (M7) (Microbiological Associates), continuous lines from rhesus monkey kidney (MA104, P54; LLC-MK2, P315), green monkey kidney (Vero, P135; CV-1, P38; BSC-1, P67), buffalo green monkey kidney (BGM, P133), mouse fibroblast (L929, P566), and rabbit kidney (RK13, P146) from Flow Laboratories (Rockville, Md.) or as previously described (M. K. Estes and D. Y. Graham, *Proceedings of the 2nd International Symposium on Neonatal Diarrhea*, in press). Cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, 2% BME vitamins, 0.03% glutamine, 0.25% glucose, 0.075% sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of gentamicin per ml. Cells were maintained in the same medium containing 2% fetal bovine serum.

Virus assays. Virus assays were performed in MA104 cells using the plaque technique under an agar overlay containing pancreatin and DEAE-dextran as previously described (10a). Assay cultures were incubated at 37°C, and plaques were counted after 4 days of incubation.

Virus growth analysis. Cultures of monkey cells were washed three times with room-temperature Tris buffer (TBS), pH 7.2, which contained 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 6 mM glucose, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$. SA11 (2 PFU per cell) was adsorbed for 90 min at 37°C. Unadsorbed virus was removed by three washes with TBS, and the cells were incubated at 37°C in Eagle medium lacking fetal bovine serum. Duplicate cultures were monitored for cytopathic development and harvested at specified intervals by quick-freezing in dry

ice-ethanol. Cultures that exhibited cytopathic effect (CPE) were photographed with a Nikon camera adapted for photography through an inverted microscope. Virus-containing supernatants were assayed by the plaque technique after cell disruption by sonic oscillation for 1 min at 10 kc (Branson Sonifier, Branson Instruments, Danbury, Conn.) and low-speed centrifugation to remove cell debris. To determine the cell-associated virus, infected cells were gently scraped into the culture medium with a sterile rubber policeman. After low-speed centrifugation (2,000 × *g* for 5 min), the supernatant was removed for assay of released virus. The cell pellet was resuspended in the same volume of medium and assayed for cell-associated virus after sonication and clarification of cell debris by low-speed centrifugation. All assays from each growth experiment were performed simultaneously in duplicate with the same lot of cells.

Immunofluorescence tests. Monolayers of cells growing on 15-mm round cover slips were washed three times with TBS and inoculated with SA11 (2 PFU per cell). At intervals after infection, cover-slip cultures were washed three times with TBS (4°C), fixed for 10 min in −20°C ethanol, and stained by the indirect immunofluorescence test as previously described (Estes and Graham, in press).

Antiserum to SA11 prepared by hyperimmunization of guinea pigs with CsCl-purified, pooled double-shelled and single-shelled SA11 particles was employed as the primary antiserum in indirect immunofluorescence tests. The secondary antiserum was fluorescein-labeled goat anti-guinea pig immunoglobulin (Hyland Laboratories, Costa Mesa, Calif.). Fluorescein-labeled antibody was absorbed with mouse liver powder and SA11-infected cell extracts to remove nonspecific fluorescence. The specificity of the guinea pig anti-SA11 antiserum was established by (i) removal of specific immunofluorescence after absorption with SA11-infected cells and (ii) agglutination of purified SA11 particles by immune electron microscopy. This antiserum showed no reaction by indirect immunofluorescence or complement fixation tests against uninfected cells, reovirus type 1-infected cells, or poliovirus type 1-, 2-, or 3-infected cells.

Cytopathogenicity. CPE were visualized by direct microscopic examination of monolayers in 25-cm^2 flasks inoculated with varying multiplicities (0.01 to 10 PFU per cell) of virus. Control and infected cells grown on cover slips were also fixed in Bouin's fixative and stained with hematoxylin and eosin (6). Alternatively, cover-slip cultures were fixed in Carnoy fixative and stained with acridine orange (7). Acridine orange-stained cover slips were observed with a Zeiss filter set for quinocrine mustard (catalogue no. 48 77 05) by reflected-light fluorescence.

RESULTS

Cytopathic response of various monkey kidney cells to SA11 infection. A wide range of rotavirus-cell interactions was noted, depending upon the cell type infected with SA11 (Fig. 1). Infection of MA104, CV-1, and BSC-1 cells (2 to 4 PFU per cell) resulted in a highly cytolytic response. CPE in these continuous monkey kidney cell lines was first detectable 8 to 10 h postinfection. SA11-infected MA104 cells totally degenerated with time. The earliest change noted was cell contraction and rounding; increased definition of cell borders which were previously indistinct was followed by detachment of a portion of the cell from the monolayer. Infected cells developed eosinophilic cytoplasmic or perinuclear inclusions, or both, with cytoplasmic vacuolization appearing in some cells. CPE in these highly cytolytic infections was maximal at 48 h postinfection, with 100% of the cells being detached from the monolayer. Staining of MA104-infected cells with acridine orange revealed cytoplasmic yellowish-green staining, often in inclusions, suggesting the presence of double-stranded nucleic acid.

Infection of primary AGMK or RhMK cells (2 to 4 PFU per cell) resulted in less extensive CPE, with only 50% of the cells showing CPE at 48 h postinfection. The cytopathic development in these cells was similar to that in MA104 cells, with the predominant feature being the appearance of cells hanging from the monolayer. Infection of the continuous rhesus monkey kidney cell line, LLC-MK2, resulted in areas of cell clumping without the destruction of the monolayer. Infection of Vero cells did not change the appearance of the monolayer compared to control cultures.

SA11 virus growth in monkey kidney cells. SA11 virus growth was monitored in a variety of monkey kidney cells by several parameters (Table 1). A wide range of total virus yields was obtained, depending on the monkey cell line infected (3×10^5 to 2×10^8 PFU per 10^6 cells). The percentage of cells infected at 24 h also varied (4 to 36%). In general, the cell lines which exhibited greatest cytolytic response resulted in the greatest virus yields. A unique cell response was observed with BGM cells, in which high yields of virus were produced, and only a small percentage (10%) of the cells demonstrated synthesis of viral antigen. An intermediate cell response occurred in primary RhMK, continuous rhesus monkey kidney, and primary AGMK cells. CPE was not extensive, and less than 10% of the cells synthesized viral antigen(s). Thin sections of infected primary cells confirmed that the percentage of cells infected in these cultures was much reduced when compared to MA104 cells. Virus multiplication was minimal in Vero cells, with only 2 to 4% of the cells synthesizing viral antigen.

The percentage of virus remaining cell-associated at 48 h postinfection also varied among the cell lines tested. In general, more virus was

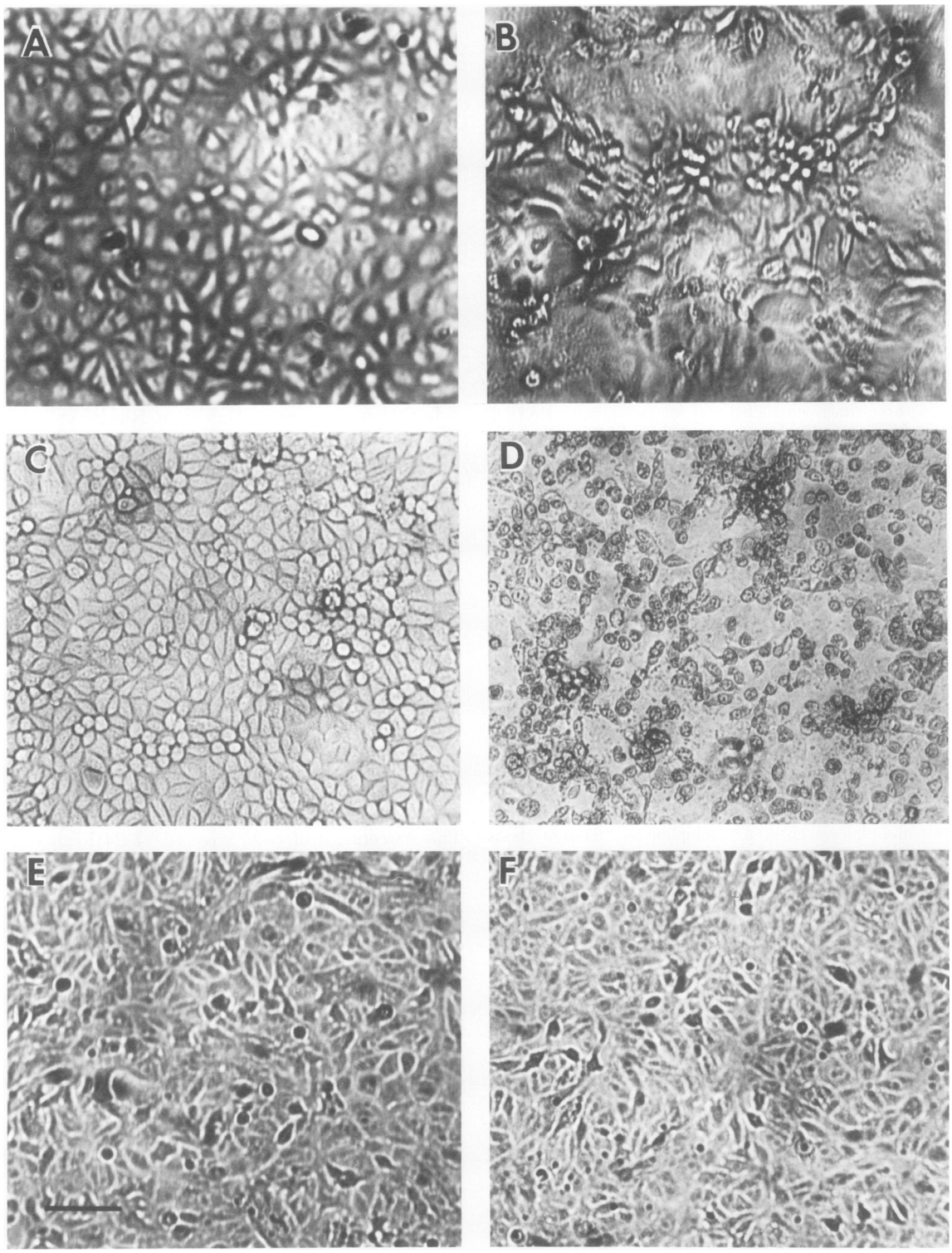

FIG. 1. *Appearance of MA104, CV-1, and Vero monkey kidney cells, respectively, 72 h after inoculation with TBS (A, C, E) or SA11 rotavirus (B, D, F). Bar represents 0.1 mm.*

TABLE 1. *Response of various monkey kidney cell cultures to infection with SA11*

Cell cultures	CPE[a]	Viral antigen synthesis (%)[b]	Total virus yield (PFU/10^6 cells)[c]	Cell-associated virus (%)[d]	Ability to plaque SA11; titer of stock (PFU/ml)
MA104[e]	4+	35	1.4×10^8	80	Yes (3.6×10^6)
CV-1[f]	4+	32	2.0×10^8	78	Yes (1.2×10^6)
BSC-1[f]	4+	36	2.0×10^8	85	No
AGMK[f]	3+	8	9.0×10^7	75	Yes (1.8×10^5)
RhMK[e]	2+	7	3.0×10^6	65	No
BGM[f]	2+	10	2.0×10^8	55	No
LLC-MK2[e]	1+	9	3.0×10^6	80	Yes (9.5×10^4)
Vero[f]	−	4	3.0×10^5	45	No

[a] CPE at 72 h. 1+, 2+, 3+, 4+ = destruction of 25, 50, 75, or 100%, respectively, of the monolayer. − = not detectable.

[b] Percentage of cells synthesizing viral antigen determined by indirect immunofluorescence tests at 24 to 30 h postinfection.

[c] Total virus yields determined at 48 h postinfection.

[d] Percentage of infectious virus remaining cell associated at 48 h postinoculation.

[e] Rhesus monkey kidney cultures.

[f] Green monkey kidney cultures.

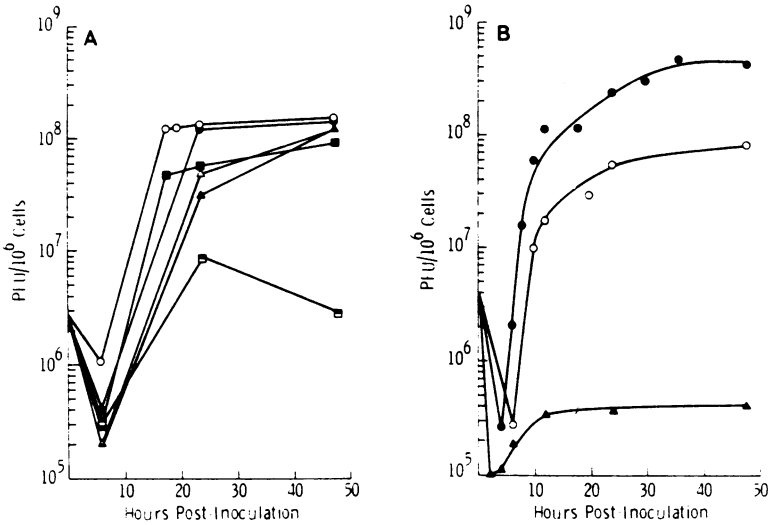

FIG. 2. *Growth of SA11 in monkey kidney cell lines. Confluent cell monolayers (10^6 cells) were washed with TBS and infected with SA11 at a multiplicity of 2 to 4 PFU per cell for 90 min at 37°C. The inoculum was removed, the monolayers were washed with TBS, and medium without serum was added. Cultures were harvested as described in the text at the specified intervals, and viral infectivity was assayed by the plaque technique. (A) ○, BSC-1 cells; ●, MA104 cells; △, ▲, CV-1 and BGM cells; ■, primary AGMK cells; ◪, primary RhMK and LLC-MK2 cells. (B) ●, MA104 cells inoculated with 4 PFU per cell; ○, MA104 cells inoculated with 0.4 PFU per cell; ▲, Vero cells inoculated with 2 PFU per cell.*

released into the supernatant from cells in which minimal CPE appeared. Lastly, Table 1 shows the ability of these various monkey cell lines to support the development of plaques. Plaques developed on monolayers of MA104, LLC-MK2, CV-1, and primary AGMK cells. MA104 and CV-1 cells were the most sensitive hosts for plaque assays, as demonstrated by the titers obtained with the same virus stock. Plaques appeared on day 10 in the primary AGMK cells and were small and difficult to read.

Viral replication in monkey kidney cell cultures. Growth curves of viral replication in monolayers of the various monkey cell cultures are shown in Fig. 2. Typical virus growth curves were observed, and, although the yields of virus varied, these studies confirmed that SA11 replicated in each of the cell lines tested.

Examination at shorter (2-h) intervals (Fig. 2B) revealed a virus eclipse phase of about 4 h, with the first detectable virus being produced at approximately 6 h postinfection. It is of interest

TABLE 2. *Effect of multiplicity of infection on virus yields from monkey kidney cell lines*

Cell line	Total virus yield (PFU/10^6 cells)[a] at:		
	0.01[b]	1.0	10
MA104	7.0×10^7	3.1×10^8	2.9×10^8
BSC-1	5.5×10^7	9.5×10^7	ND[c]
BGM	2.0×10^7	2.5×10^7	ND
LLC-MK2	8.5×10^4	1.5×10^6	3.0×10^7
Vero	1.5×10^4	7.0×10^5	1.9×10^6

[a] Virus yields determined 72 h postinfection.
[b] Multiplicity of infection.
[c] ND, Not determined.

that with both primary and continuous RhMK cells, virus titers declined between 24 and 48 h postinfection (Fig. 2A).

A variety of factors which might influence viral yields was investigated, including content of media, temperature, and multiplicity of infection. Virus titers obtained from MA104 cells were similar whether the cells were incubated at 34, 37, or 39°C. Virus grew to high titers in MA104, BSC-1, and BGM cells by 72 h postinfection (Fig. 2), irrespective of the multiplicity of infection (Table 2). In contrast, the restricted growth of SA11 in Vero cells was confirmed and accentuated by differences in viral yields obtained from inoculation of monolayers with 100-fold differences in multiplicity.

The inhibitory effect of serum observed in plaquing rotavirus (10a) was confirmed in growth studies in MA104 and BSC-1 cells. In these cells, 24-h virus yields were decreased 5- and 10-fold, respectively, by the inclusion of 2% heat-inactivated fetal calf serum (shown to be free from rotavirus antibody by complement fixation tests) in the medium.

Replication of SA11 in heterologous cell cultures. The replication of SA11 in heterologous cell cultures of nonprimate origin was also investigated. SA11 replicated poorly in epithelial or fibroblastic primary human embryonic cells and in rabbit kidney or mouse fibroblast cells (Table 3).

DISCUSSION

The present studies demonstrate that the simian rotavirus SA11 exhibits a wide and varied host range. These studies were performed using a tissue culture-adapted rotavirus whose infectivity could be quantitated by plaque assay. The plaque assay allowed highly reproducible and quantitative analyses of these varied rotavirus–cell interactions. There was no direct correlation noted between viral titers obtained and the degree of CPE observed in the cells. Rather, the presence of cytopathogenicity appeared to reflect the percentage of infected cells in a culture. Virus yields varied greatly; similar titers were produced by MA104 and BGM cells, in spite of the fact that only one-third as many BGM cells synthesized rotavirus antigens as demonstrated by indirect immunofluorescence tests. This observation suggests that BGM cells are more efficient at supporting viral replication, yielding higher virus titers per infected cell.

The use of the plaque assay allowed the demonstration of viral replication in all the cell lines examined. Although the specific factors regulating cell permissiveness to rotavirus infection remain unknown, the description of cell lines which exhibit varying susceptibilities will allow these factors to be addressed. Among the factors to be considered are (i) the presence or absence of specific viral receptors on host cells, (ii) the contribution of expression of proteolytic enzymes by the cells, (iii) the possible induction of antiviral factors by infected cells, or (iv) blockage of viral replication after adsorption and penetration.

Our previous studies have shown that SA11 will not plaque in the presence of fetal bovine serum. In the present study we confirmed that SA11 replication is significantly inhibited (5- to 15-fold) by 2% fetal bovine serum even when the serum was prescreened by complement fixation tests and shown to lack rotavirus antibodies. If these observations are applicable to other rotavirus species, the practice of preparing fecal extracts in fetal bovine serum before cell culture inoculations as well as the practice of placing samples in fetal bovine serum for transportation may have unknowingly hampered the ability of previous investigators to grow rotavirus. It was recently reported that rotavirus can be successfully cultivated from fecal samples after trypsin treatment (1, 2, 11). The inhibitory effect of fetal bovine serum may be related to the strong antiproteolytic activity of serum, which suppresses

TABLE 3. *SA11 replication in cells of nonprimate origin*

Cell line	CPE	Virus yields (PFU/10^6 cells)[a] at:	
		4 h	24 h
HEK	1+	3.8×10^4	6.0×10^5
HEL (M7)	1+	1.3×10^4	5.8×10^4
RK13	–	2.0×10^5	3.5×10^5
L929	–	ND[b]	2.5×10^5

[a] Cell cultures were infected with SA11 at a multiplicity of 2 PFU per cell. Viral yields were determined by plaque assay of duplicate harvests taken at the indicated times.
[b] ND, Not determined.

proteases necessary for rotavirus replication.

McNulty et al. (9) infected MDBK cells with a North Ireland isolate of the calf rotavirus (0.5 50% tissue culture infective dose per cell) and reported an eclipse time of approximately 3 h, followed by maximum virus excretion at 20 h. Our results with SA11 are similar: the eclipse time was approximately 4 h, with synthesis of infectious progeny virus first detectable at approximately 6 h postinfection. Synthesis of viral antigen was successfully detected 8 to 10 h postinfection by indirect immunofluorescent techniques. Maximal virus production generally occurred between 18 and 24 h postinfection, but depended upon the cell line examined.

The present studies demonstrate that SA11 does grow, although not well, in heterologous cell lines, including those of human, rabbit, and murine origin. Because the present studies were performed using virus stocks grown in monkey (MA104) cells, it is possible that, with passage, the virus could be adapted to grow to high titers in these cells. The Cody strain of the bovine rotavirus has also been successfully propagated in heterologous cell cultures (4).

LLC-MK2 cells are a continuous cell line that has been used by several investigators to detect the replication of human, monkey, and calf rotaviruses (3). The present studies demonstrate that it is a relatively insensitive host for the growth of SA11 when compared to other continuous monkey kidney cell lines. These studies suggest that the use of additional cell lines might increase the sensitivity of detecting rotavirus infections by the indirect immunofluorescent technique. MA104 cells, for example, are more sensitive for SA11 replication, and tissue culture-adapted strains of both calf and porcine rotavirus will grow and plaque in these cells (unpublished observations).

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