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Rotavirus Isolation and Cultivation in the Presence of Trypsin

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Rotaviruses are generally difficult to isolate and culture in vitro; therefore, virus isolation has not been used as a method of diagnosing this group of agents. The present report describes a simple procedure for isolating bovine rotaviruses directly from feces after pretreatment of fecal samples with trypsin. This procedure resulted in virus isolation from five of five samples that contained virus particles, as demonstrated by electron microscopy, and four of seven samples where virus particles could not be observed but were considered positive by the presence of immunofluorescent-staining cells in feces. Virus could not be isolated from "normal" feces. If the virus was not passaged in the presence of trypsin, the infectivity was gradually lost, but infectivity could be restored again if trypsin was added, resulting in increased virus spread and concomitant increase in virus yield. The application of this technique as a diagnostic tool for bovine and other rotaviruses is briefly discussed.

Rotaviruses were first described in 1969 as the etiological agent of neonatal calf diarrhea. Since that time rotaviruses have been identified as one of the major causes of diarrhea in other animals, including humans (5, 6, 10, 13, 15, 16, 19, 22, 27). The viruses infecting different species all appear to be serologically related, so that reagents (antigen or antibody) prepared against one virus have been used to detect virus antigen or antibody of another species (12, 21, 28). These reagents are often prepared from virus obtained from fecal material; consequently, stringent purification is essential to obtain adequate reagents (13, 20). Since it would be much more convenient to use tissue culture-grown virus as a source of antigen, numerous attempts have been made to culture the agents in vitro. These attempts have, in some instances, been successful with both the bovine and human rotaviruses (1, 4, 8, 9, 18, 26, 29); however, the degree of replication was often limited, adaptation took a long time (G. Woode, personal communication), or there was loss of infectivity after continued passage (29). Because of the problem encountered in culturing rotaviruses, virus isolation has not been used as a routine diagnostic tool. Secondly, it has been difficult both to analyze these agents biologically and biochemically and to compare viruses of different species to understand the evolution and transmission of rotaviruses from one species to another. The present report describes a simple procedure for the isolation and cultivation of high-titer bovine rotaviruses that should simplify many biological studies on this group of agents.

MATERIALS AND METHODS

Cells. Monkey kidney (BSC-1) cells were cultured in Eagle minimal essential medium (MEM) as described previously (3). Each liter of medium was supplemented with 2 mmol of glutamine, 10 ml of nonessential amino acids (Grand Island Biological Co., Grand Island, N.Y., no. 114), 2.5 mg of gentamicin, and 2.5 g of sodium bicarbonate. For growth, the medium contained 10% heat-inactivated fetal calf serum.

Virus growth and isolation. Confluent cultures (100-mm petri dishes) of BSC-1 cells were washed twice in Hanks balanced salt solution to remove the fetal calf serum before infection with rotavirus (strain C-486 in passages 10 to 15 after isolation; multiplicity of infection, 0.5) in the presence of 10 μg of trypsin (Difco Laboratories, Detroit, Mich., 1:250) per ml. Some of the properties of this virus have been reported previously (M. Fauvel, L. Spence, L. A. Babiuk, R. Petro, and S. Bloch, Intervirology, in press). After a 1-h adsorption period at 37°C, MEM, containing 0.5% bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) and 5 μg of trypsin per ml, was added to each plate. When the cells showed extensive cytopathic degeneration, the supernatant fluids containing detached cells and extracellular virus were removed, and the cells remaining attached were treated with 5 ml of 0.5% trypsin-Versene solution. This solution was allowed to remain in contact with the cells for 30 s and then removed. The cells were incubated at 37°C for 5 min and then suspended in culture fluids used for virus growth. The cells and virus were subjected to two freeze-thaw cycles. The cellular debris was removed by centrifugation at 3,000

× g for 10 min, and the virus present in the supernatant fluid was used as stock virus. In BSC-1 cells, the average titer of the stock virus was 1.8×10^5 tissue culture infective units. Diarrheic fecal samples were obtained from calves distributed throughout Saskatchewan during a 3-year period (1975 to 1977) and designated FD 1 through 15. "Normal" feces were obtained from clinically healthy week-old calves.

Virus isolation from feces was achieved by treating a 1/10 dilution of feces with 500 μg of trypsin per ml at 37°C for 15 min. The feces were then diluted (1/20) and added to confluent BSC-1 cell cultures that had been washed to remove traces of fetal calf serum as described above. After a 2-h adsorption period, the fecal material was removed and MEM + 0.5% bovine serum albumin containing 5 μg of trypsin per ml was added. Forty-eight hours later all cultures were trypsinized and passaged again onto fresh BSC-1 cells. The presence of virus was detected by electron microscopy, immunofluorescence, and cytopathic degeneration as demonstrated by detachment of elongated crescent-shaped cells.

Immunofluorescence. BSC-1 cells were grown in glass slides (Lab-Tek chamber slides), infected with virus, air dried, and then fixed with cold ethanol (−70°C) for 15 min. The fixed-cell monolayers were then reacted for 30 min with fluorescein-labeled rabbit anti-rotavirus conjugate (supplied by C. A. Mebus), washed three times with phosphate-buffered saline (pH 7.2), mounted, and observed with the aid of a Wild-Leitz microscope equipped with a fluorescence attachment.

Radioimmunoassay. Detection of rotavirus antigens by a solid-phase radioimmunoassay has been described previously (3). Briefly, BSC-1 cells were grown to confluency in microtiter plates (Falcon plastics no. 3040) and infected with various dilutions of virus. Some of the wells were incubated in the presence of 10 μg of trypsin per ml, whereas parallel wells did not contain trypsin. Forty-eight hours postinfection, when the cultures were showing cytopathic effect, the monolayers were fixed with ethanol, washed, and reacted for 1 h at 37°C with bovine anti-rotavirus serum. The cultures were washed and reacted with ^{125}I-labeled rabbit anti-bovine immunoglobulin G (IgG). Controls were reacted with fetal calf serum prior to treatment with ^{125}I-labeled rabbit anti-bovine IgG. The amount of radiolabel bound was determined in a Nuclear-Chicago Gamma Counter, and the binding ratio was calculated as follows: BR = cpm bound by positive serum/cpm bound by control serum, where BR represents the binding ratio and cpm represents the counts per minute. The titer was considered as the dilution of virus that gave a binding ratio of 2 or more. This value was chosen since it generally represented 10 times the standard error above the control.

Treatment of virus. Trypsin (Difco; 1:250) and soybean trypsin inhibitor (no. T9003, Sigma) were diluted in MEM before use. Various concentrations of virus were incubated at 22°C for 30 min with trypsin (100 μg/ml), trypsin followed by soybean trypsin inhibitor (1 mg/ml), or soybean trypsin inhibitor alone. Controls included untreated virus. After treatment, the virus was allowed to adsorb to confluent monolayers as described above, and the number of virus-infected cells was assayed by immunofluorescence.

Electron microscopy. Electron microscopic detection of rotaviruses has been described previously (Fauvel et al., Intervirology, in press). Briefly, 1 drop of tissue culture or fecal sample fluids was placed on a Formvar-carbon-coated grid situated on a bacteriological agar plate. The liquid phase was allowed to diffuse into the agar before the grid was stained for 30 s with a drop of 2% phosphotungstic acid, pH 7.0. The excess fluid was removed from the grid with absorbent paper, and the grid was examined on a Hitachi HU-12 electron microscope.

RESULTS

Rotavirus infectivity after serial passage and effect of trypsin. Many low-passage isolates of rotavirus do not grow very efficiently in tissue culture, and the virus is often lost if it is passaged by conventional methods. The previous observations that the infectivity of other viruses, including reovirus, can be increased by trypsin treatment prompted us to explore whether trypsin could enhance rotavirus infectivity. Table 1 illustrates that the treatment of rotavirus with trypsin and incorporation of low levels of trypsin into the medium resulted in a significant increase in both the number of infective foci observed by specific fluorescence and the size of each infected focus. Thus, if trypsin was included, there were 52 foci that contained over 20 infected cells per focus, whereas no foci of over 20 infected cells were observed if trypsin was omitted. The results are better depicted in Fig. 1B, where the trypsin-treated cultures showed many large foci of infected cells as well as some individual infected cells. If trypsin was omitted, fewer foci of infected cells were observed, and the degree of spreading was greatly reduced. Similarly, if a solid-phase radioimmunoassay was used to determine the titer of the rotavirus isolate, it was shown that the addition of trypsin increased the infectivity titer approximately 1.5 logs over the untreated cultures (Fig. 2).

In an attempt to determine whether trypsin acted directly on the virus or whether its effect was on the cells, virus was first treated with trypsin and then the trypsin was inactivated by soybean trypsin inhibitor. Figure 1C and Table 1 clearly demonstrate that pretreatment of the virus did significantly increase the number of foci but it did not greatly increase the degree of spread. Soybean trypsin inhibitor itself did not alter the number of infective foci nor the degree of spread (Fig. 1D), suggesting that the increase in infective foci was due to trypsin and not soybean trypsin inhibitor. Similar results were obtained by the radioimmunoassay (Fig. 2).

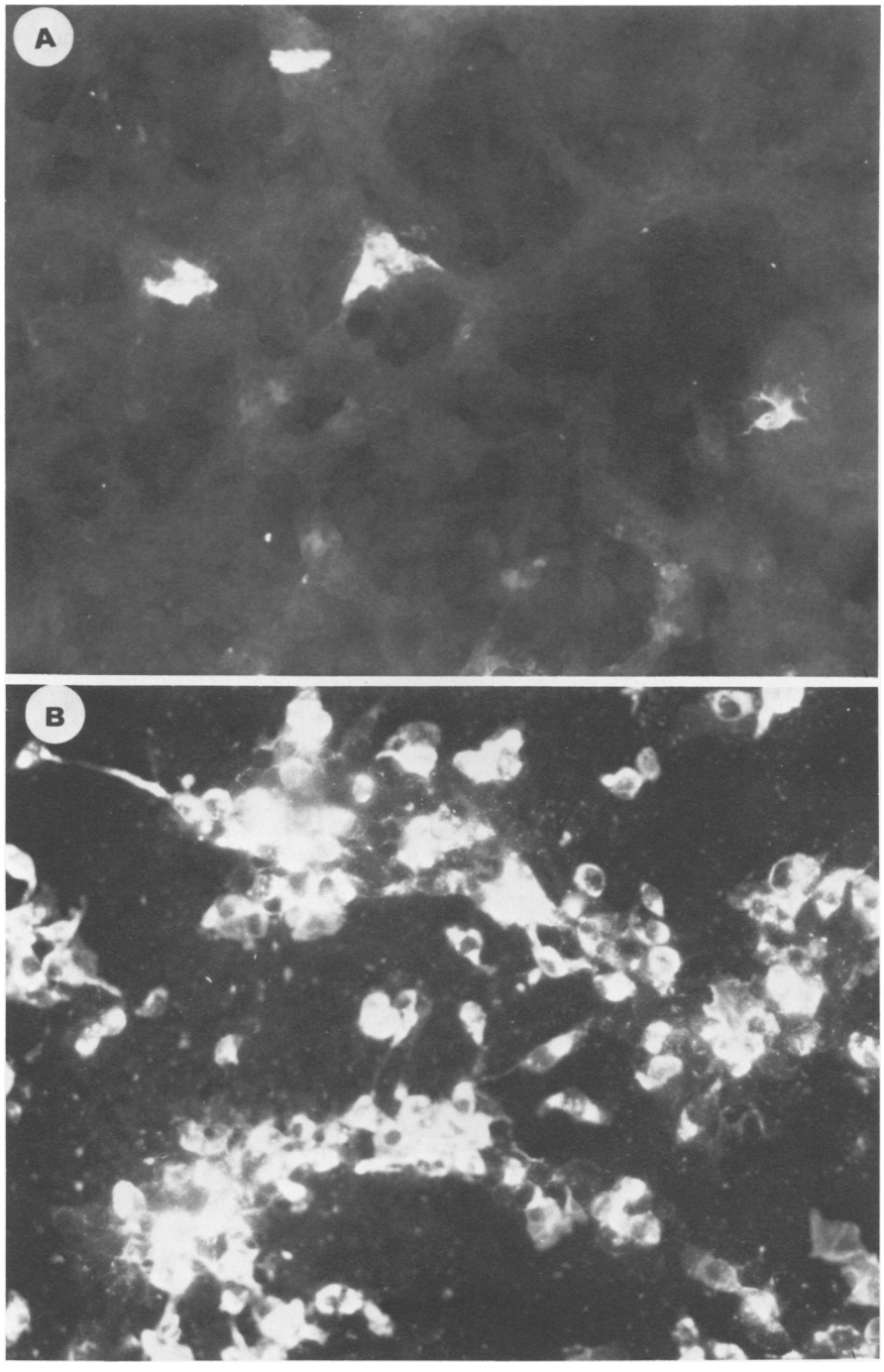

FIG. 1. *Effect of trypsin and soybean trypsin inhibitor on rotavirus replication and spread in vitro. Rotavirus strain C486 was adjusted to 10,000 infectious units/0.1 ml prior to treatment so that a 2-log dilution would yield approximately 100 infectious foci/0.1 ml of inoculum. (A) BSC-1 cells were infected with rotavirus without prior treatment, and trypsin was not added to the medium for virus growth. (B) Virus was treated for 30 min at 22°C with 100 μg of trypsin per ml, diluted, and cultured in MEM containing 10 μg of trypsin per ml. (C) Virus was treated as in (B), but the trypsin was inhibited by the addition of soybean trypsin inhibitor (1 mg/ml), and cultured in the absence of trypsin. (D) Virus treated with soybean trypsin inhibitor alone. All cultures were fixed and stained 48 h postinfection.*

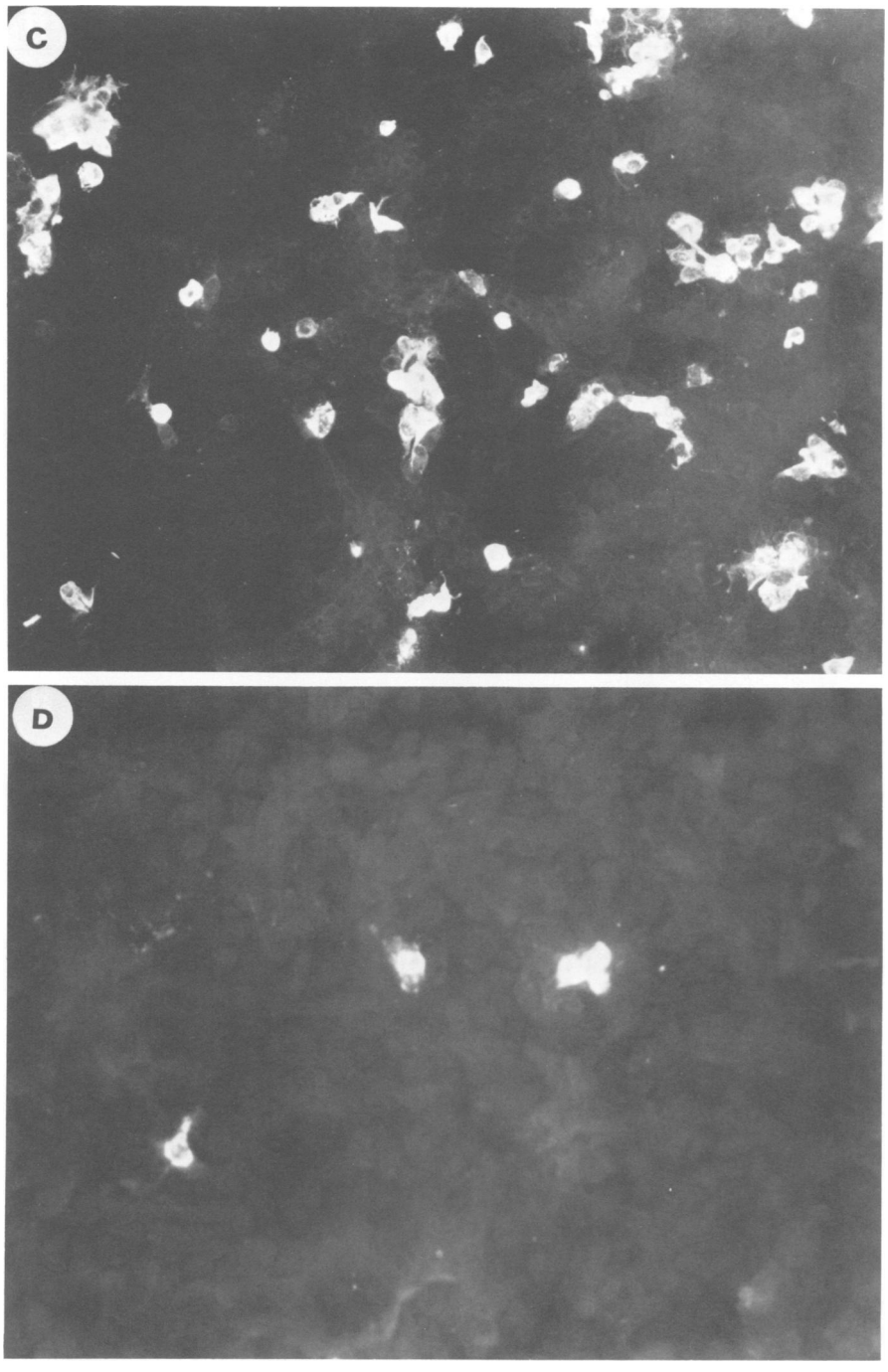

FIG. 1.

Loss of rotavirus infectivity after in vitro passage. To determine the rate at which rotavirus infectivity would decrease following passage in the absence of trypsin, a virus isolate was passaged five times in the presence of 10 μg of trypsin per ml and then passaged in the absence of trypsin. The quantity of infectious virus gradually decreased with passage and was eventually lost after nine passages (Fig. 3). However, if at the sixth passage, a time when low

TABLE 1. *Effect of trypsin on rotavirus replication in vitro*

Treatment	No. of infected foci[a]				
	1 cell/ focus	2-10 cells/ focus	11-20 cells/ focus	20+ cells/ focus	Total
Virus	79	24	4	0	107
Virus + trypsin[b]	972	180	49	52	1,253[c]
Virus + trypsin + inhibitor[d]	206	53	3	0	262[e]
Virus + inhibitor[f]	73	19	6	0	98

[a] Forty-eight hours postinfection cells were fixed and stained, and the number of fluorescent cells were enumerated.

[b] Rotavirus was treated for 30 min at 22°C with 100 μg of trypsin per ml, diluted to a final concentration of approximately 100 infectious units, added to BSC-1 cells, allowed to adsorb for 1 h, removed, and incubated at 37°C for 48 h before fixing, staining, and enumerating.

[c] Significant at 0.01% level from the untreated virus (107) as determined by the *t* test.

[d] As in *b* except prior to addition to BSC-1 cells the trypsin was neutralized with 1 mg of soybean trypsin inhibitor per ml.

[e] Significant at 0.05% level.

[f] Virus treated with 1 mg of soybean trypsin inhibitor per ml prior to infection.

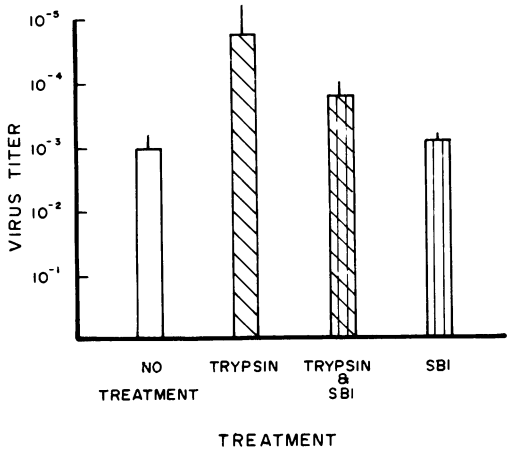

FIG. 2. *Effect of trypsin and soybean inhibitor (SBI) on rotavirus replication as detected by a solid-phase microradioimmunoassay. Virus was treated essentially as in Fig. 1 except that 48 h postinfection the cultures were fixed, reacted for 1 h with bovine antirotavirus serum, washed, and further reacted with ^{125}I-labeled rabbit anti-bovine IgG. The virus titer was calculated as the virus dilution that resulted in binding of radioactivity which was greater than twofold over the background of quadruplicate cultures as described in Materials and Methods.*

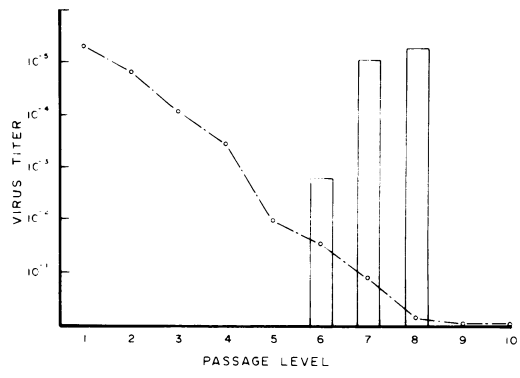

FIG. 3. *Loss of rotavirus infectivity following in vitro passage and restoration of infectivity by trypsin. Decrease in infectivity (O) following passage in the absence of trypsin. Beginning at the sixth passage, as indicated by the histogram, the virus was passaged in the presence of trypsin, and infectivity was restored to the original level after just two passages. The virus titer was determined by the radioimmunoassay as described in the legend to Fig. 2.*

levels of virus were present, the virus was treated and passaged in the presence of trypsin, the virus titer was restored to the original level.

Isolation of rotavirus. The enhancement of rotavirus replication in the presence of trypsin prompted us to determine whether pretreatment of fecal material with trypsin would be an efficient method of isolating virus directly from field samples. Table 2 illustrates that trypsin pretreatment allowed detection of rotavirus in a larger number of fecal samples than did elec-

TABLE 2. *Comparison of fluorescent-antibody, electron microscopy, and virus isolation techniques for detecting bovine rotavirus in feces*

No. of samples[a]	Fluorescent antibody[b]	Electron microscopy	Virus isolation	
			With trypsin	Without trypsin
1-5	5/5	5/5	5/5	1/5
6-12	7/7	0/7	4/7	1/7
Total (1-12)	12/12	5/12	9/12	2/12

[a] Fecal samples were derived from diarrheic calves. All samples were positive by specific rotavirus fluorescence.

[b] Number of fecal samples obtained from diarrheic calves that contained rotavirus immunofluorescent-positive cells in the feces per number of fecal samples tested. Test performed as described in Materials and Methods.

tron microscopy. Thus rotavirus could be isolated from all five samples that were positive by both fluorescence and electron microscopy and from four of seven fecal samples where virus particles could not be detected by electron microscopy but where immunofluorescence cells were present. In no instances was virus isolated from the four normal fecal samples.

DISCUSSION

Only recently has it been demonstrated that rotaviruses are one of the most common causes of nonbacterial gastroenteritis in humans and animals (5, 6, 10, 13, 15, 16, 19, 22, 27). One of the main reasons for the previous lack of association of this virus with neonatal diarrhea was the inability to isolate virus from feces by conventional methods; therefore, diagnosis has relied on counterimmunoelectrophoresis or electron microscopic detection (6, 23) and on fluorescence microscopy once specific antibody was produced against the virus. Although the use of electron microscopic techniques and immunofluorescence has demonstrated that the agent is extremely prevalent, it has been difficult to fully assess the epidemiology, mode of transmission, carrier states, and reservoir hosts, as well as the various immunological events, responsible for recovery from these viruses without being able to isolate and culture the virus. In the present report we acted on the observation that the infectivity of some viruses (2, 11), including reovirus (24, 25), can be enhanced with trypsin treatment. Using this technique, we have been able to isolate virus with relative ease from fecal samples obtained from diarrheic calves to the extent that we could isolate virus from 75% of feces that were positive by immunofluorescence, even though virus particles could be observed by electron microscopy in only 42% of the cases. If trypsin treatment was omitted, then virus replication could be detected in only two cases. In both instances virus-specific antigens could only be detected in a few individual cells and could not be repassaged unless the cells were trypsinized and passaged in the presence of trypsin.

Although the number of fecal samples studied to date is rather limited, the results do suggest that diagnosis of rotavirus by virus isolation, following trypsin treatment, from feces may be more efficient than detection by electron microscopy. An additional advantage of the isolation techniques is that many diagnostic laboratories, which do not have access to electron microscope facilities, could detect and diagnose rotavirus infections. An advantage of virus isolation over fluorescence is that the number of false positives might be reduced; however, a disadvantage is that virus isolation usually takes 48 h, whereas electron microscopy and fluorescence can be done the same day that samples are submitted.

Initially we felt that a possible reason for the inability to isolate rotavirus from fecal material in the absence of trypsin was that low levels of antibody were present in the intestine that would neutralize the virus. Trypsin might remove this antibody, thereby increasing the viral infectivity. This possibility may be partially correct during the initial isolation attempts; however, antibody is probably not responsible for the decrease in infectivity following in vitro passage in cells (fibroblast and epithelial) unable to synthesize antibody. Therefore, the decrease in infectivity following in vitro passage may be due to: (i) the production of a large number of defective particles; (ii) the production and release of some inhibitory substance (interferon) into the medium; or (iii) the presence of some external virus or host-cell component associated with the virus particle which prevents binding of the virus to the host cell or subsequent uncoating and replication once inside the cell. The first possibility was excluded by the observation that trypsin could not only enhance the spread of virus but also increase the number of infectious particles in a virus preparation (Table 1, Fig. 1 and 2). Unless the defective particles were more sensitive to trypsin than were infectious particles, one would not expect trypsin to exert the observed effect. Secondly, if defective interfering particles were responsible for inhibition, one would expect that dilution of the virus would permit a few rounds of replication to occur before the levels of defective particles once again reached such quantities where they would interfere. This was not observed. The second possibility appears feasible especially since other members of the reovirus family have been shown to be sensitive to interferon as well as good inducers of interferon. If rotaviruses could induce high levels of interferon quickly and the virus was susceptible to its action, then it is possible that an infected cell could secrete sufficient levels of interferon to protect contiguous uninfected cells and make them refractory to infection, thus preventing spread and consequently reducing the size of the foci and the yield of virus. Trypsin present in the culture medium could digest the interferon and, thus, allow virus spread and plaque formation (17). Support for this hypothesis comes from the recent findings that rotaviruses do induce interferon and are also susceptible to its action (manuscript in preparation). Secondly, Matsuno et al. (17) have recently demonstrated that rotaviruses could be plaqued if trypsin was included

in the overlay, suggesting that the spread of virus can be enhanced by such treatments.

The previous observation that the outer capsid of reovirus types 1, 2, and 3 is not essential for infectivity and in some instances may reduce infectivity (24, 25) suggested that a similar phenomenon might occur in the rotaviruses. At present this possibility appears remote since "decapsidated" rotavirus particles do not appear to be infectious (7; Woode, personal communications). However, to totally dismiss this possibility, various methods of decapsidation must be assessed and correlated with enhanced infectivity as well as with other biological functions. These possibilities are presently under investigation.

Although we have not fully elucidated the mechanism whereby trypsin enhances the infectivity of bovine rotaviruses, the present technique does make it possible to isolate and culture bovine rotaviruses with ease. We are presently using similar procedures in an attempt to isolate and culture rotaviruses from other species including humans, so that we can compare various rotaviruses with respect to their biological and antigenic properties and the host's immune response to these agents, so that prevention and control measures could be implemented.

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