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Enhancement of Antigen Incorporation and Infectivity of Cell Cultures by Human Rotavirus

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Infection of cell cultures with human rotavirus preparations was attempted, and the effects of trypsin and low-speed centrifugation on antigen incorporation, as demonstrated by immunofluorescence and radioimmunoassay, were determined. In addition, the effect of viral aggregation on antigen incorporation was investigated by filtering viral preparations. Four strains of human rotavirus were employed, and the results were compared to those obtained with two tissue culture-adapted animal rotaviruses. Centrifugation and trypsin appeared to have little or no effect on infectivity of the tissue culture-adapted (simian rotavirus) or -adaptable (Nebraska calf diarrhea virus) strains, whereas centrifugation and viral aggregation appeared to be essential for the human viruses. In addition, trypsin enhanced antigen incorporation of the human strains to some extent. Infectivity for cell cultures and in vitro human rotavirus protein formation was demonstrated by [^{35}S]methionine incorporation, and the specificity of this human viral protein was established by radio-immunoprecipitation.

Rotaviruses have been established as a major etiological agent of diarrheal disease in the young of many animals and in humans (16). Serologically distinct viruses have been obtained from a variety of animals; however, only six strains are readily cultivable in cell culture (16). The relative ease of propagation of one of these strains, the Nebraska calf diarrhea virus (NCDV), has enabled a vaccine to be developed for use in immunoprophylaxis of calf rotavirus diarrhea (9).

The first reported successful infection of cell cultures with human rotavirus involved the use of low-speed centrifugation of virus-containing stool suspensions onto monolayers and the demonstration of specific intracytoplasmic immunofluorescence (IF) (3). The presence of rotavirus antigen in the cytoplasm of different cell cultures infected with human rotavirus without the use of centrifugation also has been described (1, 11, 13, 16), including successful carriage of specific IF through 6 (1) to 24 (16) passages. The technique of centrifugation and IF has been utilized for the diagnosis of human rotavirus infection (4), and blocking of fluorescence has been used to define serotypes of human rotavirus and to distinguish between human and animal strains as well (15). In addition to centrifugation, trypsin has also been shown to enhance rotavirus infectivity for cell cultures of calf and pig rotaviruses (2, 8, 14).

In this report we describe the effects of centrifugation and trypsin on intracytoplasmic human rotaviral antigen incorporation in cell culture and their effect on infectivity as demonstrated by labeled-methionine uptake.

MATERIALS AND METHODS

Viruses. Four human rotavirus strains and a strain of NCDV were derived from diarrheal stools of experimentally infected gnotobiotic calves (10). The stools were prepared as 20% (wt/vol) suspensions in phosphate-buffered saline (PBS; pH 7.4), homogenized with glass beads, and clarified by centrifugation at 3,200 rpm for 20 min in a Sorvall RC-3 centrifuge. Simian rotavirus (SA 11) was derived from tissue culture fluid harvested 1 week after infection of African green monkey kidney cell cultures.

Cells. Cells from an African green monkey kidney cell line, LLC-MK$_2$, grown in six-well plastic trays (Linbro) were obtained from a commercial source (Flow Laboratories Ltd., Rockville, Md.).

Infection of cells. When confluent, the growth medium was removed, the plates were washed three times with PBS, and 1 ml of the appropriate stool suspension, diluted 1:4 in Eagle minimal essential medium (MEM) or SA 11 tissue culture fluid, was inoculated per well. After centrifugation in an International Equipment Co. (IEC) centrifuge at 2,600 rpm for 1 h at 37°C, the inoculum was removed and replaced with fresh MEM. The plates were incubated at

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37°C in an atmosphere of 5% CO_2. Incorporation of antigen was determined by immunofluorescence (IF) or radioimmunoassay 96 h after inoculation unless otherwise noted.

IF. The monolayers were washed three times with PBS, and the cells were then scraped off the bottom of the plates with a glass pipette. Drops of the cell suspensions were placed into holes in Teflon-coated slides (Cooke). The slides were air dried, fixed in cold acetone, and stained with hyperimmune goat anti-human rotavirus globulin conjugated with fluorescein isothiocyanate. This serum was left on the slides for 1 h at 37°C and then washed off with PBS. The slides were mounted in buffered glycerol (pH 8.0), and the cells were examined for fluorescence. For quantitation, 300 cells were counted in each hole, and the percentage of positive cells was calculated.

RIA. Radioimmunoassay (RIA) was performed as described previously by Kalica et al. (5) and was used to detect antigen in supernatant fluid, cell lysates, and cell culture preparations. Cell lysates were prepared by removing the supernatant fluid, washing the cells three times with PBS, and adding 0.5 ml of 0.5% Tergitol P-40 (Sigma Chemical Co., St. Louis, Mo.) per well to lyse the cells. The cell culture preparations were obtained by three cycles of freeze-thawing of the cells with their supernatant fluids.

Trypsin treatment. To determine its effect on antigen incorporation, trypsin (1:250; Difco Laboratories, Detroit, Mich.) was added to the inoculum in a final concentration of 10 μg/ml of MEM. After centrifugation, the inoculum was removed and replaced with trypsin-free MEM. In addition, one of the human strains, 76-51, was also preincubated with 10 μg of trypsin per ml 1 h or 24 h before inoculation. Immediately before inoculation, virus was washed free of trypsin with 40 volumes of PBS, using an Amicon Diaflo ultrafilter XM 100A. The virus was then inoculated onto the cell cultures in trypsin-free medium as described above.

Trypsin pretreatment of cells. The cell cultures were preincubated for 1 h before incubation with 15 μg of trypsin per ml. Immediately before inoculation with virus in trypsin-free medium, the cells were washed three times with PBS.

Variation of centrifugation time. Three of the human rotaviruses and NCDV were inoculated onto the cell cultures at a dilution of 1:4 in MEM, to which trypsin was added to a final concentration of 10 μg/ml. The plates were then either left uncentrifuged or were centrifuged for various periods of time from 5 to 60 min. In all cases, the inoculum remained on the cells for 60 min and was replaced with fresh trypsin-free MEM.

Filtration of virus. The effect of aggregation of virus particles on antigen incorporation was investigated for the human and animal strains. Virus preparations diluted 1:4 in MEM supplemented with 10 μg of trypsin per ml were filtered through a Swinnex no. 25, 450-nm filter which had been pretreated with 1% bovine serum albumin in PBS. RIA was performed on specimens before and after filtration to determine any loss of virus by adhesion to the filter membrane. After filtration, the preparations were inoculated onto tissue culture, and antigen incorporation was determined as described above.

Labeled-methionine incorporation. Viral protein synthesis after antigen incorporation was determined by measuring [^{35}S]methionine uptake. Human rotavirus strains 76-51, 76-52, and 77-26 were inoculated at a 1:4 dilution in MEM containing 10 μg of trypsin per ml onto LLC-MK_2 cells, as described above. After centrifugation, the inocula were removed and replaced with 2 ml (per well) of methionine-free medium to which 0.25 μg of actinomycin D (Sigma) per ml was added. To each well, 20 μCi of [^{35}S]methionine (Amersham/Searle, Arlington Heights, Ill.) was added, and the cells were then incubated for 96 h. After three cycles of freeze-thawing, viral particulates were extracted with an equal volume of trichlorotrifluoroethane (Genetron 113, Allied Chemical Corp.). Viral particulates in the extract were pelleted by centrifugation at 25,000 rpm for 60 min in a Beckman SW40 rotor, and the pellet was suspended in 2.0 ml of 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0). The suspended pellet was layered onto a preformed 40 to 55% (wt/vol) cesium chloride density gradient and centrifuged for 18 h at 35,000 rpm in a Beckman SW40 rotor. Fractions of 0.5 ml were collected, [^{35}S]methionine uptake was measured in a Beckman liquid scintillation counter, and antigenicity was determined by RIA as described above. An uninfected cell control was treated in an identical manner, with saline being substituted for the stool preparations.

Radio-immunoprecipitation. Fractions showing maximum uptake were diluted to yield a count of 2,000 cpm in a 5-μl sample. The preparations were centrifuged at 10,000 rpm for 2 min in an Eppendorf Brinkmann microcentrifuge. The supernatant fluid was removed and mixed with an equal volume of a 1:200 dilution of preinoculation or convalescent serum from a chimpanzee infected with human rotavirus. After being held overnight at 4°C, 100 μl of goat anti-human globulin (Antibodies Inc., Davis, Calif.) was added to each sample to amplify specific precipitation. The tubes were incubated at 37°C for 6 h, then centrifuged at 10,000 rpm for 2 min in the microcentrifuge. The fluid in the upper and lower halves of the tubes was counted separately in the liquid scintillation counter, and the percentage of the precipitable counts was calculated (Table 5).

RESULTS

Effect of trypsin on infectivity. Addition of trypsin to the inoculum prior to centrifugation slightly enhanced infectivity, as determined by the percentage of IF cells, of all the human strains but not NCDV or SA 11 virus (Table 1). However, the degree of antigen incorporation, as determined by the highest dilution of cell lysate positive by RIA, was not significantly different for the trypsin-treated or untreated human rotavirus specimens (Table 1).

Preincubation of the virus with trypsin, followed by washing (prior to centrifugation), resulted in the same degree of enhancement of antigen incorporation as under conventional

conditions in which trypsin was incorporated into the inoculum (Table 2). Preincubation of the cells with trypsin, however, produced no enhancement of antigen incorporation.

Effect of duration of centrifugation on infectivity. In the absence of centrifugation, IF-detectable antigen incorporation was not detected with the human strains and was somewhat less with NCDV (Table 3). The degree of antigen incorporation generally increased with centrifugation time, however. For each of the human strains, a maximum was reached at 40 min after which no increase was observed.

Antigen incorporation was not detected by RIA in lysed-cell preparations inoculated with human virus that was not centrifuged onto the cell sheet. However, lysates were positive when centrifugation was performed for 5 min, became more positive at 10 min, and then remained essentially unchanged from 10 to 60 min. With NCDV, centrifugation had no appreciable effect upon the degree of antigen incorporation as measured by RIA. On the other hand, centrifugation for up to 30 min produced increased NCDV antigen uptake as measured by IF, and centrifugation for 40 min resulted in a further increase in IF NCDV antigen uptake.

Effect of viral aggregation. Prior to centrifugation, filtration through a 450-nm filter resulted in a dramatic reduction in antigen incorporation of the human strains, usually to levels undetectable by IF (Table 4). In contrast, SA 11 showed no change, and NCDV only a slight reduction in IF-detectable antigen incorporation after sonication and filtration. The reduction of infectivity did not appear to be due to adherence of particles to the filter, as indicated by the failure of filtration to diminish the quantity of antigen in the filtrate as measured by RIA.

Labeled-methionine incorporation. Analysis of fractions from a cesium chloride gradient for [^{35}S]methionine incorporation showed a monophasic or biphasic peak with all three strains of human rotavirus at densities varying from 1.21 to 1.26 g/cm^3 (Fig. 1). RIA of the fractions showed a peak at a density higher than that of the [^{35}S]methionine peak, as well as two further peaks at densities of 1.39 and 1.30 g/cm^3 where [^{35}S]methionine uptake was not detected. The latter two peaks probably represent input virus. The [^{35}S]methionine uptake peak was analyzed further by radio-immunoprecipitation.

Radio-immunoprecipitation. Antigenic specificity of the [^{35}S]methionine incorporation peaks was indicated by the percentage counts precipitated by the convalescent chimpanzee serum (Table 5).

TABLE 1. *Effect of trypsin on rotavirus infection*

Virus	% cells positive by IF		Titer (reciprocal) of cell lysate by RIA	
	Trypsin	No trypsin	Trypsin	No trypsin
Human strain				
76-51	87	48	320	320
76-52	15	—[a]	160	160
76-56	56	35	160	160
76-69	29	15	320	160
NCDV	80	75	ND[b]	ND
SA 11	59	58	ND	ND

[a] —, Occasional cell positive.
[b] ND, Not done.

TABLE 2. *Effect of trypsin pretreatment of virus and cells on antigen incorporation by IF*[a]

Treatment	Cells positive by IF (%)
1 h preincubation of virus	50
24 h preincubation of virus	63
1 h preincubation of cells	11
Non-pretreated control[b]	60

[a] Virus consisted of a 1:16 dilution of 20% (wt/vol) stool suspension of human rotavirus 76-51.
[b] Control consisted of a 1:16 dilution of stool suspension inoculated in MEM containing 10 μg of trypsin per ml, as described in the text.

TABLE 3. *Effect of varying duration of centrifugation on antigen incorporation*

Virus	IF and RIA values after centrifugation for:[a]													
	Uncentrifuged		5 min		10 min		20 min		30 min		40 min		60 min	
	IF	RIA	IF	RIA	IF	RIA	IF	RIA	IF	RIA	IF	RIA	IF	RIA
Human strain														
76-51	0	3	13	18	17	40	15	33	12	46	36	52	33	38
76-54	0	2	5	15	8	26	5	27	7	24	18	34	16	34
76-69	0	2	5	9	8	26	20	30	19	28	37	33	37	50
NCDV	8	54	20	45	20	51	22	43	12	49	34	36	40	34

[a] Centrifugation was at 2,600 rpm in an IEC centrifuge. IF values indicate percentage of cells positive by IF technique; RIA values are the ratio (P/N) of the counts per minute in virus-treated cells (P) and the counts per minute in untreated cells (N).

TABLE 4. *Effect of filtration on antigen incorporation*

Virus	Cells positive by IF (%)	
	Untreated	Filtered
Human strain		
76-51	87	8
76-52	15	Neg[a]
76-54	54	Neg
76-69	29	Neg
Bovine NCDV	80	50
Simian SA-11	59	57

[a] Neg, No immunofluorescent cells seen.

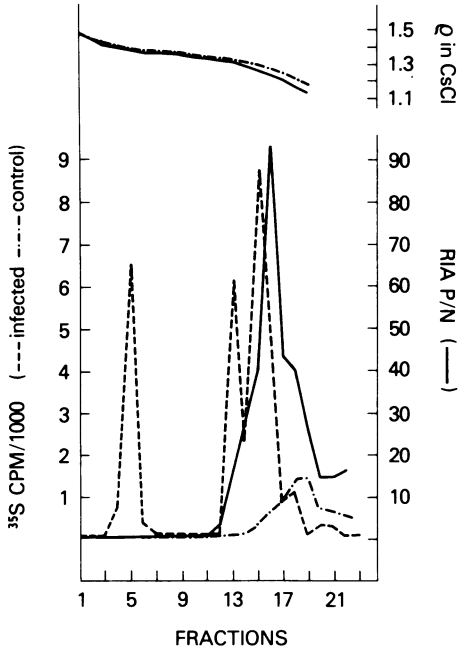

FIG. 1. *Uptake of labeled methionine in cell cultures infected with human rotavirus 76-52 and uninfected control cells. Radioactive uptake was monitored in 10-μl samples from each fraction, and antigenicity of each fraction was monitored by RIA. Peak [^{35}S]methionine uptake in infected cell preparation was at a density of 1.25 g/cm^3 and in the uninfected cell control was at 1.18 g/cm^3. RIA peaks were at densities of 1.39, 1.31, and 1.26 g/cm^3.*

DISCUSSION

In the present report, three factors were found to influence the degree of human rotaviruses antigen incorporation into cell cultures. Firstly, low-speed centrifugation of virus-containing material onto the cell culture monolayers proved to be essential for demonstrable antigen incorporation to occur. As little as 5 min of centrifugation resulted in some detectable antigen in the cells; however, an optimum was reached after 40 min for IF-detectable antigen. The enhancing effect of centrifugation may be due to concentration of heavy viral aggregates onto the cell surface. By duplicating these conditions of centrifugation, i.e., 2,600 rpm for 60 min, from 70 to 93% of the counts present in the [^{35}S]methionine incorporation peaks could be precipitated in V-bottom microtiter plates (B. D. Schoub et al., unpublished data).

TABLE 5. *Radio-immunoprecipitation of fractions of human rotavirus showing maximum [^{35}S]-methionine uptake*

Strain	Fraction no.	Buoyant density (g/cm^3)	[^{35}S]methionine precipitated by chimpanzee sera (%)[a]	
			Preimmune	Convalescent
76-51	15	1.27	−2	18
76-52	15	1.25	2	17
77-26	17	1.23	3	13

[a] Values are expressed as the percentage of the counts in the lower half of the tube minus 50.

The second enhancing factor was found to be trypsin. The effect of trypsin was on the virus rather than on the cell; pretreatment of the virus before inoculation enhanced antigen incorporation whereas pretreatment of the cells did not. This agrees with the findings of Babiuk et al. (2) with calf rotavirus. Exposure to trypsin prior to centrifugation did not appear to be essential for antigen incorporation. In the absence of trypsin, some intracellular IF antigen was produced, and the amount of RIA-detectable antigen produced did not appear to be influenced by trypsin treatment. Whether trypsin enhancement is the result of inhibition of trypsin-sensitive viral inhibitors such as the variola virus inhibitor (7) or is due to cleavage of viral-coded precursor proteins into functional proteins as with enteroviruses and Sindbis virus (12) remains to be elucidated.

The third factor found to be of importance for efficient antigen incorporation of human rotaviruses was the aggregation of virus particles. Virus material in a form small enough to pass through a 450-nm filter failed to be incorporated into the cells to any demonstrable degree. The importance of viral aggregation may be the result of the formation of material heavy enough to be concentrated onto the cell surface by low-speed centrifugation. Infection may take place only if a sufficiently high concentration of virus is present on the cell surface, as occurs when heavy viral aggregates are centrifuged onto the cell membrane.

The cell culture-adapted SA 11 and cell cul-

ture-adaptable NCDV viruses differed from the human rotaviruses in that the three enhancing factors had little or no effect on infectivity. The pattern of pathogenesis of infection, however, also appears to be quite different, as evident by growth curve studies and cytopathology as viewed by IF at various stages of infection (B. D. Schoub et al., manuscript in preparation).

In vitro synthesis of human rotavirus protein in infected cells was demonstrated by [^{35}S]methionine incorporation under conditions of centrifugation and addition of trypsin. This synthesized viral protein banded in cesium chloride at densities between 1.23 and 1.26 g/cm^3. This protein was demonstrated to be specific by precipitation of the counts by convalescent chimpanzee antirotavirus serum, although the latter had a fairly low antirotavirus titer. The density of this material is even lower than that of empty rotavirus particles which band at 1.29 g/cm^3 (6), and its composition remains to be determined.

We have thus demonstrated that some transcription and translation occur when human rotavirus infects cell cultures under conditions of centrifugation and exposure to trypsin.

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