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REOVIRUS GROWTH INHIBITION BY ROTAVIRUS FOLLOWING COINFECTION

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Abstract

Interference between the two members of reoviridae family, which multiply inside the cytoplasm of the host cell, was investigated. Monkey kidney cells (BSC-1) were coinfected with reo and rota viruses and the amount of virus produced was determined by TCID50 and Fluorescent Focusing Assay (FFA). Upon coinfection the growth of reovirus was reduced considerably in presence of rotavirus. Electron microscope examination of viruses extracted from mixed infected cells revealed that only rotavirus particles were present. This observation was confirmed by examining thin section of coinfected cells in which only cytoplasmic rotavirus like particles were visualized. Interference between rotavirus and reovirus was not at the level of adsorption and receptor attachment competition. Analysis of viral RNA extracted from the coinfected cells revealed that RNA genomes of both viruses were synthesized but after the first passage only rotavirus RNA was present in the infected cells. Similarly viral proteins of both viruses were produced in coinfected cells as was shown by polyacrylamide gel electrophoresis, followed by immunoblotting and by the immunofluorescence staining. Since there was no inhibition of viral macromolecular synthesis in mixed infected cells it was concluded that inhibition of reovirus maturation in presence of rotavirus was probably at the level of virus assembly.

Keywords: Interference; Rotavirus; Reovirus; Coinfection

Introduction

The phenomenon of viral interference or the inhibition of virus growth by another virus has been well known for several viruses and the mechanism of such phenomenon has been partly described [1].

Interference has been described in certain systems such as: serial passage of viruses at high moi, resulting in accumulation of defective interfering viruses [2-4], mixed infection of wild type viruses with certain temperature-sensitive (ts) mutant [5-9], coinfection of cells with different wild type virus isolates [10-13] and

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coinfection of cells with different genus of viruses belonging to the same or different family of viruses. This kind of interference has significant application value in certain systems. An example of such interference is the effect of enterovirus infection with live-attenuated poliovirus vaccine, which, slows down replication of vaccine virus inside the cells, resulting in inefficiency of vaccine-induced protection [14-16]. The other example is the effect of reovirus on environmental detection of enteroviruses [17].

Interference between different reovirus strains would cause one of the strains to reduce the yield of progeny RNA from the other parental strains upon coinfection [11]. In another study it was shown that bovine-human reassortant strain containing ten human rotavirus gene segments and segment 4, encoding VP4, of bovine rotavirus were isolated from an infected infant during cell culture adaptation and this reassortant virus suppressed replication of other rotaviruses in coinfected cells [18]. In a different system, combination of hepatitis A and B vaccine has been shown that the hepatitis B portion of the vaccine did not produce clinically acceptable antibody due to immunologic interference [19,20].

In this study we have examined coinfection of cells with reovirus and rotavirus. Both of these viruses belong to the family of reoviridae, containing double-stranded segmented RNA (ds RNA) enclosed in a double protein shell [14,21].

The genomes of reovirus and rotavirus consist of 10 and 11 segments of ds RNA respectively [14]. These viruses can coexist in alimentary tract and their interference might have some impact on the outcome of their infection. We report the result of experiments obtained from intracellular coinfection of cells with reovirus and rotavirus.

Materials and Methods

Cells and Viruses

Mouse L929 cells were used for reovirus propagation. Stock culture of L929 cells were grown in monolayers in Joklik modified Minimal Essential Medium SMEM (GIBCO BRL) supplemented with 5% Fetal Bovine Serum (FBS, GIBCO BRL). BSC-1 cells were grown in Dulbecco's Minimal Essential Medium D-MEM (GIBCO BRL) containing 8% (vol/vol) FBS and used for growth and propagation of rotavirus. Since these cells (BSC-1 cells) were susceptible to both rota and reovirus they were used for coinfection experiments during this study. All cells were grown at 37°C in an atmosphere of 5% CO_2.

Reovirus type 3 (T3D) stocks were propagated in L929 cells and assayed by fluorescent focusing assay (FFA) and TCID50. Simian rotavirus (SA11) was propagated in BSC-1 cells in the absence of FBS and in presence of 3µg trypsin/ml and titrated by FFA and TCID50 methods.

Viral Coinfection

BSC-1 cells were used for coinfection because they support the growth of both reo and rotaviruses. Monolayers of cells were coinfected with equal volume of reovirus and trypsin treated rotavirus at moi of 50. Infection was carried out in the absence of FBS for 1 hr. For control, monolayers of cells were similarly infected separately with reovirus and rotavirus. All the infected cells were incubated in DMEM without calf serum at 37ºC in an atmosphere of 5% CO_2. After 24-48 h. infected cultures were harvested and freezed-thawed 3 times. After brief centrifugation the supernatant was saved for viral assay.

Virus Titration

The titer of viruses was determined by the methods of TCID50 and FFA. For TCID50 cells were grown in 24-wells tissue culture dishes and the test was performed according to the Reid & Muntch method.

For Fluorescent Focusing Assay, monolayers of BSC-1 cells grown on round coverslips in 24-wells dishes were infected with serial dilutions of viruses. Sixteen hour post-infection cells were fixed and stained indirectly using the fluorescent staining technique. The cells on the entire coverslip were scanned and the stained cells were counted.

Virus Purification

For animal immunization purified viruses were used. Rotavirus (SA11) was purified on sucrose cushion followed by CsCl density gradient centrifugation as previously described by Shahrabadi [22]. Reovirus was extracted from the infected cells using Freon extraction, followed by CsCl density gradient centrifugation according to the method of Furlong *et al.* [23].

Preparation of Specific Antisera

Reovirus and rotavirus bandings in purified forms were mixed with Freund's adjuvant and used to inoculate female New Zealand white rabbits. Three injections were given at weekly intervals and the last booster was given three weeks later. Sera were collected 10 days after the last injection.

Immunofluorescent Staining

Cells were grown in monolayer on glass coverslips in 24-wells tissue culture dish. Cells were infected either by reo and rota viruses separately or coinfected by a mixture of the two viruses. 16 hr post infection, coverslips were removed, washed in PBS and fixed in acetone at 4°C. They were stained indirectly with fluorescent conjugated anti-rabbit IgG using specific viral antisera.

Receptor Binding Competition

S_{35} labeled reovirus was prepared by exposing the infected cells to methionin free medium containing 50µc/ml of S_{35} methionin at hour 8 postinfection. The virus-infected cells were harvested at hour 24 Virus was extracted and purified by CsCl density gradient centrifugation as described.

Monolayers of BSC-1 cells grown in 35 mm plastic dishes were washed twice with 3 ml of MEM. A volume of 0.25 ml of rotavirus with moi of 200 FFU/cell was added to the monolayers. Cells were kept at 4°C for 1 h then were washed 3 times with cold PBS. A volume of 0.25 ml of S_{35} labeled reovirus with moi of 200 FFU/cell was added and incubated at 4°C for an additional hour. The unadsorbed virus was removed and the cells were washed as above then lysed with 0.5% of Triton X100. Control cells without addition of rotavirus were similarly exposed to labeled reovirus and were prepared similarly.

In another experiment rotavirus was mixed with S_{35} labeled reovirus with equal moi and were added to the monolayers. The control cells were also exposed to labeled reovirus alone and allowed to adsorb at 4°C for 1 h then were processed as above. Samples were counted in a liquid scintillation counter.

Analysis of Viral Proteins by SDS Polyacrylamide Gel Electrophoresis

Cells were coinfected with reovirus and rotavirus as described above. Infected cells were harvested 16 hr. postinfection. The cells were scraped off the bottles and centrifuged for 10 min at low speed. The pellet was lysed by addition of equal volume of lysis mix and boiled for 5 min. Samples were electophoresed on 10% polyacrylamide gel according to the method of Lammeli [24]. Purified viruses were mixed with equal volume of lysis mix buffer, boiled for 5 min and similarly applied to the gel. After completion of electrophoresis the gels were fixed and stained with Commassie blue.

For immunoblotting the protein bands in acrylamide gel were blotted onto nitrocellulose paper using a dry blotter at 30 mA for 1 h The blotted proteins were treated with specific antisera to either rota or reovirus and stained with anti-rabbit peroxidase conjugate using TMB as substrate.

Analysis of Viral RNA by Electrophoresis

Sixteen hour infected cells were pelleted by low speed centrifugation. Cells were lysed by treatment with SDS, and proteinase K and then RNA was extracted by phenol chlorform according to the standard method. Extracted RNA was electrophoresed on 10% acrylamide gel at 10 mA overnight. The RNA bands were stained by ethidium bromide and visualized by UV light. Purified viruses were briefly heated at 90°C in lysis mix and electrophoresed as above.

Electron Microscopy

Cells in monlayers were infected with viruses as described above. At various times after infection they were fixed in 3% glutaraldehyde at 4°C for 3 hr. then post fixed in 1% osmium tetroxide. After dehydration in serial dilutions of ethanol, they were embedded in araldite. Sections were cut, stained with uranyl acetate followed by lead citrate and examined in a Zeiss EM 10 electron microscope. Negative staining of virus particles was performed using purified virus and 1% PTA as described by Shahrabadi [25].

Results

Virus Yield in Coinfected Cells by Reo and Rota Viruses

Cells were infected simultaneously with reovirus and rotavirus as described. Infected cells were incubated in serum free medium. Control infected cells which were inoculated separately with the same moi by each of the two viruses were incubated similarly. After 48 h cultures were harvested and the amount of virus yield was determined by the methods described in materials and methods. As it is shown in Figure 1a, after the first passage there was a decrease in reovirus production of about 4 logs as assayed by TCID50 method. Similar results were obtained by FFA method, which demonstrated a marked reduction in reovirus titer (Fig. 1b).

When the viruses obtained in coinfected cells were used to infect the cells (first passage), the reovirus yield was diminished whereas rotavirus titer remained unchanged. In infected cells inoculated only by individual viruses, there was no decrease in virus titer in serial passages.

Determination of Virus Yield by Ultracentrifugation

The viruses produced in coinfected cells were used as mixed viral seed to infect BSC-1 cells as the first passage. The infected cells were harvested and virus was extracted and purified as described. The viral bands were taken from the tube and after dialysis were examined by electron microscope using negative staining technique. In Figure 2a the particles obtained from the upper band were typical double-shelled rotaviruses and those obtained from the lower band were the single-shelled rotaviruses (Fig. 2b). There was no visible reovirus particles observed by electron microscope. The results showed that no reovirus was produced in coinfected cells after the fist passage and rotavirus was the dominant virus in mixed infection.

Cell Attachment Competition between Reovirus and Rotavirus

In order to determine if the inhibitory effect of rotavirus on reovirus multiplication upon coinfection was due to cell attachment competition, cells were exposed to rotavirus and S_{35} labeled reovirus. The amount of labeled virus attached to the cells in presence and absence of rotavirus was determined. As it is shown in Table 1 there was no significant difference in adsorption of reovirus in presence and absence of rotavirus. When rotavirus was allowed to adsorb first to the cells for 1 h then labeled reovirus was added, again no difference in attachment was noticed (Table 1).

The results indicated that upon coinfection both viruses were able to adsorb to the cells and their attachment to receptors was not affected in mixed infection.

Viral Macromolecular Synthesis in Coinfected Cells

To determine the extent of viral RNA and viral protein synthesis, RNA from coinfected cells was extracted and analyzed on 10% polyacrylamide gel. As it is shown in Figure 3, coinfected cells contained all the RNA genome segments of the both reovirus and rotavirus (Fig. 3 Lane 3). However, after the first passage, 11 segments of rotavirus genome were present and there was no apparent RNA band of reovirus (Lane 4, Fig. 3).

Viral proteins of both reovirus and rotavirus were synthesized and accumulated in coinfected cells. The results in Figure 4 show percentage of coinfected cells in which viral proteins were stained with fluorescin conjugated antibodies using specific antisera for both viruses. Reovirus antigens appeared as intensely stained bodies inside the cytoplasm (Fig. 5a) and rotavirus antigens were homogenously distributed inside the cytoplasm (Fig. 5b). About 85% of coinfected cells contained both reo and rota virus proteins, whereas after the first passage, cells containing reovirus proteins was decreased to less than 15% (Fig. 4).

Proteins of coinfected cells were also analyzed by polyacrylamide gel electrophoresis followed by immunoblotting. The results showed that the coinfected cells contained both reo and rota virus antigens and in subsequent passages the only detectable viral protein bonds belonged to rotavirus (Fig. 6).

Electron Microscopy of Coinfected Cells

In order to determine the morphological properties of intracellular viral particles produced upon co-infection, cells were fixed at various times after infection and prepared for electron microscopy. At 12 hr. post infection the only morphological changes, which could be observed in coinfected cells, were presence of granular dark staining viroplasm, which is characteristic of rotavirus intracellular multiplication (Fig. 7a). Some virus particles resembling rotavirus were also in peripheral area of the viroplasm. At the late stage of infection (16 h) many rotavirus particles appeared inside the cytoplasm, some of which were in process of budding from the endoplasmic reticulum (Fig. 7b). Sections of cells infected with only reovirus contained intra-cytoplasmic inclusions in which reovirus particles were present (Fig. 8). There were no such particles and inclusions observed in mixed infected cells.

Table 1. Monolayers of BSC-1 cells were exposed either to S_{35} labeled reovirus alone or a mixture of labeled reovirus and rotavirus, and processed as described in Material and Methods.

Sample	CPM
Reovirus	28900
Reovirus+Rotavirus	27300
Rotavirus+Reovirus*	27800

* Cells were first exposed to rotavirus for 1 h at 4°C. They were washed and superinfected with reovirus. The amount of radioactive labeled virus attached to the cells was determined.

Discussion

Interference between viruses belonging to the same family or between members of unrelated ones upon coinfection of cells has been reported [11,26].

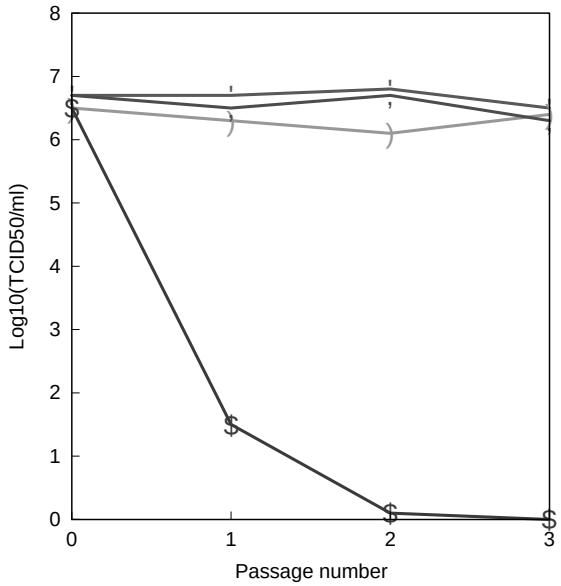

Figure 1a. Virus yield in coinfected cells. Cells were coinfected by reo and rota viruses as described in methods. After 48 h the amount of viruses produced was determined by the TCID50 method. ● Rotavirus in coinfected cells, □ reovirus in coinfected cells, ▲ control rotavirus, ■ control reovirus.

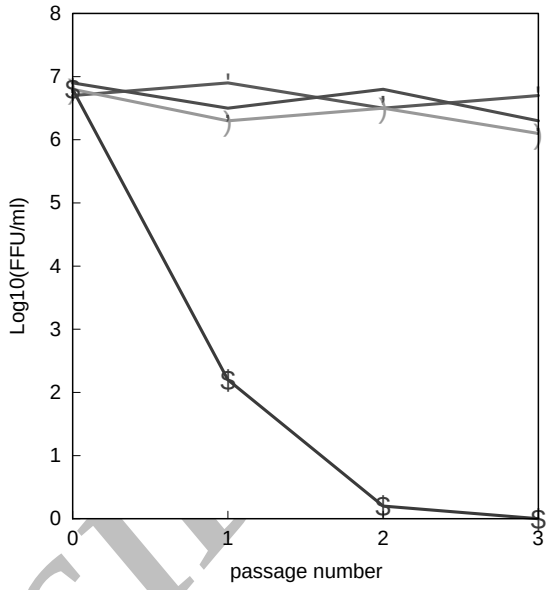

Figure 1b. Virus yield in coinfected cells as in Figure 1a and assayed by the fluorescent focusing assay. ● Rotavirus in coinfected cells, □ reovirus in coinfected cells, ▲ control rotavirus and ■ control reovirus.

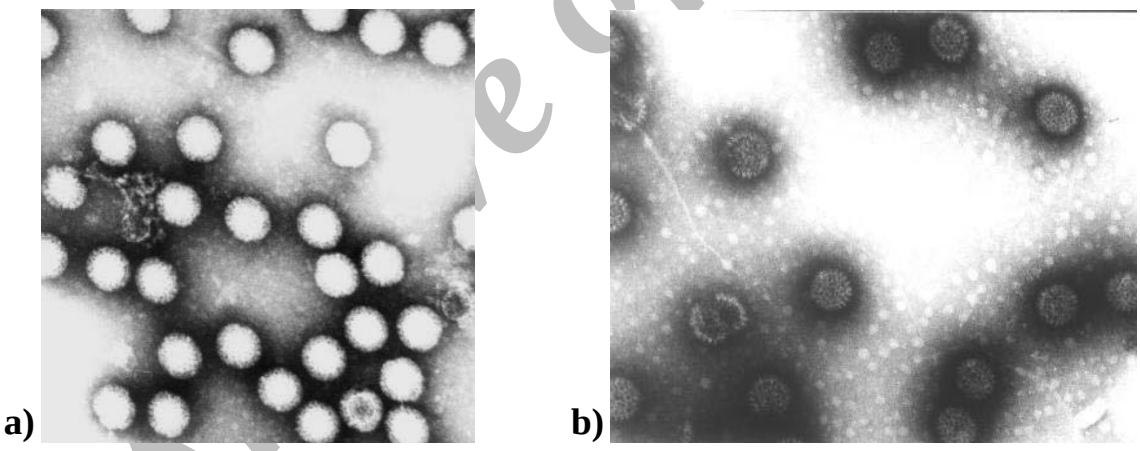

Figure 2. Electron microscopy of purified virus from coinfected cells, prepared by the negative staining technique. Virus particles from the upper band of the tube are complete double shelled rotavirus (a). The viruses from the lower band are single shelled rotavirus (b) (160000).

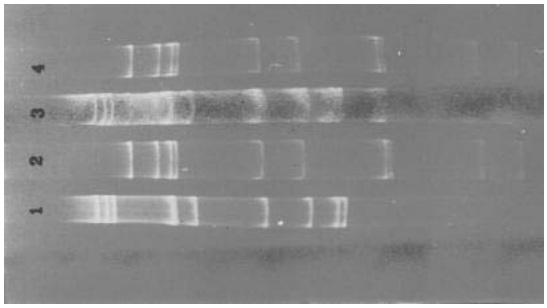

Figure 3. Analysis of viral RNA extracted at 24 h post infection. Lane 1, RNA from purified reovirus. Lane 2, RNA from purified rotavirus. Lane 3, RNA from mixed infected cell after first passage showing presence of both rota and reo virus RNA. Lane 4, coinfected cell after first passage showing presence of only rotavirus RNA bonds.

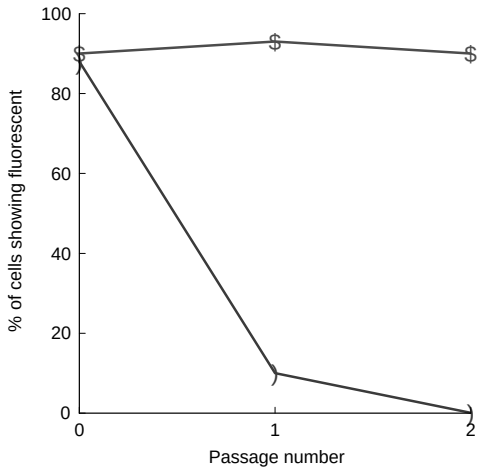

Figure 4. Cells were coinfected with reo and rota viruses and stained by fluorescin conjugate using viral specific antisera. Percent of cells showing fluorescence was determined in subsequent passages. ● Cells with reovirus antigen, □ cells with rotavirus antigen.

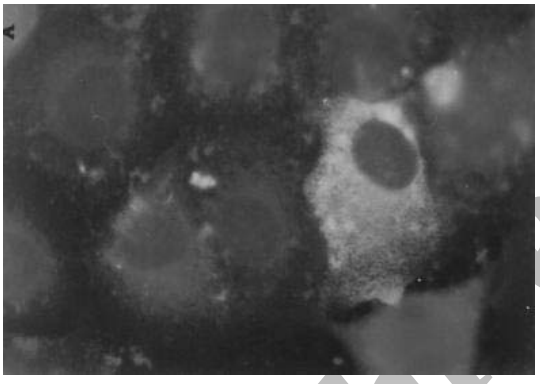

Figure 5a. Reovirus antigens in coinfected cells. Cells were stained by fluorecsein-conjugated antibody, using reovirus specific antisera. Viral antigens appear as intensely stained spots inside the cytoplasm.

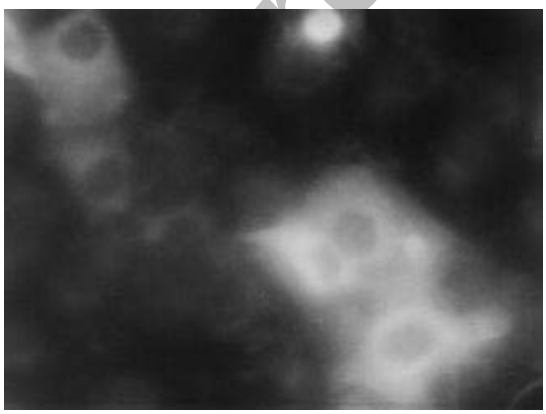

Figure 5b. Rotavirus antigens in coinfected cells. Cells were stained, using rotavirus specific antisera. Viral antigens are uniformly distributed inside the cytoplasm.

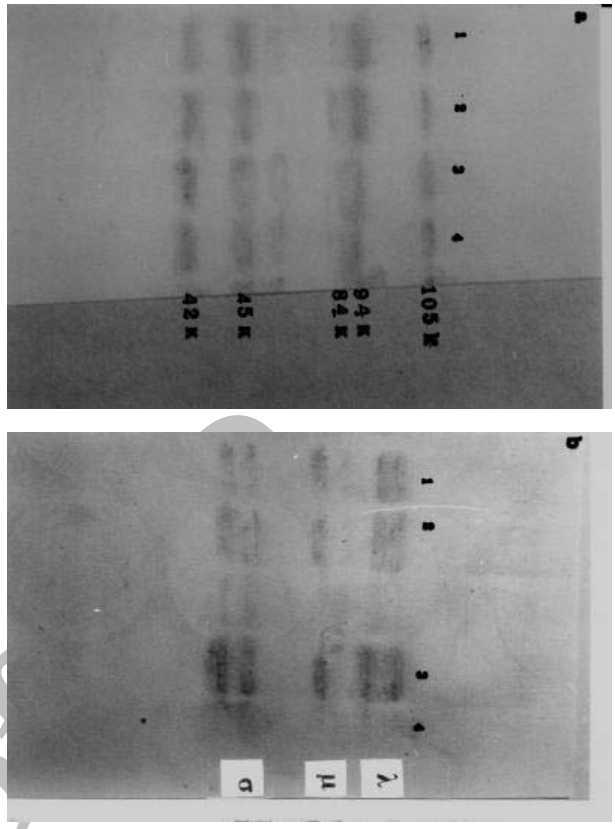

Figure 6. Immunoblotting of viral proteins from 16 h coinfected cells. a) proteins were electrophoresed in 10% polyacrylamide gel and blotted onto nitrocellulose paper. Protein bands were stained using rotavirus antibody. Lane 1, proteins from infected cells with rotavirus as control. Lane 2, proteins from purified rotavirus. Lane 3, proteins from coinfected cells. Lane 4, rotavirus proteins after the first passage. b) protein bonds were stained using reovirus antibody. Lane 1, infected cells with reovirus. Lane 2, purified reovirus. Lane 3, proteins from coinfected cells showing presence of reovirus proteins. Lane 4, proteins from coinfected cells after first passage, no reovirus protein is present.

In general, Infection of a cell with two viruses could result in growth and maturation of both viruses, which might be beneficial to one of the viruses such as coinfection by adenovirus, and adeno-associated viruses. Coinfection could also end up in growth inhibition of one of the viruses by the other such as infection of cells with enteroviruses, which would cause growth inhibition of poliovirus [16,19].

In our study we wanted to test the extent of interference between reovirus and rotavirus, which can coexist in alimentary tract. Since rotaviruses are host specific and grow only in certain cells; therefore we used BSC-1 cells, which support growth of both viruses.

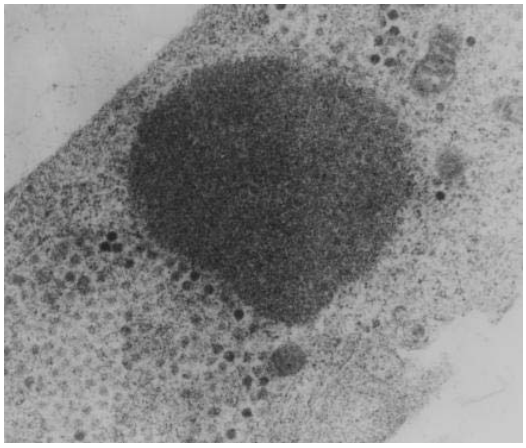

Figure 7a. Electron micrograph of thin section from a cell coinfected with reo and rota viruses. Cells were harvested at 12 h postinfection showing dark staining viroplasm surrounded by rotavirus particles (40000).

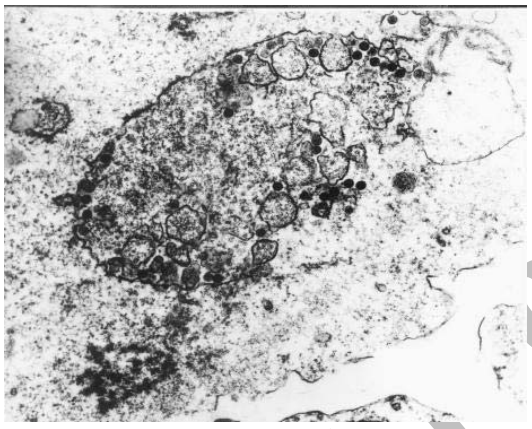

Figure 7b. Electron micrograph of 16 h mixed infected cells similar to Figure 7a. Many rotavirus particles are in process of budding (40000).

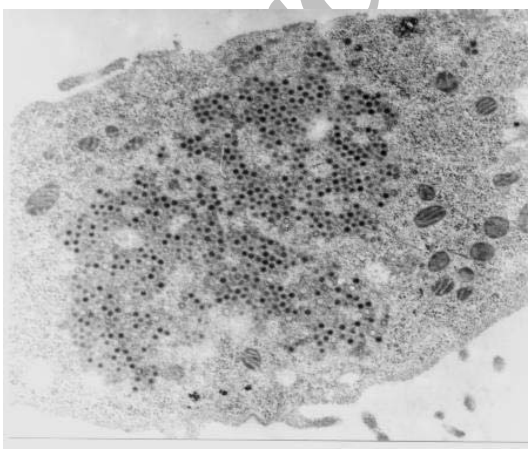

Figure 8. Electron micrograph of thin section from a reovirus infected cell 16 h post infection. Many reovirus particles maturing within dark staining inclusions are seen (20000).

The amount of infectious reovirus production was reduced significantly. This assay was performed by two independent assay systems. The moi of 50 was used to ensure that all cells became infected with the both viruses. The results indicated that infectious reovirus formation was inhibited in mixed infected cells. When the lysate of coinfected cells were used for reinfecting cell monolayers, it was found that only rotavirus was present in lysate. In electron microscopy examination of negatively stained preparations from coinfected cells after the first passage, only particles which were morphologically similar to rotavirus were observed. The cells infected with mixed viruses contained RNA and proteins of both rota and reo viruses as was shown by SDS PAGE analysis, immunofluorescent staining and blotting experiments which indicated viral macromolecules of both viruses were synthesized. However, when the lysate of mixed infected cells was utilized to reinfect the cells, only rotavirus macromolecules were synthesized. These observations with the results of direct visualization of viruses by electron microscope revealed that formation of mature reovirus particles was curtailed in coinfected cells. Interference in mixed infected cells by different mutant and wild types of reovirus has been reported by several investigators [6,11]. They found that the mutant inhibition of protein synthesis was dominant. These investigators suggested that the factors produced by the wild type might have been replaced by defective factors synthesized by the ts mutant. In our study inhibition of macromolecule synthesis of viruses did not seem to occur upon coinfection. In order to determine the possible stages of interference between the two viruses, receptor binding competition experiment was carried out and revealed that even at high moi, the adsorption of viruses to the cells was not affected in mixed infection. It was concluded that inhibition of reovirus growth in presence of rotavirus was not at the stage of virus adsorption and penetration.

Superinfection exclusion has been described in some viruses such as Blue tongue virus [27] and vesicular stomatitis virus [28]. This phenomenon has been described to occur not at the level of adsorption but perhaps at the some stages of the early period of infection. In our study it was found that in coinfected cells, viral genome and structural proteins of both viruses were made but formation of infectious reovirus particles was blocked. It seemed that inhibition of reovirus growth was at the stage of assembly. This possibility was supported by the observation of sections from coinfected cells in which only particles resembling rotavirus were observed and no reovirus like particles were seen. This observation was made by examining

many sections of mixed infected cells at various times intervals. The lysate from coinfected cells did not have any reovirus infectivity after the first passage.

From our study it appeared that in coinfection of cells with rota and reovirus the dominant rotavirus inhibited the growth of reovirus. We have examined this phenomenon at simultaneous coinfection. The question of how superinfection at various times will affect this interference and what is the molecular mechanism for such interference remains to be determined.

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