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Simian Rotavirus SA-11 Plaque Formation in the Presence of Trypsin

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Incorporation of 5 μg of trypsin per ml of the overlay (Eagle minimal essential medium–0.7% Ionagar no. 2) was found to be necessary for plaque formation by simian rotavirus SA-11. Plaques of 3 to 4 mm in diameter were produced in MA-104 cells after 5 days of incubation at 37°C. Plaque size was even larger (5 to 6 mm) in monolayers of African green monkey kidney cells. Addition of diethylaminoethyl-dextran, protamine sulfate, or 5-bromodeoxyuridine to the trypsin-containing overlay did not improve plaque formation by the virus. Incorporation of beef extract or yeast extract to a final concentration of 0.5% in the trypsin-containing overlay inhibited plaque formation. On the other hand, the presence of lactalbumin hydrolysate or peptone at a similar concentration in the overlay did not inhibit plaque formation. When methylcellulose was used instead of the agar as the solidifying agent in the overlay, no plaques were seen. SA-11 is a useful model for the study of human rotaviruses, and this relatively simple plaque assay system should further enhance its usefulness in this regard.

Rotaviruses are now known to be a major cause of diarrhea in humans and animals (7, 18, 29). Although they are excreted in very large numbers in the feces of infected individuals, most rotaviruses cannot yet be readily propagated in vitro (18). Success in the in vitro cultivation of bovine (18, 19) and simian (16) rotaviruses has, however, been achieved. The presence of trypsin in the maintenance medium has been shown to greatly facilitate the growth of bovine rotaviruses in cell culture (1, 3, 4). Matsuno et al. (17) found that trypsin was also required for plaque formation by the Lincoln strain of the neonatal calf diarrhea virus (NCDV). On the other hand, Wyatt et al. (R. G. Wyatt, R. M. Chanock, and W. D. James, Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/8) reported that trypsin or other proteolytic enzymes were not needed for the plaque formation of the UK strain of bovine rotavirus.

It has also been reported that simian rotavirus SA-11 (16) does not depend on proteolytic enzymes either for enhanced growth in cell cultures (B. D. Shoub and D. M. Bertran, Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/10) or for the formation of plaques (Wyatt et al., Proc. 4th Int. Congr. Virol., The Hague, Abstr. no. W35/8). When our initial attempts to plaque SA-11 virus in cell cultures in the absence of trypsin failed, we initiated the investigation described here.

MATERIALS AND METHODS

Cells. MA-104 cells, an established line derived from rhesus monkey kidneys, were kindly supplied to us by H. Malherbe of the University of Texas at San Antonio. Cells were routinely cultivated as monolayers in 75-cm^2 plastic tissue culture flasks (Flow Laboratories) with Eagle minimal essential medium (MEM) in Earle base (Autopow; Flow Laboratories). Each 450 ml of the medium was supplemented with 25 mg of gentamicin (Schering Corp.), 13.5 ml of a 5.6% solution of sodium bicarbonate, 5.0 ml of a 200 mM solution of L-glutamine (Flow Laboratories) and 50 ml of virus- and mycoplasma-tested fetal bovine serum (Microbiological Associates).

Monolayers were trypsinized with 2.0 ml of a mixture of trypsin (0.25%) and Versene (0.5%) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. A split ratio of 1:4 was generally used for the passage of the cells, and cultures for plaque tests were prepared in 25-cm^2 plastic flasks (Flow Laboratories).

Primary African green monkey kidney (AGMK) cells were purchased in suspension from Connaught Laboratories, Toronto. The cells were initially cultivated in 75-cm^2 flasks. When monolayers were formed, they were trypsinized, and by using a split ratio of 1:2, secondary cultures for plaque tests were prepared in 25-cm^2 flasks.

Virus. Simian rotavirus SA-11 (strain H 96) was also kindly supplied to us by H. Malherbe. The virus pool used in this study was prepared in MA-104 cells. Because of the inhibitory action of fetal bovine serum on the infectivity of rotaviruses (4), it was necessary to wash the monolayers at least twice with Earle balanced salt solution (EBSS) before virus inoculation.

After virus adsorption, for 1 h at 37°C, maintenance medium (MEM without serum and trypsin) was added to the cultures and they were incubated at 37°C. When nearly 75% of the monolayer showed virus cytopathic effects, cultures were frozen (−20°C) and thawed three times. After centrifugation at 1,000 × *g* for 15 min, the supernatant fluid was dispensed in 0.5-ml volumes and frozen at −80°C. Electron microscopic examination of the pool confirmed the presence of rotavirus particles.

Trypsin. A 0.1% stock solution of crystalline trypsin (1:250; GIBCO Laboratories) prepared in EBSS was sterilized by passage through a 0.22-μm membrane filter (Nalge; Sybron Corp.) and kept frozen in 1.0-ml volumes at −20°C.

Solidifying agents. Agar (Difco), Noble agar (Difco), Oxoid agar no. 1 (Oxoid), Ionagar no. 2 (Oxoid), and methylcellulose (BDH) were tested for their suitability as solidifying agents in the overlay medium. The appropriate amount of the solidifying agent was suspended in a measured volume of deionized water and sterilized by autoclaving.

Additives for overlay medium. Diethylaminoethyl (DEAE)-dextran (Pharmacia), protamine sulfate (Nutritional Biochemicals Corp.), and 5-bromodeoxyuridine (GIBCO Laboratories) were prepared as 1% stock solutions in deionized water. Bovine serum albumin fraction V (Sigma Chemical Co.), beef extract (Oxoid), lactalbumin hydrolysate (GIBCO Laboratories), yeast extract (GIBCO Laboratories), and peptone (Difco) were prepared as 20% stock solutions in EBSS. Tryptose phosphate broth (Difco) and nutrient broth (Difco) were rehydrated in deionized water in accordance with the manufacturer's instructions. All the solutions were sterilized by filtration before storage at 4°C.

Plaque testing. After the growth medium was removed, cell monolayers were washed twice with about 5 ml of EBSS. Each culture received 0.5 ml of the appropriately diluted virus inoculum. For virus adsorption, inoculated cultures were kept at 37°C for 1 h. Excess inoculum was then removed, and 5 ml of the overlay medium under test was added to each flask. Generally, the cultures were left to incubate for a period of 5 days. When they were examined for plaques, each monolayer was first fixed for at least 1 h in 3 ml of a 10% solution of Formalin in normal saline. After the removal of the overlay and fixative, the cultures were thoroughly washed in tap water and stained with a crystal violet solution (21). A 3- to 5-min exposure to the stain was necessary before a final wash in tap water.

RESULTS

Need for trypsin in the overlay. Even in the absence of trypsin, SA-11 virus grows and produces pronounced cytopathic changes in monolayer cultures of MA-104 cells. But when an overlay consisting of MEM and 0.7% agar (Difco) was used, the virus failed to form detectable plaques even after 7 days of incubation at 37°C. However, when trypsin at a final concentration of 5 μg/ml was incorporated in the overlay, small (about 1 mm in diameter) plaques could be seen after 5 days at 37°C. Longer incubation did not lead to any further increase in plaque size, but resulted in rapid thinning and deterioration of the monolayers. Increase in the amount of trypsin in the overlay also produced premature and nonspecific cell degeneration.

Effect of agar type on SA-11 plaque formation. In an effort to further improve the size and appearance of the plaques, Oxoid agar no. 1, Noble agar, and Ionagar no. 2 were compared as solidifying agents for the overlay. Table 1 shows that the presence of 0.7% Ionagar in the trypsin-containing overlay gave plaques of the largest (3 to 4 mm in diameter) size in monolayers of MA-104 cells after 5 days of incubation. Similar results were reported in an earlier investigation with NCDV (17). Figure 1 shows the appearance of SA-11 plaques on an MA-104 monolayer along with a virus-inoculated culture which had received the same overlay but without trypsin. On the basis of these observations, Ionagar no. 2 was chosen as the solidifying agent for the overlay in subsequent experiments.

Effect of protein-supplemented overlays on SA-11 plaque formation. The presence of protein supplements in the overlay is considered helpful in the proper and prolonged maintenance of cell monolayers. Since serum has been shown to be inhibitory to rotaviruses (4), beef extract, yeast extract, lactalbumin hydrolysate, and peptone were incorporated separately in the trypsin-containing overlay at a final concentration of 0.5%. The results of these experiments are summarized in Table 2. The presence of beef extract and yeast extract in the overlay resulted in no plaque formation by the virus. Plaques were formed in the presence of peptone or lactalbumin hydrolysate but, when compared with cultures with unsupplemented overlay, there was no detectable improvement in the size and number of plaques produced.

Effect of overlay containing DESE-dextran, protamine sulfate, or 5-bromodeoxyuridine. Matsuno et al. (17) reported that along

TABLE 1. *Effect of the type of agar on rotavirus SA-11 plaque formation in MA-104 cells*

Type of agar[a]	No. of plaques per 0.5 ml[b]	Plaque diam (mm)
Difco	32	1
Oxoid #1	34	1
Noble	32	1
Ionagar #2	38	3–4

[a] Overlay consisted of MEM, 5 μg of trypsin per ml, and 0.7% of agar under test.

[b] Each flask was inoculated with 0.5 ml of stock virus diluted 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

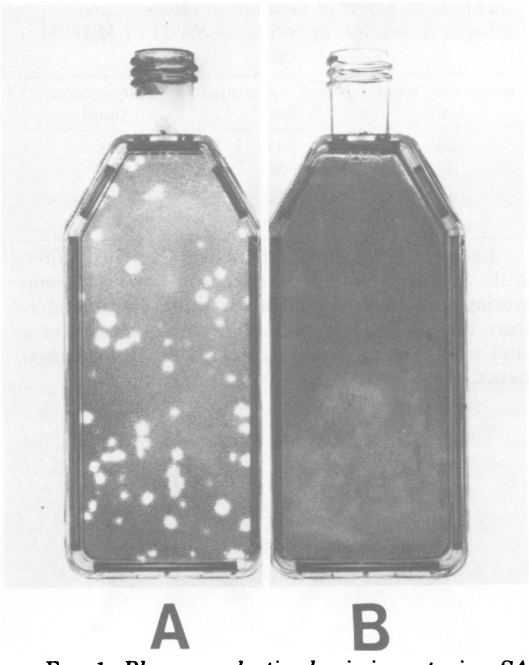

FIG. 1. *Plaque production by simian rotavirus SA-11 in MA-104 cells after 5 days of incubation at 37°C. (A) Monolayer with 5 μg of trypsin per ml of the overlay. (B) Virus-inoculated monolayer with overlay containing no trypsin.*

TABLE 2. *Effect of protein-rich supplements in the overlay on plaque formation by rotavirus SA-11 in MA-104 cells*

Type of supplement[a]	No. of plaques per 0.5 ml[b]	Plaque diam (mm)
None	36	3–4
Beef extract	0	—
Yeast extract	0	—
Lactalbumin hydrolysate	30	3–4
Peptone	34	3–4

[a] Overlay consisted of MEM, 5 μg of trypsin per ml, 0.7% Ionagar no. 2, and 0.5% of supplement under test.

[b] Each flask was inoculated with 0.5 ml of stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

with trypsin, DEAE-dextran was needed in the overlay for optimal plaque production by the Lincoln strain of NCDV in MA-104 cells. In our experiments (Table 3) with SA-11 and MA-104 cells, the presence of DEAE-dextran up to 50 μg/ml in the overlay did not contribute to the improvement of plaque size and numbers. Addition of 100 μg or more of the substance per ml of the overlay resulted in a decrease in the size and number of SA-11 plaques.

The presence of protamine sulfate in the overlay has been shown to enhance plaque formation by certain viruses (28). Addition of 50 to 300 μg of protamine sulfate per ml of the overlay had no noticeable effect on plaque formation by SA-11 (Table 3). Similar results were obtained when 25 to 200 μg of 5-bromodeoxyuridine was incorporated per ml of the overlay (Table 3).

Effect of overlay with methylcellulose. Agars of various types are known to contain sulfated polysaccharides which can inhibit plaque formation by certain viruses (25). To avoid this possibility, use of methylcellulose as a solidifying agent in overlays has been recommended (11). The use of methylcellulose as a possible substitute for Ionagar no. 1 was tested. When an overlay medium consisting of MEM, 5 μg of trypsin per ml, and 1.0% methylcellulose was used, SA-11 virus plaque formation was completely inhibited. Variation in the amount of trypsin from 2.5 to 10 μg/ml of the overlay did not allow plaque formation in the presence of methylcellulose. Even when the methylcellulose-containing overlay was supplemented with either 10 to 25 μg of DEAE-dextran or 10 to 50 μg protamine sulfate per ml no plaque formation occurred. Any further increase in the amounts of these cationic polymers made the overlay cytotoxic.

TABLE 3. *Effect of chemical additives in overlay on rotavirus SA-11 plaque formation in MA-104 cells*

Additive and concn (μg/ml) in overlay[a]	No. of plaques per 0.5 ml[b]	Plaque diam (mm)
None	38	4
DEAE-dextran		
50	32	4
100	30	2
200	28	2
300	28	2
Protamine sulfate		
50	36	4
100	38	4
200	34	4
300	34	4
5-Bromodeoxyuridine		
25	38	4
50	36	4
100	38	4
200	38	4

[a] Overlay consisted of MEM, 5 μg of trypsin per ml, 0.7% Ionagar no. 2, and the desired concentration of additive under test.

[b] Each flask was inoculated with 0.5 ml of stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

Effect of virus diluent-suspending medium. For the experiments described above EBSS was used as the virus diluent as well as the suspending medium during adsorption to cell monolayers. It has, however, been shown that the presence of protein-rich additives in the diluent and during virus adsorption enhances the plaquing efficiency (9). To test this for SA-11 virus, bovine serum albumin, tryptose phosphate broth, or nutrient broth was added to EBSS to a final concentration of 0.5%. The presence of these protein-rich additives in virus diluent-suspending medium did not alter the size and number of SA-11 virus plaques in MA-104 cells (Table 4).

Effect of incubation temperature. After virus adsorption to the cells at 37°C and introduction of the overlay, separate lots of the cultures were placed at 33, 35, 37, and 39°C for 5 days; after which plaques were examined and counted. Incubation at 37°C was found to be optimal with regard to both plaque size and number (Table 5).

SA-11 plaque formation in African green monkey kidney cells. Wyatt et al. (Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/8) reported that the presence of trypsin or other proteolytic enzymes in the overlay was not necessary for SA-11 plaque formation in primary cultures of AGMK cells. This observation was tested in the present study. Because primary cultures often do not produce uniform and satisfactory monolayers for virus plaquing, the AGMK cells were used as secondary cultures. After adsorption of SA-11 virus to the cells they were covered with overlay either with (5 μg/ml) or without trypsin. They were examined for plaques after 5 days of incubation at 37°C. As can be seen from Fig. 2, no plaques could be seen in cultures receiving the overlay without trypsin,

TABLE 4. *Effect of virus diluent on plaque formation by rotavirus SA-11 in MA-104 cells*

Diluent	No. of plaques per flask[a]	Plaque size (mm)
EBSS	34	3-4
0.5% Tryptose phosphate broth in EBSS	34	3-4
0.5% Nutrient broth in EBSS	28	2-3
0.5% Bovine serum albumin in EBSS	30	3-4

[a] Each flask received 0.5 ml of stock virus diluted to 10^{-5} in the diluent under test. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

TABLE 5. *Effect of incubation temperature on plaque formation by rotavirus SA-11 in MA-104 cells*

Incubation temp (°C)	No. of plaques per flask[a]	Plaque diam (mm)
33	44	2
35	36	2-3
37	42	4
39	24	2-3

[a] Each culture received 0.5 ml of stock virus diluted to 10^{-5} in EBSS and after virus adsorption (37°C) and overlaying were incubated at the appropriate temperature for 5 days. Numbers represent the mean of a total of 10 counts obtained in two separate experiments.

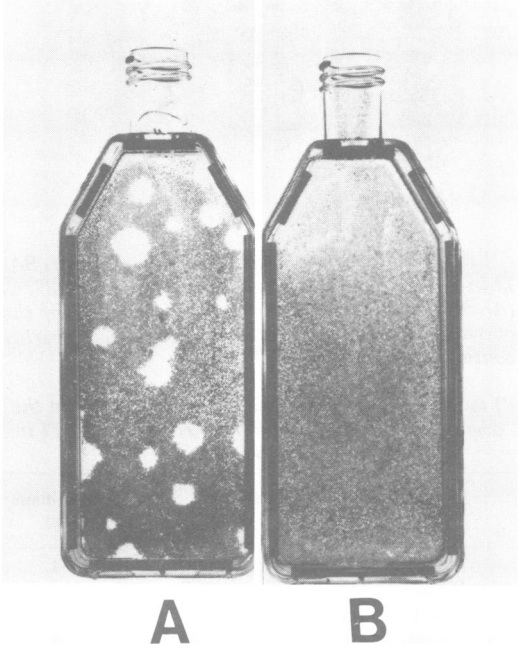

FIG. 2. *Plaque production by simian rotavirus SA-11 in AGMK cells after 5 days of incubation at 37°C. (A) Monolayer with 5 μg of trypsin per ml of the overlay. (B) Virus-inoculated monolayer with overlay containing no trypsin.*

whereas, in the presence of trypsin in the overlay, plaques of 5 to 6 mm in diameter were produced.

DISCUSSION

Trypsin has been shown to enhance the infectivity of reoviruses (23) and influenza viruses (13, 14) in vitro, to permit plaque formation by influenza viruses (2, 26), and to enhance the size and number of vaccinia virus plaques (8, 27). A similar potentiating effect on rotavirus infectivity in cell cultures has been demonstrated (1, 3,

4), but there have also been reports of the lack of enhancement of rotavirus infectivity in cell cultures in the presence of trypsin (B. D. Schoub and D. M. Bertran, Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/10; Wyett et al., Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/8).

The present study showed that trypsin was essential for plaque formation by simian rotavirus SA-11 in cultures of MA-104 and AGMK cells. Preliminary work in our laboratory has shown similar results for NCDV strain C-486. This is contrast to an earlier report by Wyatt et al. (Proc. 4th Int. Congr. Virol., The Hague, 1978, Abstr. no. W35/8) where trypsin or other proteolytic enzymes were found to be unnecessary for plaque formation by SA-11 and the UK strain of bovine rotavirus. Our findings are, however, in agreement with the observations of Matsuno et al. (17), who reported that the Lincoln strain of NCDV produced plaques in MA-104 cells only when trypsin was incorporated in the overlay. They also observed that addition of DEAE-dextran to the trypsin-containing overlay increased the number of plaques without any increase in their size. In our experiments with SA-11 virus and MA-104 cells, the need for DEAE-dextran in the overlay was not indicated. In fact, DEAE-dextran in amounts greater than 100 μg/ml of overlay resulted in a slight reduction in the size and number of SA-11 plaques. The increase in the number of NCDV plaques (17) in the presence of dextran may possibly have been due to disaggregation of virus clumps.

Incorporation of protamine sulfate in the trypsin-containing overlay did not alter either the number or the size of rotavirus plaques. This may have been due to the digestion of protamine sulfate by trypsin (28).

The absence of SA-11 plaques in the presence of beef extract and yeast extract could have been due to nonspecific virus inhibitors associated with them. It is also possible that these protein-rich substances may have interfered with the proteolytic activity of trypsin essential to virus plaque development.

Although several possible explanations have been suggested (1, 3), the exact mechanism by which trypsin enhances rotavirus infectivity in cell cultures is not yet clearly understood. Preliminary studies in our laboratory indicate that for SA-11 plaque formation the presence of trypsin is necessary throughout the incubation period. Work to further elucidate this point is now in progress.

The use of methylcellulose in the overlay led to a total inhibition of SA-11 plaque formation, which could not be reversed by an increase in the amount of trypsin or the addition of cationic polymers such as DEAE-dextran or protamine sulfate. The basis for this phenomenon is not clear at this stage.

A suitable virus diluent is among the factors considered necessary for optimal plaque production (9, 27). Proteinaceous substances in virus diluents not only exert a protective effect on virus infectivity, but they have also been known to disaggregate virus clumps (9). In the experiments with SA-11, addition of protein-rich substances to the diluent (EBSS) was not found to alter the number and size of plaques.

Addition of halogenated pyrimidines to virus-infected cell cultures has been shown to overcome interferon production (10). Activation of the synthesis of both RNA and DNA viruses has also been reported in the presence of 5-bromodeoxyuridine (20, 24). Incorporation of this substance in the overlay produced no noticeable effect on the plaque-forming ability of SA-11 virus in MA-104 cells.

There have been repeated and convincing demonstrations of the close similarities between SA-11 and human rotaviruses (7, 18, 29). Therefore, until easy and reliable means for human rotavirus cultivation and quantification in vitro become available, SA-11 will continue to serve as a very useful substitute. For example, SA-11 virus has been employed as an antigen in the serodiagnosis of human rotavirus infections (12). The possible application of SA-11 virus in immunization of humans against rotaviruses has also been suggested (22). Because of the absence of a simple and sensitive plaque assay system for SA-11, some earlier investigations (6, 16) with this virus resorted to the use of cumbersome and less sensitive means of infective virus quantitation.

Even in certain advanced and industrialized countries there has been an increase in recent years in the number of waterborne outbreaks of gastroenteritis (5). Many of these outbreaks are suspected to have a viral etiology. Although the exact role of rotaviruses in such outbreaks is not known, the spread of these viruses through sewage-polluted waters has been reported (15). The direct demonstration of rotaviruses in water samples has, however, not yet been achieved. This is mainly due to the absence of proper techniques for their concentration from the water environment. The availability of a plaque system for SA-11 should permit the development and testing of such techniques and their eventual application in the investigation of waterborne outbreaks of rotavirus gastroenteritis.

SA-11 virus is a very suitable model for the study of rotaviruses in general and human rota-

viruses in particular, and the plaque system reported here should further enhance the usefulness of this virus in this regard.

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