Production of High-Titer Bovine Rotavirus with Trypsin†

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Titers of bovine rotavirus in excess of 10^9 immunofluorescent infectious units per ml of culture fluids have been produced, using trypsin treatment of the virus. Infectivity of preparations of the virus can be increased with as little as 1 ng of trypsin per ml, with maximum increases of 1 to 2 $\log_{10}$ with 1 μg of trypsin per ml. The virus grows to titers in excess of 10^8 immunofluorescent units per ml in MDBK, LLC-MK₂, MA-104, and HeLa cells. When MDBK cells are infected with a multiplicity of infection of 20, maximum yields of cell-associated, trypsin-enhanceable virus are obtained 4 to 8 h postinfection. Maximum yields of cell-free, trypsin-enhanceable virus are produced 16 to 20 h postinfection. The results presented here indicate that trypsin can be used to produce high-titer stocks of bovine rotavirus.

The study of rotaviruses isolated from various species of animals (13) has been delayed by the lack of cell culture-adapted strains of virus which grow to high titers. Most success has been with bovine rotavirus, of which there are four culture-adapted strains currently available (4, 7, 12). Two other rotaviruses, the simian rotavirus SA-11 and the O-agent have also been successfully grown in cell culture (5).

Although the above strains of rotaviruses have been adapted to in vitro growth, all of the strains isolated to date reproduce to low levels, with titers of virus produced generally in the range of 10^4 to 10^7 infectious virions per ml of cell culture fluids. These low titers make studies of the events during virus replication difficult, if not impossible.

Early work on the effect of proteolytic enzymes on the infectivity of reovirus by Spendlove et al. (10) established that certain proteolytic enzymes could markedly increase reovirus infectivity. These workers found that at least eight different proteolytic enzymes will enhance the infectivity of reovirus preparations. It was hypothesized that proteolytic enzymes remove or alter viral capsid structures, which leads to enhanced infectivity (9).

The work of Spendlove et al. (8-10) led us to wonder whether proteolytic enzymes might affect the infectivity of a strain of bovine rotavirus in a similar manner. Subsequent to the initiation of our study, several reports dealing with the effect of trypsin on the infectivity of rotaviruses have appeared. Matsuno et al. (6) showed that

bovine rotavirus could be successfully plaqued by using overlay media containing trypsin and diethylaminoethyl-dextran. The successful isolation of bovine (1) and porcine (11) rotaviruses by trypsin treatment of virus-containing fecal material has also been reported. The results presented in this paper extend the results obtained by others and show that trypsin treatment of bovine rotavirus can effectively produce high-titer stocks of the virus.

MATERIALS AND METHODS

Cells. Bovine kidney (MDBK), monkey kidney (LLC-MK₂), MA-104 (Microbiological Associates), and human cervical carcinoma (HeLa) cells were grown in Eagle minimum essential medium (MEM) in Earle's balanced salt solution (BSS) (KC Biologicals) containing penicillin (25 U/ml), streptomycin sulfate (25 $\mu\text{g}/\text{ml}$), bacitracin (2.5 U/ml), and sodium bicarbonate (2 mg/ml). Heat-inactivated fetal bovine serum (Sterile Systems, Inc.) was added to the medium to a final concentration of 10%. Cells were grown at 36°C in a 5% CO₂ atmosphere.

Virus. The virus used in this study, the vaccine strain of the Lincoln isolate of bovine rotavirus, was kindly provided by Norden Laboratories, Lincoln, Nebr. The virus was originally adapted to cell culture by Mebus et al. (7) and has been partially characterized in this laboratory (2).

Trypsin treatment of virus. Trypsin (ICN, 1:250; or type XI, Sigma Chemical Co.) was prepared in Earle's BSS at a concentration of 1 mg/ml. To enhance virus infectivity, samples of bovine rotavirus in MEM (pH 7.6) were treated routinely with trypsin at 10 $\mu\text{g}/\text{ml}$ and incubated at room temperature for 15 to 30 min.

Virus growth. In studying the effect of trypsin on bovine rotavirus infectivity, it was found that most lots of fetal bovine serum tested contain rotavirus inhibitors (E. D. Neas, B. B. Barnett, and R. S. Spend-

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love, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S342, p. 268). Thereafter, cells to be infected were washed free of fetal bovine serum with Earle's BSS before infection. Washed monolayers of cells were infected with bovine rotavirus that had been treated with trypsin at 10 μ g/ml. After adsorption for 1 h at room temperature, the inoculum virus was removed, and the cells were washed with Earle's BSS. Virus was grown in MEM without fetal bovine serum. Infected cultures were incubated at 36°C for various times.

High-titer stocks of bovine rotavirus were produced by infecting washed monolayers of MDBK cells with trypsin-treated virus at a multiplicity of infection of 20. After adsorption at room temperature for 1 h, MEM without serum was added to the cultures. The infected cultures were incubated at 36°C. Extensive degeneration of the cell sheets was observed 12 to 24 h postinfection, when approximately 80 to 90% of the cells were detached. Infected cells and culture fluids were extracted in a Virtis 60K homogenizer for 2 min at 10,000 rpm with Freon 113 (0.26 ml per ml of culture fluids). The aqueous phase which contained the virus was separated from the Freon phase by centrifugation and stored at -20°C.

Immunofluorescent virus assay. The procedure for assaying virus by using the fluorescent cell-counting assay has been described (3). Briefly, monolayers of MDBK cells were grown on circular cover slips to confluency. The cover slips were washed twice with Earle's BSS before infection with 0.02 ml of virus. The infected cell monolayers were incubated at 36°C for 16 to 20 h and air dried. The cover slips were then fixed with cold acetone and stained with fluorescein-conjugated anti-bovine rotavirus rabbit immunoglobulin G. The stained cells were viewed under ultraviolet illumination, and the fluorescing (infected) cells were counted.

RESULTS

Early work by Spendlove and Schaffer (10) established that the infectivity of reovirus, a virus structurally similar to rotavirus, could be significantly increased by treatment with several proteolytic enzymes. To determine whether the infectivity of a bovine rotavirus could be altered in a similar manner, we treated samples of virus with trypsin. The results (Fig. 1) confirm that the infectivity of bovine rotavirus can be increased with trypsin treatment. The titer of this sample was increased approximately threefold with as little as 10 ng of trypsin per ml. Maximum increases in infectivity of 45-fold were achieved with trypsin concentrations of 1 μ g or greater per ml.

With the striking increase in infectivity of bovine rotavirus with trypsin treatment, the effect of trypsin on cultivation of the virus was studied. MDBK cells were infected with Norden Laboratories production seed (lot 13) with and without trypsin treatment. The titer of inoculum virus was 10^4 fluorescent cell-forming units (FCFU) per ml when assayed in the presence of

FIG. 1. Effect of trypsin on a preparation of bovine rotavirus. An infected-cell extract of bovine rotavirus was incubated at room temperature with various concentrations of trypsin for 15 min (—). The control represents the same sample not treated with trypsin (-----). Titer, FCFU determined by fluorescent-antibody assay as described in the text.

trypsin. Infectious virus was below the limit of detection without trypsin treatment. It was found (Fig. 2) with trypsin treatment of inoculum virus that titers in cell culture extracts increased from 6×10^4 FCFU/ml after 24 h to 2×10^8 FCFU/ml in 72 h when assayed in the presence of trypsin, an increase of 3,000-fold. Without trypsin treatment, the titer increased slightly from 10^4 FCFU/ml in 24 h to 9×10^4 after 72 h. It was also found that serially passaging virus with trypsin treatment of the inocula led to the production of virus with a titer in excess of 10^9 FCFU/ml in two passages, when assayed in the presence of trypsin (not shown). These high-titer stocks of bovine rotavirus were used to further study the growth characteristics of the virus.

Growth kinetics of bovine rotavirus were studied by infecting monolayers of MDBK cells with a multiplicity of infection for trypsin-treated virus of 20. Cell-free and cell-associated samples were assayed at various times with and without trypsin treatment. Following the nomenclature of Spendlove et al. (8), virus that is infectious only in the presence of trypsin is referred to as potentially infectious (PIV), whereas virus infectious without trypsin treatment is referred to as infectious virus (IV). It was found that cell-associated PIV reached maximum titers of 3×10^9 to 4×10^9 /ml 4 to 8 h postinfection, which represents the production of approximately 4,000 virions per cell (Fig. 3). Cell-free PIV reached a maximum titer of 2×10^8 /ml in 16 h or 200 virus particles per cell. Maximum yields of cell-associated IV were obtained 24 h postin-

FIG. 2. Effect of trypsin on cultivation of bovine rotavirus. Norden laboratories bovine rotavirus production seed (lot 13) was used to infect confluent monolayers of MDBK cells with or without trypsin treatment before infecting the cells. Infected cultures were harvested at 24, 48, and 72 h postinfection with three cycles of freeze-thaw. Samples were assayed with and without trypsin. P-1 and P-2 refer to the first and second passage of the virus on MDBK cells, respectively.

fection with a titer of 5×10^7 /ml or 50 virions per cell. Cell-free IV reached maximum titer late in the infection cycle, yielding a titer of 10^7 /ml, which represents approximately 10 virions per cell. It should be pointed out that the curves labeled PIV and IV in Fig. 3 represent differences found when cell-free and cell-associated virus samples were incubated with (PIV) and without (IV) trypsin before assay. If monolayers of MDBK cells grown on cover slips are infected with the same multiplicity as in the growth experiment, viral antigen can be detected 2 h postinfection (not shown).

The ability of various cell lines to support the growth of bovine rotavirus was studied by infecting each cell line with a multiplicity of infection for trypsin-treated virus of 10. The cells were harvested by two cycles of freeze-thaw at 24 and 48 h. Each sample was assayed on MDBK cells and represents combined cell-free and cell-associated virus populations. Maximum yields of 10^9 were produced in MDBK cells 48 h postinfection (Table 1). Infecting MDBK cells with virus not treated with trypsin led to the production of approximately 100-fold less virus than when the virus was treated with trypsin. MA-104, LLC-MK₂, and HeLa cells produced approximately 10-fold less virus than did MDBK cells, but yielded maximum titers in 24 h.

DISCUSSION

Although the structure, morphogenesis, and involvement of rotaviruses in nonbacterial gas-

troenteritis has been extensively studied (13), the molecular events in rotavirus replication are essentially unknown, due to the lack of high-titer, cell culture-adapted strains of the virus. Cultivation of the virus has been difficult, and the strains thus far adapted grow to relatively low titers. The results presented in this paper show that high-titer stocks of bovine rotavirus can be produced by trypsin treatment of inoculum virus.

Working with diarrheic fecal samples, Theil et al. (12) and Babiuk et al. (1) have shown that porcine and bovine rotaviruses, respectively, can be adapted to cell culture growth by trypsin treatment. Although both groups have shown that these two rotaviruses can be efficiently adapted to cell culture, yields of virus were low, in the range of 10^5 infectious virions per ml of culture fluids. The fact that we have produced virus with titers in excess of 10^9 per ml may be due to several factors. Results in our laboratory indicate that the majority of lots of fetal bovine serum contain rotavirus inhibitors at levels sufficient to reduce titers of virus 80 to 90% (Neas et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978). We have also found that concentrations

FIG. 3. Growth of bovine rotavirus in MDBK cells. Confluent monolayers of MDBK cells were infected with trypsin-treated virus at a multiplicity of infection of 20. Cell-free (CF) and cell-associated (CA) samples were taken at various times and assayed with (PIV) and without (IV) prior trypsin treatment at $10 \mu\text{g}/\text{ml}$.

TABLE 1. *Growth of bovine rotavirus in various cell lines*

Cell line	Time postinfection ^a (h)	Titer with trypsin ^b	Titer without trypsin ^c
LLC-MK ₂	24	1.5×10^8	3.4×10^6
	48	1.4×10^8	7.5×10^5
MA-104	24	2.7×10^8	1.3×10^6
	48	1.2×10^8	1.4×10^6
MDBK	24	6.1×10^8	6.3×10^6
	48	1.3×10^9	2.5×10^7
MDBK (with-out trypsin)	24	5.9×10^7	3.1×10^5
	48	1.8×10^7	1.2×10^5
HeLa P-1	24	1.0×10^8	1.2×10^6
	48	7.8×10^7	8.4×10^5

^a Cells were infected with trypsin-treated virus at a multiplicity of infection of 10. Infected cultures were harvested by three cycles of freeze-thaw at 24 and 48 h postinfection.

^b Virus assayed in the presence of 10 μ g of trypsin per ml.

^c Virus assayed without prior trypsin treatment.

of ethylenediaminetetraacetic acid as low as 10 mM can destroy the infectivity of the virus (unpublished data). Considering the above results, it is possible that other workers have not had our success because their virus was exposed to either fetal bovine serum or ethylenediaminetetraacetic acid.

The mechanism of trypsin enhancement of bovine rotavirus infectivity is unknown. Work in our laboratory (unpublished data) and by Babiuk et al. (1) indicates that trypsin acts on the virus and not the infected cell. In both cases, it was found that the increase in infectivity obtained with trypsin treatment remains unaltered if the trypsin is inhibited with soybean trypsin inhibitor before infecting cells. Spendlove et al. (9) found that chymotrypsin removes the outer capsid of reovirus and that, under carefully controlled conditions, the ratio of physical particles to infectious particles approaches 1 after chymotrypsin treatment. This observation suggests that there are two populations of virus in reovirus preparations and that conversion of the chymotrypsin-enhanceable form to the infectious form involves removal of virion surface polypeptides. We have found that potentially infectious bovine rotavirus (PIV) not treated with trypsin still attaches to cells at a rate identical to IV (unpublished data). It is possible that trypsin enhances the infectivity of bovine rotavirus by rendering virus infectious that would otherwise be aborted by the cell. It is also possible that

trypsin increases the infectivity of samples of bovine rotavirus merely by dispersing viral aggregates. These possibilities are currently under investigation. We are also studying the effect of trypsin on bovine rotavirus structure.

In studying the growth of infectious and potentially infectious reovirus, Spendlove et al. (8) noted that the titer of cell-free and cell-associated PIV drops rapidly after maximum titers are reached. This drop in titer was thought to be due to thermal inactivation and was discussed in terms of the time infected cultures should be harvested for maximum yield of virus. In contrast to reovirus, the titer of potentially infectious cell-free and cell-associated bovine rotavirus remains essentially constant throughout the growth experiment reported in this paper. For practical purposes, infected cultures may be harvested any time from 12 to 45 h postinfection to achieve maximum yields.

In summary, high-titer bovine rotavirus can be obtained with trypsin treatment. The virus produced can grow to high titers in various cell lines. Virus produced in this manner will be of great value in studying the replication of rotaviruses.

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