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Effect of actinomycin D on simian rotavirus (SA11) replication in cell culture

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Abstract

Rotaviruses are the major cause of viral diarrhea in humans and animals. Actinomycin D (Act D) is an antibiotic that intercalates DNA and therefore inhibits DNA-dependent transcription. The current study was carried out to assess the influence of Act D on the replication of simian rotavirus (SA11) in cell culture. Virus-infected MA-104 cell cultures were studied in the presence of Act D at concentrations of 1.25 and 2.5 µg/ml. Treatment of rotavirus-infected cells with 2.5 µg/ml Act D 48 h post-infection reduced the cytoplasmic metachromasia after staining with acridine orange by 25%. Viral RNA labeled with ^{3}H-uridine in the presence of the drug was separated by polyacrylamide gel electrophoresis. Viral RNA replication was not affected by Act D, but increased ^{3}H-uridine uptake was demonstrable by infected cells in the presence of the drug. This possibly was due to the inhibition of cellular RNA synthesis by Act D, which thus enhances incorporation of the radionuclide into the viral RNA. Act D reduced the number of infected cells presenting virus-specific fluorescence 48 h post-infection by more than 50%. These data suggest that Act D may have complexed with viral RNA and prevented newly synthesized mRNA from being translated, but may not have prevented early replication.

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- Replication
- Cell culture

Rotaviruses are classified as a genus within the family Reoviridae. These viruses are the major causal agents of acute diarrhea in mammals and fowl. The virions are nonenveloped 75 nm in diameter, and the genome consists of eleven segments of double-stranded RNA that encode six structural (VP1-VP4, VP6 and VP7) and five nonstructural (NSP1-NSP5) proteins (1). They have a triple-layered double capsid and replicate in the cytoplasm. Virion uncoating is followed by penetration into susceptible host cells and productive infection depends on the function of viral transcriptase in the synthesis of mRNA and double-stranded RNA segments of the progeny. The minus RNA strands act as templates for the synthesis of full-length plus strands (2,3). Both newly synthesized and pre-existing plus strand RNA act as templates for minus strand synthesis.

Actinomycin D (Act D) is an antibiotic that binds noncovalently to DNA and strongly inhibits transcription in eukaryotic and prokaryotic cells (4,5). Due to its toxicity, Act D has not been used clinically as an

antimicrobial agent, but is used as an antitumor agent (6). The structure of Act D consists of a phenoxazone ring linked to two identical cyclic pentapeptides. It binds tightly to double-stranded DNA but not to single-stranded DNA or RNA, double-stranded RNA, or RNA-DNA hybrids (7). Act D inhibits the replication of DNA- and RNA-containing viruses.

Vaccinia virus double-stranded DNA replication was 99% inhibited by Act D at 0.1 µg/ml; however, the replication of mengovirus single-stranded RNA was not affected by the drug at 1.0 µg/ml (8). At 1-10 µg/ml the drug inhibited by 99% the replication of fowl plague virus segmented single-stranded RNA. In contrast, Newcastle disease virus single-stranded RNA synthesis was not affected (8,9). Reovirus segmented double-stranded RNA replication was inhibited by up to 98% in the presence of 0.1-5.0 µg/ml of the drug in L cells (10). Scheiber et al. (11) demonstrated that human coronavirus, strain 229 E, single-stranded RNA was not affected by Act D. However, experiments with two feline strains of coronavirus with different tropism and virulence revealed that only the virulent strain was inhibited by the drug (12). Fish pancreatic necrosis virus segmented double-stranded RNA replication was inhibited by the drug at very low concentrations (13). Poliovirus, as well as several retrovirus single-stranded RNA were also shown to be inhibited by the drug (14-16). Therefore, Act D has been used as a tool for studying the *in vitro* replicative process of several viruses and its interaction with functions encoded by the host cell (11,17).

The present study assessed simian rotavirus (strain SA11) replication in MA-104 cells maintained with Act D at concentrations that inhibit cellular transcription.

MA-104 cell cultures (Rhesus monkey kidney fetal cells) were used at 70% confluence throughout the experiments. Simian rotavirus (SA11) pretreated and maintained in cell culture with crystalline trypsin (Gibco, Gaithersburg, MD, USA) at 30 and 10 µg/ml, respectively, was used at multiplicity of infection of approximately 1. A stock solution of Act D (Sigma, St. Louis, MO, USA) was prepared at 2.5 mg/ml in sodium phosphate-buffered saline (PBS), pH 7.3, and maintained at 4ºC.

The Act D cytotoxicity test was carried out by submitting cell cultures to drug-containing medium at the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.25, 2.5 and 5.0 µg/ml at 37ºC, for 7 days. Cells were observed daily for morphological changes.

The effect of Act D on viral RNA synthesis was monitored by acridine orange staining and ^{3}H-uridine incorporation. Cell cultures grown in 10 x 100 mm glass tubes were inoculated in triplicate and, after adsorption for 1 h at 37ºC, inocula were removed, and the cultures were refed with fresh medium containing Act D at final concentrations of 1.25 and 2.5 µg/ml, and 5 µCi/ml of ^{3}H-uridine. Infected untreated and mock-infected cell cultures with and without the drug were used in parallel as controls. At intervals of 24, 36, 48, 60 and 72 h post-infection cultures were harvested and submitted to three cycles of freezing and thawing. The cell homogenates were concentrated by dialysis to one third of the original volume and submitted to polyacrylamide gel electrophoresis (17). A human strain was used for control. The strips of the gel between the limits of the largest and the smallest segments of viral RNA (3.30-0.66 kb) were cut out and dissolved in 2.0 ml of hydrogen peroxide/ammonium hydroxide solution (19:1) (18). One milliliter of aqueous scintillation solution (Sigma) was added and counting was performed with a beta counter (Beckman LS 6000 SC).

For the metachromasia assay virus-infected cell cultures treated with 0, 1.25 and 2.5 µg/ml Act D were harvested at 48 h post-infection fixed with Carnoy's fixative for 5 min and stained with 0.02% (w/v) acridine orange (Merck, Darmstadt, Germany) ac-

cording to Rovozzo and Burke (19). Cultures were observed under ultraviolet light and results were expressed as the mean counts of cells with cytoplasmic metachromasia in ten fields.

Viral protein synthesis was monitored by immunofluorescence staining. Briefly, cell cultures grown on coverslips in Leighton tubes were inoculated as above. After adsorption, excess inoculum was removed and the monolayers were washed with PBS and refed with fresh maintenance medium containing 1.25 or 2.5 µg/ml of the drug. Infected monolayers in the absence of drug and noninfected monolayers with and without the drug were maintained as controls. At intervals of 48, 52, 56, and 72 h post-infection, coverslips were removed and washed with PBS and monolayers were fixed with cold acetone for 20 min. Coverslips were overlaid with mouse anti-porcine rotavirus monoclonal antibody directed at VP6, diluted at 1:40. A FITC conjugate goat anti-mouse immunoglobulin (Sigma) diluted at 1:40 was used. Results are reported as the mean counts of fluorescent cells showing specific fluorescence in ten fields. MA-104 cell cultures treated with 0.1 µg/ml Act D did not show any detectable morphological alterations, whereas at higher concentrations morphological changes were observed 72 h post-treatment.

The treatment of virus-infected cultures with 2.5 µg/ml Act D reduced by 25% the number of cells presenting cytoplasmic metachromasia at 48 h post-infection, whereas at 1.25 µg/ml the reduction was under 5%. The kinetics of ^{3}H-uridine uptake (Figure 1) demonstrated that viral RNA synthesis was not affected; however, increased counts were observed in infected cells in the presence of the drug.

The immunofluorescence assay demonstrated a reduction of the number of fluorescent cells when the drug was used at the concentrations of 1.25 or 2.5 µg/ml (Table 1). At 48 h post-infection, 54 fluorescent cells were detected in infected cultures in the absence of drug as compared to 24 and 22 cells in infected cultures treated with the

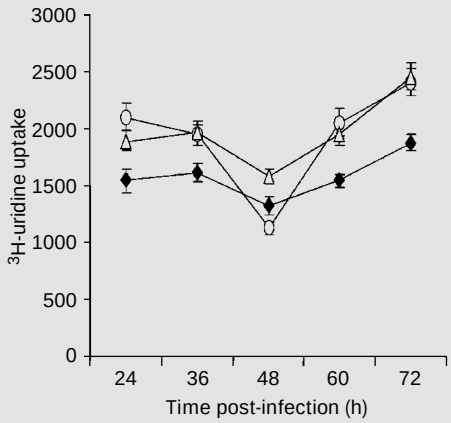

Figure 1. Effect of actinomycin D on rotavirus RNA synthesis. Kinetics of 5 µCi/ml ^{3}H-uridine uptake by SA11-infected MA-104 cells in the presence of actinomycin D at the concentrations of 1.25 (circles) and 2.5 µg/ml (triangles) and by infected untreated cells (lozenges). At the times post-infection indicated cell homogenates were submitted to PAGE, and the labeled viral RNA was eluted and radioactivity counted. Data are reported as means ± SEM for 3 experiments.

Table 1. Reduction of fluorescent cells upon actinomycin D (Act D) treatment.

Time post-infection (h)	Number of cells with specific fluorescence after treatment with Act D			Percent reduction in the number of cells with specific fluorescence after treatment with Act D	
	0	1.25	2.5	1.25	2.5
48	54 ± 8.5	24 ± 1.7	22 ± 2.0	55.6	59.9
52	42 ± 8.0	29 ± 7.0	23 ± 2.0	31.0	45.0
56	62 ± 13.5	35 ± 7.1	26 ± 4.0	43.6	58.0
72	48 ± 7.4	26 ± 3.6	24 ± 4.0	46.0	50.0

MA-104 cells were inoculated with rotavirus (SA11) in the presence of Act D at the concentrations of 1.25 and 2.5 µg/ml. Viral proteins were detected by immunofluorescence at the times post-infection indicated. Data are reported as means ± SD for 3 experiments.

drug at concentrations of 1.25 and 2.5 µg/ml, respectively, corresponding to reductions of 55.6 and 59.9%. At 52 h post-infection, infected cultures in the absence of drug showed 42 fluorescent cells in comparison to 29 and 23 in cultures treated at concentrations of 1.25 and 2.5 µg/ml, respectively, representing reductions of 31 and 45%. Similar results were obtained at 56 and 72 h post-infection with reduction of fluorescent cells of 43.6 and 58%, and 46 and 50%, for 1.25 and 2.5 µg/ml Act D, respectively.

Act D has been used to study the interaction of functions encoded by the host cell in the replication process of several viruses (10,15,16). In the current study we demonstrated an increase of ^{3}H-uridine uptake by cells infected with simian rotavirus in the presence of Act D in comparison to untreated cells. It is reasonable to expect that at the drug concentrations used, inhibition of most of the synthesis of cell RNA was achieved, favoring the uptake of radioactivity into viral RNA. Shatkin (10) showed an increase in ^{14}C-uridine uptake in viral RNA in L929 cells (mouse fibroblast cells) infected with reovirus and treated with Act D, with 92% inhibition of the synthesis of L929 cell RNA. Barry et al. (9) demonstrated that Act D at concentrations of 1.0 to 10.0 µg/ml inhibited the replication of influenza virus (segmented single-stranded RNA). Similar results were obtained for fowl plague virus. However, Act D did not interfere with the replication of Newcastle disease virus non-segmented single-stranded RNA (8). The sites of replication of these viruses in the host cells are different. Fowl plague virus replicates in the nucleus and Newcastle disease virus in the cytoplasm, and, in addition, fowl plague virus uses cellular RNA as primer for the initiation of transcription.

The reduction in the number of cells presenting cytoplasmic metachromasia in the present study could represent an inhibition of viral RNA synthesis, or more likely, the inability of acridine orange to intercalate with viral RNA which had already intercalated with Act D, as recently shown for Borna disease virus single-stranded RNA (20). The maximum inhibition of rotavirus replication (59.9%) demonstrated by immunofluorescence was detected when the drug was used at the concentration of 2.5 µg/ml, 48 h post-infection. After this period of time, lower levels of inhibition were observed. This effect could be explained by the consumption or breakdown of the drug, or both, because the drug was not replenished throughout the experiment. The experiments were carried out from 24 h post-infection onward because viral RNA and antigen could not be demonstrated before, probably due to the low multiplicity of infection used. Nevertheless, the inhibition of virus replication proved to be dose dependent. Since at the early stages of rotavirus replication positive strands of parental double-stranded RNA are used as templates to produce negative strands which originate the double-stranded genome of the progeny and mRNA (1), we suggest that Act D may complex with positive strands soon after transcription. This complex can prevent newly synthesized mRNA from being translated. Interestingly, double-stranded RNA synthesis was not inhibited by the drug. It is possible that inhibition could occur later during the infection at a time after that evaluated. Nevertheless, intercalation of Act D in transient double-stranded RNA at the time of mRNA transcription may be suggested, even though it does not explain why the synthesis of progeny double-stranded RNA is not inhibited. Instability of the double-stranded RNA/Act D complex could be the reason for the inability of the drug to prevent synthesis of the progeny double-stranded RNA. Although binding of Act D to double-stranded RNA was not confirmed, we demonstrated the inhibition of the number of cells presenting virus-specific fluorescence, suggesting a reduction of mRNA synthesis by the drug.

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