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Inhibition of Coronavirus 229E Replication by Actinomycin D

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The yields of human coronavirus 229E grown in L132 cells were markedly inhibited by actinomycin D, the 50% inhibitory dose being 0.1 μg/ml. Inhibition was maximal during the early phase of virus replication, did not appear to involve viral RNA synthesis per se, and was shown to be dependent on the input multiplicity of infection.

Recent biochemical studies have revealed that the single-stranded RNA of several coronaviruses is polyadenylated, indicating that it is probably of positive polarity (4, 6, 9-11), and the genome of infectious bronchitis virus has been reported to be infectious (5, 10), supporting evidence that the coronavirion RNA has a message function. These findings imply that coronaviruses would not require host nuclear functions for replication and that addition of inhibitors of host RNA synthesis, such as actinomycin D (act D), would facilitate the study of virus replication.

However, the effect of act D on the replication of coronaviruses has been examined in several instances with conflicting results. Parker et al. (9) reported that 1 μg of act D per ml had no effect on the replication of rat coronavirus, as measured by induction of cytopathic effect or of development of viral antigen detectable by fluorescent antibody. Mallucci (7) grew mouse hepatitis virus to normal titers in mouse macrophage cultures in the presence of 0.01 and 0.05 μg of the drug per ml. On the other hand, Lomniczi (Abstr. 3rd Int. Congr. Virol., C120, p. 231, 1975) reported not only that the drug reduced the yield of infectious bronchitis virus but also that the extent of reduction depended on the nature of the host cells. A 0.02-μg amount reduced the yield to 3% of controls when tested in chicken lung cells, but 10-fold-greater concentrations were needed to produce the same degree of inhibition in kidney cells. Clarke (2) grew transmissible gastroenteritis virus in the presence of 0.2 μg of act D per ml and observed an 84 to 92% reduction in yield of virus at 18 h postinfection (p.i.), depending on the mode of exposure; but, although Mishra and Ryan have reported that transmissible gastroenteritis virus-specific RNA synthesis was insensitive to 0.1 to 0.2 μg/ml and was maximum at 8 h p.i., they did not report on the yields of infectious virus in these experiments (8).

In the studies reported here, we have obtained evidence that the replication of human coronavirus (HCV) 229E in L132 cells is specifically inhibited by act D; yields of 229E from cells incubated in the presence of various levels of this drug were depressed in comparison with yields obtained from parallel cultures not exposed to the drug. This effect was reproducible, and the inhibition was proportional to the amount of act D present during growth. The results of the initial dose-response experiments are shown in Fig. 1. Controls were included which indicated that host RNA synthesis, as expected, was inhibited in proportion to the act D concentration, whereas adenovirus multiplication was severely repressed at all concentrations tested. Thus, DNA-dependent activities in these cells were shown to be curtailed under our experimental conditions. On the other hand, vesicular stomatitis virus, insensitive to act D and independent of host nuclear activity, was able to replicate normally in the presence of 1 μg/ml, 10 times the 50% inhibitory dose for 229E, and 10 μg/ml still permitted virus replication to 63% of control (no drug) levels (unpublished data).

Because the replication of 229E was sensitive to act D, a growth curve was established (Fig. 2), and the effect of adding act D at various stages during the growth cycle was studied (Fig. 3). Inhibition of 229E was maximal when act D was present throughout the growth cycle, being proportionally less when the drug was added later in the course of replication. After an initially high level of inhibition early in growth cycle, there was a progressive decrease until about 8 to 10 h, when the effect of the drug rapidly diminished, having no further deleterious effect after 16 h. These results indicate that the target reaction for act D inhibition is maxi-

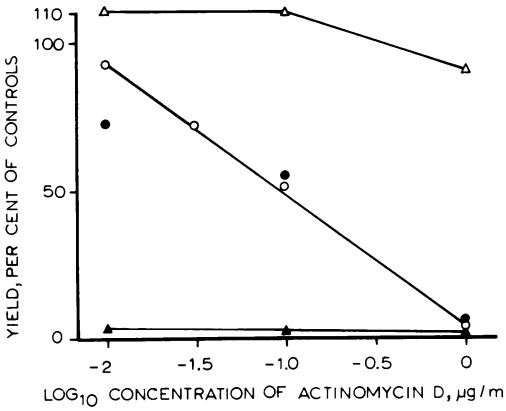

FIG. 1. *Effect of act D on HCV 229E* (○), *vesicular stomatitis virus* (△), *and adenovirus type 7* (▲) *replication and RNA synthesis* (●) *in L132 cells. Dose-response curves were obtained by infecting tube cultures with the test and control viruses under the following appropriate conditions. HCV 229E: input multiplicity, 3 PFU/cell; adsorbed 1 h, 33°C; incubation, 33°C, 40 to 42 h. Vesicular stomatitis virus: input multiplicity, 10 PFU/cell; adsorbed 1 h, 33°C; incubation, 33°C, 16 h. Adenovirus type 7: input multiplicity, 7.5 50% tissue culture dose per cell; adsorbed 2 h; incubation, 37°C, 32 h. Act D was added with the media after the adsorption period. After incubation, virus was released by three cycles of freeze-thawing and assayed in L132 cells (229E and vesicular stomatitis virus, plaque assay [3]; adenovirus type 7, end point titration). RNA synthesis was evaluated after addition of different levels of act D, by determining the amount of [5-^{3}H]uridine (specific activity, 29.65 Ci/mmol; New England Nuclear Corp.) incorporated into acid-precipitable cell extracts. After incubation for 1 h, culture medium was removed, the cells were washed twice with phosphate-buffered saline, and 1.5 ml of phosphate-buffered saline was added per tube. The cells were then disrupted by three freeze-thaw cycles. A 1.5-ml amount of 10% trichloroacetic acid was added to each tube and then incubated for 1 h at 0°C to allow maximum precipitation. Precipitates were washed twice with 5% trichloroacetic acid and then solubilized with 1 ml of NCS (Amersham/Searle Corp.) and digested for 1 h at 50°C. Digests were neutralized with glacial acetic acid (0.03 ml/ml of NCS), and 10-ml/tube scintillation cocktail (6 g of 2, 5-diphenyloxazole in toluene per liter) was added; the digests were then transferred to counting vials and counted in a Beckman LS-250 scintillation counter.*

mally sensitive in the first 10 h of the replicative cycle. Of additional interest was the finding that virus yields from cultures treated with act D later than 16 h p.i. were slightly, but consistently, higher than untreated controls; however, this observation was not explored further.

When we compared the pattern of RNA synthetic activity in uninfected L132 cells with that of cells treated either with act D or 229E or with both drug and virus by pulse-labeling appropriate groups of cultures for 1 h at hourly intervals and measuring the acid-precipitable [^{3}H]uridine, we observed several features (Fig. 4). First, infection with 229E apparently depressed the overall RNA synthetic activity in the L132 host system by as much as 12 to 20%. Second, act D progressively depressed both uninfected- and

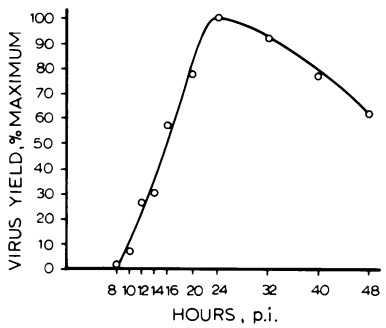

FIG. 2. *Growth curve of HCV 229E obtained by infecting L132 cells with an input multiplicity of 10 PFU/cell in tube cultures (medium 199, 0.2% bovine serum albumin, and antibiotics), removing three cultures at each of the indicated times p.i., and assaying these for virus content after freeze-thawing to aid in virus release (3). Numbers shown represent averages of repeated experiments. The virus yield was routinely 100 to 150 PFU/cell and in some cases was even as high as 300 PFU/cell.*

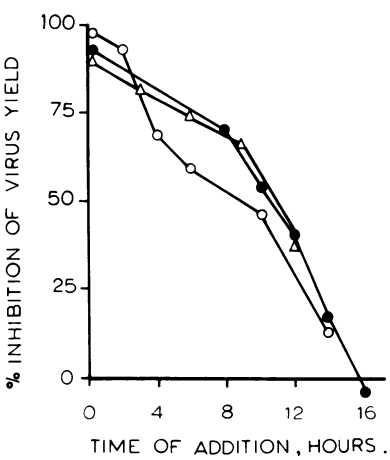

FIG. 3. *Results of three experiments* (○, ●, △) *showing the effect of the addition of act D at indicated times during the virus growth cycle. L132 cells were infected with 11 to 15 PFU of HCV 229E per cell, medium 199 with 2% bovine serum albumin was added, and the cells were incubated at 33°C. At intervals p.i., 100 μl of act D to give a final concentration of 2 μg/ml was added to replicate cultures and incubated at 33°C for the remainder of the 24-h period studied. At this time, all cultures were frozen and titrated by plaque assay. The results are expressed as the percent inhibition by act D of virus yields as compared with those obtained from the untreated controls.*

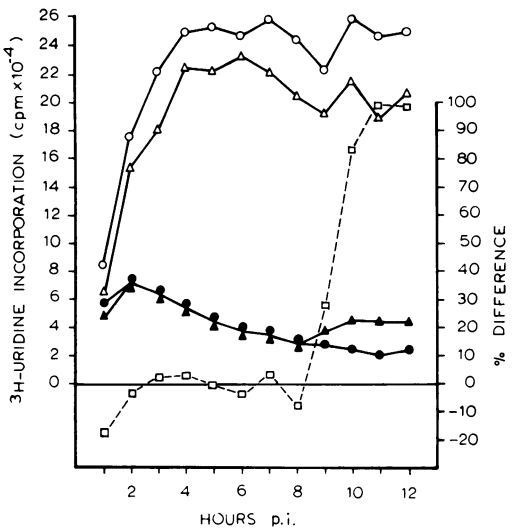

FIG. 4. *Incorporation of [^{3}H]uridine into acid-precipitable fractions of four groups of cultures was followed as a measure of RNA synthetic activity by the method of Ball et al. (1). L132 cell monolayers were prepared in glass scintillation vials, and two vials from each group were pulsed at hourly intervals for 1 h with 2.5 μCi of [5-^{3}H]uridine per vial. After each pulse, cultures from each group were placed immediately on ice, and medium was replaced with 1.5 ml of saline at 0°C. Each vial was then filled with 1.5% (vol/vol) perchloric acid (approximately 12 ml) and allowed to stand for 10 to 15 min. The perchloric acid was decanted, and the washing procedure was repeated twice. Ethanol (3 to 5 ml) was then added, swirled briefly, and poured off, and the vials were inverted to drain them completely. A 0.5-ml amount of NCS was added, and the vials were incubated for 1 h in a water bath at 50°C. After neutralizing with 0.015 ml of glacial acetic acid per vial, 10 ml of counting cocktail (6 g of 2, 5-diphenyloxazole in toluene per liter) was added, and each vial was counted in an LS-250 Beckman scintillation counter. The four groups of cultures were the following: uninfected L132 cells (○); uninfected cells + 1.0 μg of act D per ml (●); 229E-infected cells (△); 229E-infected cells + 1.0 μg of act D per ml (▲). The RNA synthetic activity at each hour p.i. is shown as counts per minute per culture. The effect of virus infection on the depression of RNA synthesis in host cells treated with act D is expressed as the percent difference (□).*

infected-cell RNA synthesis to a similar extent until approximately 8 h p.i. when, third, there appeared to be a significant increase in RNA synthesis in only the virus-infected cells, which was presumably virus-specific RNA.

Thus, it seems that although the target of act D inhibition is an early event, it does not act on viral RNA synthesis per se. These results support the finding of Mishra and Ryan (8) that transmissible gastroenteritis virus-specific RNA synthesis continued in the presence of act D. However, the depression of the yield of infectious 229E virus during the first 8 to 10 h (Fig. 3) indicates inhibition of some early virus-cell interaction. If a limiting unstable host product were involved in this phenomenon, then the degree of inhibition should vary with the virus input multiplicity since the utilization of such a product would be more efficient in the presence of a higher virus concentration, resulting in higher yields of infectious virus in the presence of act D. The effect of the input multiplicity of 229E on the degree of inhibition produced by act D was, therefore, assessed by comparing yields of virus from cultures infected at four different input multiplicities in the presence and the absence of a single level of act D (1 μg/ml). The results of these experiments are shown in Fig. 5.

The yield of virus obtained from act D-treated cultures increased with increasing virus input and at an input multiplicity of 42 PFU/cell was six times higher than that obtained after infection with 0.8 PFU/cell. However, in the control untreated cells, increasing the input multiplicity resulted in decreased virus yields, by as much as 40% of the maximum. Thus, although it is evident that the virus multiplicity used to initiate infection affected the magnitude of the sensitivity to act D, the decreasing yields obtained without act D demonstrate some type of autointerference which requires further investigation.

These results support the conclusion that a limiting unstable host product is required during the early stage of 229E replication in L132 cells. This apparent requirement for a host component during early stages of replication may have some bearing on the characteristic narrow host range of coronaviruses. The use of act D should prove to be a valuable tool in further defining the host-

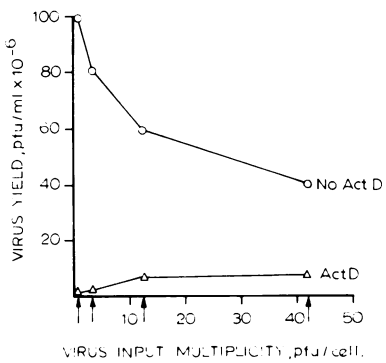

FIG. 5. *Effect of input miltiplicity of infection (0.8, 3.3, 12, and 42 PFU/cell) on virus yield at 28 h p.i. in the presence (△) and the absence (○) of 1.0 μg of act D per ml. Triplicate tube cultures of L132 cells were infected, and virus yield was assayed as described previously (3).*

virus relationship and elucidating virus-specific functions. Our studies presently involve the use of act D to preferentially produce specific virus RNA relatively free from host RNA. Future studies concerning the possibility of some host restriction on a maturation step of the virus are indicated by the observation that at 16 or more h p.i., there was an increased yield of virus in act D-treated cultures. Finally, the observed depression of virus yield due to increasing input multiplicity in untreated cultures must be clarified, especially since this phenomenon was affected by act D.

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