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Reovirus Type 2-Infection, Cycloheximide, and Cell Death.* (32337)

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Mammalian cells infected with reovirus have been shown to produce two kinds of ribonucleic acids, an early single-stranded species, followed by a double-stranded structure which is also found in the mature virion (1,2,3). The present communication describes the effect of cycloheximide, an antagonist of protein synthesis, on the production of viral RNA and of complete reovirus particles. In addition we wish to describe the unique development of extensive cytopathology occurring only in infected cell cultures which have been treated with the antibiotic. To our knowledge, this combined action between virion and antibiotic has never been previously described.

During the course of this investigation Watanabe, Kudo and Graham(4) and Shatkin and Rada(2) reported that inhibition of the production of the two kinds of ribonucleic acids by cycloheximide was dependent on when the antagonist was added. Whereas the early addition of cycloheximide to L cells infected with reovirus type 3 was found to inhibit the production of both kinds of nucleic acids, the antibiotic when added later during the infectious cycle selectively inhibited the synthesis of the double-stranded form of viral RNA.

Materials and methods. Cell cultures. The routine growth and preparation of HeLa cells have been described previously(5). The growth and basic experimental media consisted of Eagle's basal medium (EBM) plus 10% calf serum, and EBM plus 0.2% fetal calf serum, respectively.

Virus. Reovirus type 2 (strain D-5) was grown in HeLa cell cultures and titer was determined by the immunofluorescent assay method(6). The titer in RA cells was 2.1×10^8 infectious units (IU) per ml.

Infection procedure. Monolayers of HeLa cultures were infected with an exposure multiplicity of 10 to 20 IU per cell. After adsorption for 2 hours at 37°C, unadsorbed virus was removed by washing with balanced salt solution; then the experimental medium was introduced, and the culture reincubated at 37°C.

Protein and RNA analyses. Protein synthesis was determined by exposing experimental cell cultures at various intervals to tritiated leucine (New England Nuclear Corp., specific activity 5.0 C/mM) for 15 minutes. The amount of trichloroacetic acid-insoluble radioactivity incorporated was measured in a Packard liquid scintillation spectrophotometer and expressed as counts per unit of absorbancy at 280 mμ. The synthesis of RNA was determined as previously described(7).

Antibiotic. Cycloheximide (Actidione) was generously supplied by the Upjohn Company, Kalamazoo, Michigan.

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Results. Effect of cycloheximide on formation of reovirus. Cycloheximide at two concentrations (2.5 μg/ml and 5 μg/ml) was added to HeLa cell cultures at zero hours after infection. At 24 hours postinfection (p.i.) the yields of infectious virus from all cultures were determined. As seen in Table I, the antibiotic at both concentrations completely suppressed virus production.

When cycloheximide was added at various intervals beyond 4 hours after infection, partial production of the virion was obtained (Fig. 1). Maximal yields of the virion were attained only when the inhibitor was added at 10-12 hours p.i. or later.

Effect of cycloheximide on protein synthesis. Monolayer cultures of HeLa cells were treated with 5 μg/ml of cycloheximide and the synthesis of protein was followed by pulse labelling (15 min) with tritiated leucine (0.75 μc/ml). The total amount of acid-insoluble radioactivity incorporated at various intervals was determined (see *Materials and Methods*).

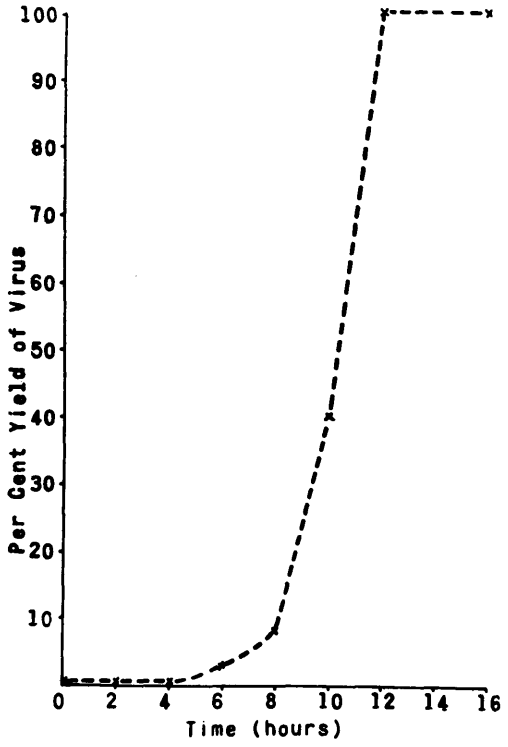

FIG. 1. Effect of cycloheximide added at different intervals of the infectious sequence. Cycloheximide (5 μg/ml) added at times indicated. Virus yields determined at 24 hr post-infection and expressed as per cent of untreated infected controls.

TABLE I. Effect of Cycloheximide on Production of Reovirus Type 2 in HeLa Cells.

Conc of cycloheximide (μg/ml)	Virus titer (infectious units/ml $\times 10^4$)	% Virus inhibition
0*	1800	—
2.5	7.5	100
5	9.0	100

* Virus titer at 0 hr after infection was 11 $\times$ 10^4 IU per ml. AU virus titer determined at 24 hr after infection.

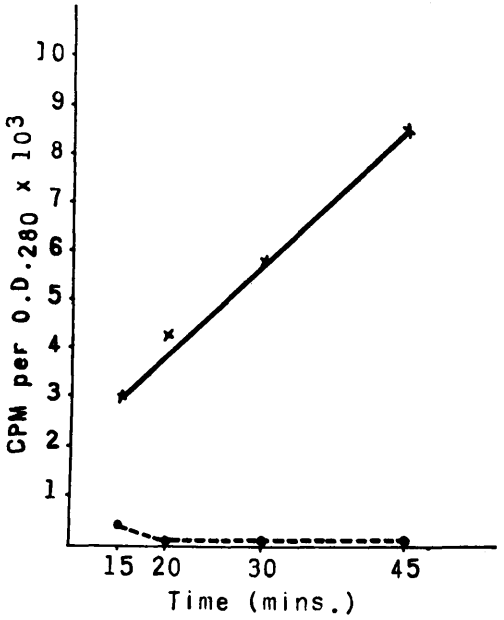

FIG. 2. Effect of cycloheximide on protein synthesis in uninfected HeLa cells. At various intervals the cell cultures were exposed to tritiated leucine for 15 min and were subsequently processed for determination of radioactivity as previously described.

The results (Fig. 2) indicate that synthesis of protein is inhibited by 87% within 15 minutes of addition of the antibiotic. After 30 minutes the inhibition of protein synthesis is almost complete (99%). At 2.5 μg/ml the suppression of protein synthesis was about 90%.

Inhibition of double-stranded viral RNA synthesis by cycloheximide. While examining the effect of the addition of cycloheximide at various intervals after infection on the production of viral RNA, it was found that addition of the antibiotic late in infection (8 to 10 hrs. p.i.) selectively inhibited the synthesis of the

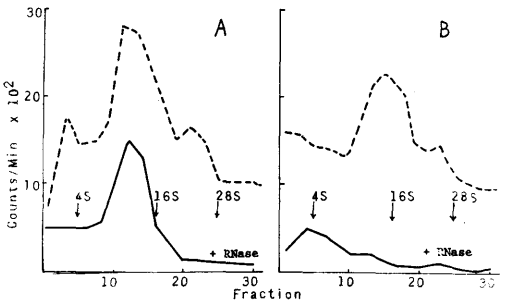

FIG. 3. Sucrose gradient analysis of RNA from cycloheximide treated and untreated HeLa cells infected with reovirus type 2. Cycloheximide (5 μg/ml) was added at 8 hr p.i. and all infected cultures were labeled with tritiated uridine (1 μc/ml) for 120 min at 10 hr p.i. Total RNA was extracted and analyzed by sucrose gradients as described(7). The RNA fractions from alternate tubes were treated with RNase at a concentration of 2 μg/ml for 30 min at 37°C. (A) No cycloheximide. (B) Plus cycloheximide.

double-stranded RNA. The inhibitor when added at zero time of infection completely suppressed the formation of both kinds of viral nucleic acids.

Monolayer cultures of HeLa cells infected with reovirus type 2 were treated at 8 hours p.i., with 5 μg of cycloheximide per ml. Untreated and antibiotic-treated infected cultures were both exposed to tritiated uridine from 10 to 12 hours p.i. The RNA was extracted and fractionated in sucrose gradients as previously described(8). As shown in Fig. 3, almost 40% of the viral RNA formed in untreated infected cultures was resistant to RNase. In contrast, no RNase-resistant component could be demonstrated in infected cultures treated with cycloheximide. In such cultures only single-stranded RNA was formed.

Cytopathology, reovirus infection and cycloheximide. Microscopic examination of infected HeLa cells treated with the antibiotic revealed the appearance of cytopathology at about 4 hours post-infection (Table II). The morphological changes became progressively marked, and by 10 to 12 hours after infection most of the cells had rounded and sloughed off. In contrast, control cell cultures treated with the antibiotic exhibited no morphologic alterations even as late as 13 hours after treatment. Furthermore, untreated-infected cultures did not show any cytopathology until 14 to 16 hours after infection.

Essentially similar results were obtained when actinomycin D. was used at concentrations (0.05 μg per ml), which selectively suppressed host cell RNA synthesis but not the production of viral RNA(7).

We have reported(9) the cytotoxic properties of UV-inactivated reovirus type 2 when added to HeLa cell cultures at high multiplicities of exposure (80 to 100). Under such conditions morphologic changes occurred as early at 3 to 4 hours after infection. Included in Table 2 are the results obtained when UV-inactivated reovirus type 2 at an exposure multiplicity of 10 is used alone and in combination with cycloheximide (5 μg per ml). In both instances cytopathology occurred as early as 4 hours after infection. However, the morphologic changes were more extensive in antibiotic-treated infected cell cultures than in untreated cultures.

TABLE II. Effect of Cycloheximide and Reovirus Infection on HeLa Cell Monolayers.

Appearance of cytopathology* (hr)	Samples: Cycloheximide†	Virus‡	Cycloheximide† + virus‡	Cycloheximide† + UV-inactivated virus§	UV-inactivated virus§
2	—	—	—	—	—
3	—	—	+	+	—
4	—	—	++	++	+
6	—	—	++	++	+
10	—	—	+++	+++	++
12	—	—	+++	+++	+++

* Cytopathology scored as follows: — = no change; + = degeneration of 25% of monolayer; ++ = degeneration of 50% or more of monolayer; +++ = degeneration of 75% or more of monolayer; ++++ = degeneration of entire monolayer.

† 5 μg cycloheximide per ml added at zero hour of infection.

‡ Reovirus type 2 at an exposure multiplicity of 10.

§ UV-inactivated reovirus type 2 at an exposure multiplicity of 10.

Discussion. The present investigation confirms the findings that the synthesis of both kinds of ribonucleic acids in reovirus-infected cells is dependent upon the formation of early protein(s). It also reaffirms the selective inhibitory action of cycloheximide when added late in the infectious sequence (8-10 hours p.i.). It was presumed that, since the double-stranded RNA component is preferentially inhibited at this late period, the continuous synthesis of another new protein(s) is required for the specific production of the RNA component.

The accelerated appearance of extensive cytopathology in the absence of virus replication in infected cell cultures treated with cycloheximide is inexplicable at present. It suggests the possibility that the antibiotic acts synergistically with some component(s) of the incoming virion in a manner yet to be defined. Several investigators have suggested that the cytotoxic effect of many virions which cause cell death in the absence of the production of infectious particles can be directly related to virus-induced metabolic depressions. Thus, the cell-killing property of EMC virus and of poliovirus is due to their ability to inhibit either cellular RNA only(10) or both cellular RNA and protein synthesis (11,12). In the poliovirus system, it was further determined that such virus-induced morphological lesions could be caused by the synthesis of new virus coat proteins, which occurred toward the end of the latent period (13). The possibility that the capsid protein from the incoming virion(12) or some other virion-related protein may be responsible for the cytopathology observed cannot be excluded. Although suppression of both cellular DNA and protein synthesis in reovirus-infected cells has been reported(1,14), it is unlikely from the evidence obtained here that a virion-induced protein is responsible for the morphologic changes since protein synthesis is almost completely inhibited by the antibiotic. That new RNA synthesis is also not involved is indicated by the appearance of morphologic lesions in infected cultures treated with actinomycin D. In such cultures cellular RNA activity is markedly suppressed. Since the inhibition of cellular DNA synthesis occurs late in the infectious cycle, it was postulated that this may well be a pathological consequence of virion replication.

Summary. The antibiotic cycloheximide, an antagonist of protein synthesis, when added early in the infectious cycle has been found to inhibit the production of the two kinds of virus-specific ribonucleic acids found in HeLa cells infected with reovirus-type 2. When added at later periods, the antibiotic selectively inhibits the formation of the double-stranded form of viral RNA. It was also noted that the addition of virus-inhibiting concentrations of the antibiotic to infected HeLa cells results in the accelerated appearance of extensive cytopathology of the monolayers. In contrast, during the same period normal cell cultures treated with the antibiotic and infected cell cultures without cycloheximide did not exhibit any morphologic changes.

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