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Effects of Actinomycin D and Ultraviolet and Ionizing Radiation on Pichinde Virus

MICHAEL F. CARTER, FREDERICK A. MURPHY, J. PIERRE BRUNSCHWIG, CHRISTINE NOONAN, AND WILLIAM E. RAWLS

Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77025, and National Communicable Disease Center, Atlanta, Georgia 30333

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Actinomycin D (0.05 μg/ml) suppresses the synthesis of ribosomal RNA of baby hamster kidney (BHK21) cells. The production of infectious Pichinde virus was enhanced in the presence of actinomycin D, although the production of virus particles was not substantially different from cultures inoculated in the absence of the drug. By prelabeling BHK21 cells with ^{3}H-uridine and then allowing the virus to replicate in the presence of actinomycin D, it was possible to show that ribosomal RNA synthesized prior to infection was incorporated into the virion. A single-hit kinetics of inactivation of Pichinde virus was observed with ultraviolet light, suggesting that the virus contains only a single copy of genome per virion. Comparison of the inactivation kinetics by gamma irradiation of Pichinde virus with Sindbis and rubella virus indicated that the radiosensitive genome of Pichinde virus was about 6×10^6 to 8×10^6 daltons. This value is greater than the 3.2×10^6 daltons which was estimated by biochemical analysis. One possible explanation considered is that the ribosomal RNA of host cell origin is functional and accounts for the differences in genome size estimated by the two methods.

The virions of the arenavirus group contain multiple electron-dense granules which resemble ribosomes (4, 17, 18). The nucleic acid of lymphocytic choriomeningitis virus (LCMV) and Pichinde virus, which are members of the arenavirus group, have been found to contain multiple RNA components (3, 19, 20, 21). The RNA of Pichinde virus contained 31, 28, 22, 18, and 4 to 6*S* components. The 28 and 18*S* RNAs were methylated and newly synthesized; however, newly synthesized 28 and 18*S* RNAs were not incorporated into virions which were grown in the presence of concentrations of actinomycin D which inhibited the synthesis of host cell ribosomal RNA (3).

The present study was undertaken to answer three questions. (i) Is the virus grown in the presence of actinomycin D infectious and, if so, is ribosomal RNA synthesized prior to infection incorporated into the virion? (ii) Is there evidence of multiple copies of genome in the virions? (iii) Does the genome size estimated by ionizing radiation correspond to the genome size estimated by chemical analysis?

MATERIALS AND METHODS

Tissue cultures. Two continuous lines of African green monkey kidney cells, Vero (31) and BSC-1 (8), and baby hamster kidney (BHK21; reference 29) were obtained from laboratory stocks. Growth medium for the three cell lines consisted of Eagle medium supplemented with 0.75 g of sodium bicarbonate per liter, 10% fetal bovine serum, 100 U of penicillin, and 100 μg of streptomycin per ml. Maintenance medium for the cells consisted of Eagle medium supplemented with 1.5 g of sodium bicarbonate per liter, 2% fetal bovine serum and antibiotics. When the cells were incubated in a 5% CO_2 atmosphere, the content of sodium bicarbonate was increased to 3 g per liter. Primary cultures of chicken embryo fibroblasts were prepared from 8- to 11-day-old chicken embryos as previously described (6).

Viruses and virus assays. Pichinde virus, strain AN 3739 (16, 30), Sindbis virus strain AR 339, and rubella virus strain R-1 (15) were used. Stocks of Pichinde virus and Sindbis virus were prepared in BHK21 cells, whereas rubella virus stocks were prepared in Vero cells. Pichinde virus was assayed in Vero cell cultures by the plaque counting method previously described (16). Sindbis virus was assayed by similar methods in chicken embryo fibroblasts, except that neutral red was incorporated into the initial agar overlay, and plaques were counted 24 to 36 h after infection. Rubella virus titers were determined in BSC-1 cells by the use of the hemadsorption negative plaque technique (23).

Preparation of labeled Pichinde virus. BHK21 cells were seeded in 16-oz (0.473 liter) prescription bottles (10^6 cells/bottle) and incubated at 37 C for 36

h. The medium was then replaced with 25 ml of growth medium containing 2 μCi of uridine-5-^3H (specific activity = 30 Ci/mmol; Amersham/Searle) per ml. After an additional 48 h of incubation, the medium was replaced with an equal volume of maintenance medium supplemented with uridine (25 μg/ml). Approximately 18 h later the cells were infected with 1 to 5 PFU per cell. After adsorption of the virus, the cells were washed once with maintenance medium, and 25 ml of fresh medium containing 0.2 μCi of uridine-2-^{14}C (specific activity $\geq$ 50 mCi/mmol; New England Nuclear Corp., Boston, Mass) per ml was added. In some experiments the maintenance medium containing the ^{14}C-uridine was supplemented with 0.05 μg of actinomycin D (Calbiochem, Los Angeles, Calif) per ml. The infected cells were incubated for 48 h at 37 C, and the extracellular fluids were harvested and clarified by centrifugation (1,100 $\times$ g for 20 min).

Concentration and purification of Pichinde virus. Pichinde virus was precipitated from clarified extracellular fluid with polyethylene glycol 6000 (Union Carbide Corp., New York) and purified by centrifugation in a 20 and 50% (wt/wt) discontinuous sucrose gradient followed by centrifugation in a continuous sucrose gradient. The details of the purification method have been described (3).

Isolation of Pichinde virus RNA. The fractions from the continuous sucrose density gradient containing maximum amount of virus (density = 1.14–1.18 g/cm^3) were pooled, and the viral RNA was isolated as previously described (3).

Isolation of RNA from cells. Confluent monolayers of HeLa cells were labeled with ^{14}C-uridine (2 μCi/ml) for 24 h. The cells were collected, suspended in 0.01 M acetate buffer (pH 5.1), and the RNA was isolated as described by Scherer and Darnell (24).

Velocity sedimentation of RNA in sucrose gradients. A 0.2-ml sample of the RNA preparation was layered on a 5 to 20% (wt/wt) linear sucrose gradient and centrifuged under conditions previously described (3). ^{14}C-labeled HeLa-cell RNA was used as reference marker. After centrifugation fractions (0.15 ml) were collected by bottom puncture of the tube onto Whatman filter paper disks, and the acid-precipitable radioactivity of each fraction was determined (2, 3). Radioactivity was measured in a Beckman (model LS-250) liquid scintillation spectrometer.

Ultraviolet (UV) and γ-irradiation of Pichinde virus. Pichinde virus was diluted 10-fold in Tris-buffered saline (pH 7.4) supplemented with 1% fetal bovine serum, and 1.0-ml fractions were delivered to 60-mm tissue culture dishes. Uncovered dishes were irradiated at an estimated dose of 3.5 ergs per mm^2 per s by using a Sylvania G15T8 germicidal lamp. After appropriate exposure time, each sample was diluted 10-fold in Eagle medium, and residual infectivity was assayed by the plaque counting method. Sindbis and rubella viruses were irradiated under identical conditions.

To measure the sensitivity to γ-irradiation, triplicate samples of lyophilized virus were irradiated for various time intervals. The source of radiation was the research reactor located at the Nuclear Science Center, Texas A&M University, College Station, Texas. The vials were placed in an aluminum container lined with Boron carbide which screened the samples from thermal neutrons. The absorbed gamma dose was measured by using Fricke dosimeters. At a dose rate of 2.35 $\times$ 10^6 rads of gamma radiation per h it was estimated that the samples were exposed to not more than 7.2 $\times$ 10^2 rads of fast neutrons per h and 5 $\times$ 10^2 mrads of thermal neutrons per h. For the samples the reactor was operated at power levels of 200 kW or 1,000 kW which delivered approximately 4 $\times$ 10^5 rads/h or 2 $\times$ 10^6 rads/h, respectively, of gamma radiation at a distance of 40 cm. The total doses delivered ranged from 0.5 $\times$ 10^5 to 20 $\times$ 10^5 rads. The lower doses, 0.5 $\times$ 10^5, 10^5, and 2 $\times$ 10^5 rads were delivered when the reactor was operated at 200 kW for 7.5, 15, and 30 min, respectively, whereas the higher doses, 5 $\times$ 10^5, 10 $\times$ 10^5, 15 $\times$ 10^5, and 20 $\times$ 10^5 rads, were delivered when the reactor was operated for 15, 30, 45, and 60 min, respectively, at a power level of 1,000 kW. After irradiation the ampules were held at 4 C until assayed for residual infectivity. Lyophilized Sindbis and rubella viruses were irradiated under identical conditions.

Electron microscopy. To obtain thin sections, BHK21 cell monolayers in 32-oz (0.946 liter) prescription bottles were washed once with phosphate buffer (Millonig's, at 4 C, pH 7.3), scraped from the glass with a rubber spatula and centrifuged at 800 $\times$ g for 20 min. The packed cells were fixed with buffered 3.0% glutaraldehyde for 60 min. The fixed cells were treated with 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in an Araldite-Epon mixture, and sections were stained with uranyl acetate and lead citrate as previously described (18). To carry out negative staining, samples from fractions of the continuous sucrose gradients used in the final step of purification were prepared by the droplet pseudoreplication method (13, 27). Osmium tetroxide vapor was used to enhance the virus particle contrast (13). The calculation of virus particles in suspension per milliliter of sample was done by using the droplet formula (13).

RESULTS

Actinomycin D and the virion. Pichinde virus replicated in the presence of 0.05 μg of actinomycin D per ml does not incorporate newly synthesized ribosomal RNA (3). To determine whether virions without newly synthesized ribosomal RNA were infectious, virus was grown in the presence or absence of actinomycin D and purified. The virus particle-to-infectivity ratio was determined in the portion of the continuous sucrose gradient which contained maximum infectious virus. The results are shown in Table 1. The yield of virus particles in cultures incubated in the presence of actinomycin D was similar to the yield observed in the absence of the drug. However, the particles were found to be more infectious when replicated in the presence of actinomycin D.

TABLE 1. *Effect of actinomycin D on Pichinde virus infectivity*

Fraction no.	Density (g/cm³)	Virus particles/ml		PFU/ml		Particles per PFU	
		Actinomycin D[a]	No drug	Actinomycin D	No drug	Actinomycin D	No drug
7	1.18	1.1×10^9	6.4×10^8	1.1×10^7	1.2×10^6	100	533
8	1.17	1.7×10^9	2.1×10^9	1.0×10^8	1.1×10^7	17	191
9	1.16	5.2×10^9	2.8×10^9	4.7×10^8	5.5×10^7	11	51
10	1.15	1.0×10^{10}	8.6×10^9	8.0×10^8	1.4×10^8	13	62
11	1.14	5.0×10^9	5.9×10^9	8.5×10^8	1.7×10^8	6	35
12	1.13	1.4×10^9	6.2×10^9	2.7×10^8	8.0×10^7	5	78

[a] Virus replicated in the presence of 0.05 μg of actinomycin D per ml.

Clearly, the lack of newly synthesized ribosomal RNA in the virions does not decrease virus infectivity.

The morphology of the virus particles did not appear to be altered by the presence of actinomycin D. BHK21 cells were infected with 1 to 2 PFU of Pichinde virus per cell and incubated for 48 h in the presence or absence of actinomycin D. Electron microscope examination of thin sections revealed that the virus particles in BHK21 cells were indistinguishable from those observed in Vero cells (18). There were no detectable differences in the number or appearance of internal granules within the virus particles replicated in the presence of actinomycin D.

The possibility that host-cell ribosomal RNA synthesized prior to infection was incorporated into the virus was examined. Cells were prelabeled for 36 h with ³H-uridine, and after an 18-h chase with cold uridine the cells were infected with Pichinde virus. The cell cultures were then incubated for 48 h in medium containing ¹⁴C-uridine, and actinomycin D (0.05 μg/ml) was added to one-half of the cultures. Figure 1 shows the profile of the purified virion RNA obtained by velocity sedimentation in a sucrose gradient. In the absence of actinomycin D, ³H-uridine was observed primarily in the major 28*S* component, while the ¹⁴C-uridine was observed in the major 28*S* peak as well as in the 22, 18, and, 4 to 6*S* components. The sedimentation patterns of the virion RNA was distinctly different when actinomycin D was added to the medium (Fig. 2). The ³H-uridine incorporated prior to infection was observed in the 28, 18 and, 4 to 6*S* regions of the gradient. However, the ¹⁴C-uridine added after infection was absent from the 28*S* component but was detected in peaks corresponding to 31, 22, and 4 to 6*S*. In four separate experiments electrophoresis of Pichinde virus RNA on 2.4% polyacylamide gels (3) also revealed only the 31 and 22*S* peaks of ¹⁴C-uridine activity (data not shown). Thus, host-cell RNA synthesized prior to infection appears to be incorporated into the virion, and this host-cell RNA corresponded in size to ribosomal RNA.

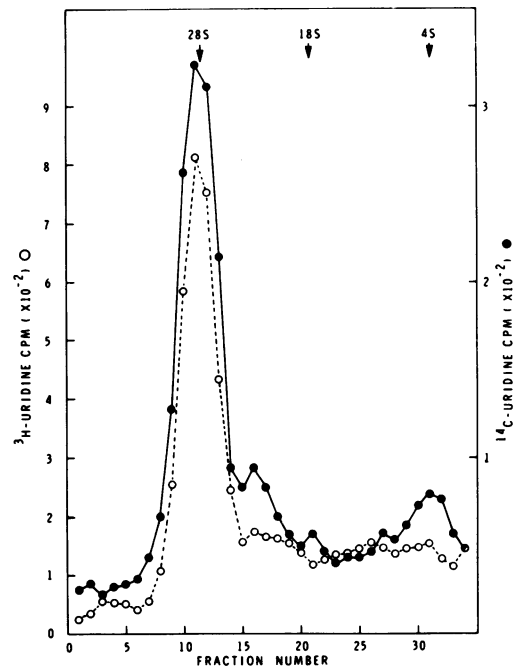

FIG. 1. *Sucrose velocity centrifugation of Pichinde virus RNA. BHK21 cells were labeled for 48 h with ³H-uridine followed by an 18-h chase with unlabeled uridine. The cells were infected and incubated for 48 h in culture medium containing ¹⁴C-uridine. Virus was purified from extracellular fluid, and the viral RNA was isolated (see Materials and Methods). A 0.2-ml sample of RNA was layered onto the top of a 5 to 20% (wt/wt) linear sucrose gradient, and centrifugation was carried out at 43,000 rpm for 3.25 h at 4 C in a Spinco SW50 rotor. Fractions were collected by bottom puncture, and acid-precipitable radioactivity of each fraction was measured. ¹⁴C-uridine-labeled HeLa cell RNA, centrifuged in a separate tube, was used as a marker to calculate sedimentation coefficients of viral RNA components (14).*

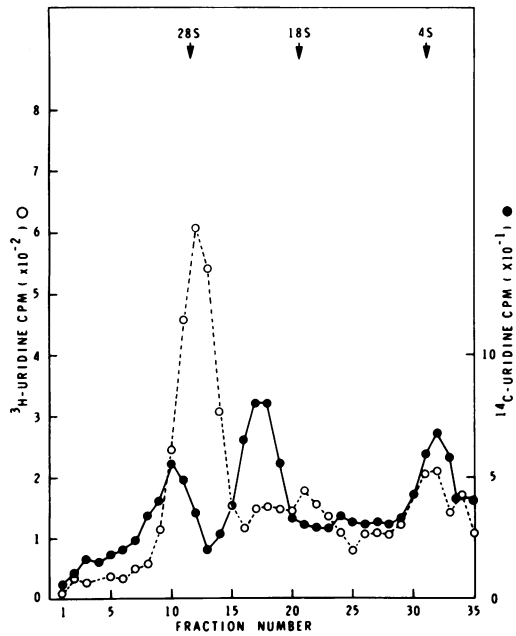

FIG. 2. *Same as Fig. 1 except that after infection cells were incubated for 48 h in culture medium containing ^{14}C-uridine and actinomycin D.*

Effects of uv and ionizing irradiation. Indirect evidence of the existence of more than one viral genome per virion was sought by examining the kinetics of inactivation of Pichinde virus with UV light. The sensitivity of Pichinde virus to UV light was measured and compared to that of Sindbis and rubella virus (Fig. 3). The linear nature of the dose-response curve indicated that inactivation of each viral preparation followed single-hit kinetics. The dose which resulted in 37% survival (D_{37}) was calculated for each virus. The D_{37} values for Sindbis (D_{37} = 140 ergs/mm^2) and rubella virus (D_{37} = 157 ergs/mm^2) were 3 to 4 times greater than that for Pichinde virus (D_{37} = 42 ergs/mm^2) which indicated the greater sensitivity of Pichinde virus to UV irradiation.

The sizes of several virus genomes have been estimated by measuring the sensitivity to gamma irradiation (1, 7, 9, 10, 12, 22). The kinetics of inactivation for Pichinde virus was measured and compared to two control viruses, Sindbis and rubella viruses, which were irradiated and treated under identical conditions. The inactivation curves contained two components, and each virus demonstrated a rapid initial inactivation which is unexplained. Figure 4 shows the portions of the inactivation curves for the three viruses which were linearly related to the dose. Comparison of the 37% survival dose for each virus tested revealed that Sindbis (D_{37} = 4.2 × 10^5 rads) and rubella virus (D_{37} = 3.8 × 10^5 rads) were approximately twice as resistant to gamma irradiation as Pichinde virus (D_{37} = 2.1 × 10^5 rads).

DISCUSSION

A common property of arenaviruses is the presence of multiple electron-dense granules within the virion instead of a discernible core (4, 17, 18). The structure and function of the nucleic acid in these viruses may thus be unique from previously studied virus groups. The dense granules could represent a nucleocapsid with helical or cubic symmetry, and the virions may contain multiple viral genomes. Alternatively it has been suggested that the granules may represent ribosomes (3, 17, 18, 20, 21). On biochemical analysis, Pichinde virus was found to contain RNA; the RNA consisted of five components with sedimentation coefficients of 31, 28, 22, 18, and 4 to 6*S*. The 28, 18, and 4 to 6*S* components were thought to be of host-cell

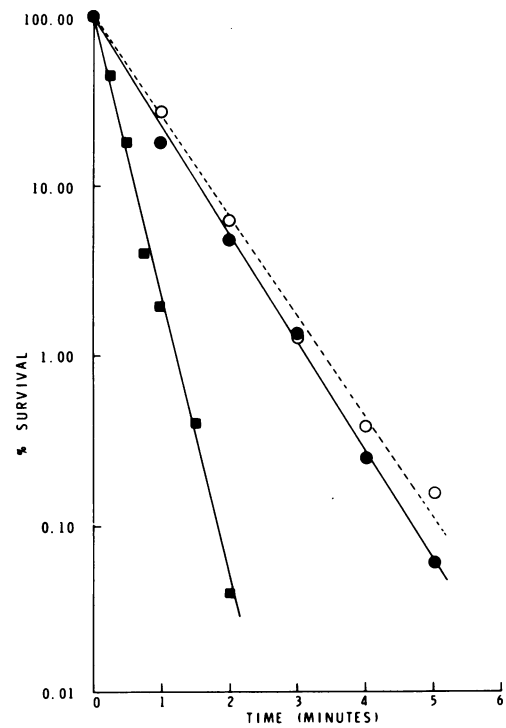

FIG. 3. *Inactivation of Pichinde virus by UV irradiation. A 1-ml amount of a 1:10 dilution of Pichinde virus in Tris-buffered saline was irradiated at different doses as described in Materials and Methods. Surviving virus was assayed by the plaque counting method. Sindbis and rubella viruses were irradiated under identical conditions. Symbols: Pichinde virus (■); Sindbis virus (●); rubella virus (○).*

origin (3). Similar observations have been reported for LCMV (20, 21).

The evidence that the 28 and 18S components of the virion are of host cell-origin consists of the following. (i) Characteristic of ribosomal RNA, the 28, 18, and 4 to 6S RNAs of the virion are methylated. (ii) The base composition of the 28 and 18S RNAs of the virion is similar to the host-cell 28 and 18S RNA. (iii) Virions replicated in the presence of concentrations of actinomycin D that inhibit 28 and 18S ribosomal RNA synthesis do not contain newly synthesized 28 and 18S RNA (3). The data of the present study indicate that ribosomal RNA synthesized prior to infection is incorporated into the virion. Actinomycin D was utilized at a concentration which inhibited ribosomal-RNA synthesis. The production of infectious particles was enhanced, although the number of electron-dense granules in the virus particles did not decrease, as observed by electron microscopy. In addition, replication of virus in the presence of actinomycin D allowed the demonstration of incorporation of host cell 28S and, to a lesser degree, 18S RNAs synthesized prior to infection. These observations indicate that either newly synthesized or preexisting host cell ribosomal RNAs can be incorporated into Pichinde virions. Pedersen (21) has reported that LCMV can also incorporate preexisting, as well as newly synthesized, host-cell 28 and 18S RNAs.

The sizes of several virus genomes have been estimated by inactivation with gamma irradiation (1, 7, 9, 10, 12, 22). Pichinde virus was more rapidly inactivated by gamma irradiation than either Sindbis or rubella virus. Comparison of the D_{37} values, which are inversely related to the size of the radiosensitive nucleic acid target (7), suggests that the functional genome of Pichinde virus is approximately twice that of Sindbis and rubella virus. Since the genomes of Sindbis and rubella virus are approximately 3×10^6 to 4×10^6 daltons (5, 25), the size of the functional genome of Pichinde virus can be estimated to be 6×10^6 to 8×10^6 daltons. The 31 and 22S RNAs of Pichinde virus correspond to molecular weights of 2.1×10^6 and 1.1×10^6, respectively. If the virion contained one molecule of each RNA species, the molecular weight of the viral genome would be 3.2×10^6 which is considerably less than the genome size estimated by gamma irradiation. The linear nature of the dose-response curves for Pichinde virus suggests that each virion contains a single copy of unique viral information. It is possible that viral-specific RNAs corresponding to either 31 or 22S may consist of a population of molecules with similar sedimentation coefficients and electrophoretic mobilities but different genetic information. Multiple segments of these RNAs per virion could then account for the greater genome size estimated by gamma irradiation.

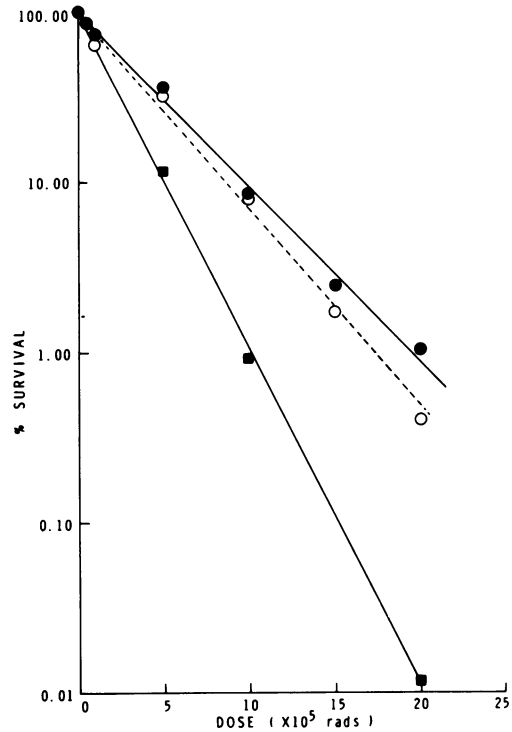

FIG. 4. *Inactivation of Pichinde virus by γ-irradiation. Triplicate samples of lyophilized Pichinde virus were irradiated at different total doses as previously described (see Materials and Methods). The samples were held at 4 C until the residual infectivity of each sample was determined. Each point represents the average surviving infectivity in the three samples. Lyophilized triplicate samples of Sindbis and rubella viruses were irradiated under identical conditions. Symbols: Pichinde virus (■); Sindbis virus (●); rubella virus (○).*

Another possibility is that the ribosomal RNA is functional. The functional integrity of *Escherichia coli* ribosomes is sensitive to gamma radiation (11). Thus, the disparity in the genome size estimated by the two methods could be accounted for by a functional role of the host-derived ribosomal RNA. Further studies will be needed to delineate between these possibilities.

Noteworthy is the replication of Pichinde virus in BHK21 cells treated with low concentrations of actinomycin D which consistently resulted in an increased yield of virus. An enhancement has also been reported for LCMV and Junin virus grown in BHK21 cells (26, 28). The increased Pichinde virus titer appeared to

be due to an increased production of infectious particles, since the total particle yield was not substantially altered. The enhanced production of infectious particles did not appear to be related to differences in the incorporation of viral RNA into the virions. It is possible that the actinomycin D suppresses a cellular function which inhibits production of infectious particles.

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