Effects of Actinomycin D on the Cytopathology Induced by Poliovirus in HEp-2 Cells

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One possible mechanism of virus-induced cell damage is that the redistributed (released) lysosomal enzymes produce the cytopathic effect during cytolytic types of infections such as poliovirus in HEp-2 cells. To determine if the lysosomal enzyme redistribution and cell damage are host-cell directed, we studied sensitivity of these events to the action of actinomycin D. By the use of actinomycin D at concentrations producing the least toxicity but maximal effectiveness in shutting down cell RNA synthesis, it was shown that the cytopathic effect and enzyme redistribution were not inhibited and, therefore, not directly controlled and induced by the cell genome in response to the virus infection. Evaluation of cytopathic effect by a phase contrast microscopy method detected changes earlier than the erythrocin B uptake method.

Lysosomal enzyme release occurs during the cytolytic poliovirus infection of HEp-2 cells (6, 12, 13, 26) and during certain other virus infections (1, 8, 14). This lysosomal enzyme redistribution has been proposed by others and ourselves to be a mechanism of cytopathology, since lysosomes contain hydrolytic enzymes capable of degrading cellular constituents and since enzyme release from lysosomes occurs concomitantly with the development of cytopathic effect (CPE) in poliovirus-infected cells. One of the questions raised concerns the control mechanism which initiates lysosomal enzyme release (redistribution) in the infected cells. The work described here attempts to determine if the enzyme release is initiated under cellular or viral control. Amako and Dales (2) proposed that the virus may code for a protein which can act on the membrane of the lysosome, and thus result in the release of hydrolytic enzymes within the cells. Alternative interpretations are that the infectious process derepresses the cell genome, allowing the production of a lysosome-damaging protein, or that the infecting virus induces a cellular protein which in turn acts on its own lysosomes. It is already established that under certain circumstances uninfected cells utilize their lysosomal enzyme system to carry on normal degradative processes (25), thus lending credibility to the latter hypothesis.

To determine whether viral-induced lysosomal enzyme release is under the genetic control

of the virus or the cell, we chose to use actinomycin D (AMD), an agent known to inhibit DNA-dependent RNA synthesis (20). By using this approach, we were able to distinguish the influence of the viral genome from that of the cell genome.

MATERIALS AND METHODS

Cell maintenance and virus production. HEp-2 suspension cultures were maintained in Erlenmeyer flasks on a gyrotary shaker, and stock suspensions of poliovirus (type 1, Mahoney) were prepared as previously described (23).

Evaluation of the CPE. To determine the extent of CPE, samples were removed from the poliovirus-infected cultures at various times postinfection (PI) and mixed with an equal volume of 0.05% erythrocin B (Matheson, Coleman and Bell, Cincinnati, Ohio) dissolved in phosphate buffered saline. The percentage of erythrocin B uptake (dead cells) was determined with the aid of a phase microscope by counting the number of red-stained and total number of cells in ten or more fields (magnified $\times 450$), each containing between 20 and 40 cells.

Inhibitor studies. A stock solution of 1.2 mg per ml of actinomycin D (Calbiochem, San Diego, Calif.) was prepared in serum-free minimal essential (MEM)-spinner medium and sterilized by filtration through a Gelman membrane filter (0.45 μ m). Incorporation studies were performed in serum-free MEM-spinner medium without phenol red. Cells (3×10^6 /ml) were exposed to various concentrations of AMD and, after 105 min, AMD was removed from cultures and cells were pulsed with 2.5 μ Ci of [5-³H] uridine per ml (specific activity, 21.7 Ci/mM; Tracerlabs, Waltham, Mass.) for 35-min periods throughout the ensuing 14 h. Pulses were terminated by transferring duplicate 1-

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ml portions to tubes (12 by 100 mm) containing 10 μ g of cold (unlabeled) uridine in 1 ml of water. Each tube received 1.0 ml of 15% trichloroacetic acid, and the precipitate was centrifuged and washed twice with 2.0 ml of 5% trichloroacetic acid. The final precipitate was dissolved in 0.5 ml of solvene (Packard Inst. Co., Downers Grove, Ill.) and digested at 50 C for 30 min. Samples were transferred to glass counting vials containing 10 ml of toluene base cocktail (0.7% 2,5-diphenyl-oxazole and 0.025% dimethyl 1,4-bis-(5-phenyloxazolyl)benzene) and counted in a Packard model 3375 Tricarb liquid scintillation spectrometer at a maximal counting efficiency of 40%.

Infectious center assays. Infectious center assays were used to determine the number of cells supporting poliovirus replication under the influence of various concentrations. Suspended HEp-2 cells, at a concentration of 6.4×10^4 cells per ml were incubated with AMD at the indicated concentrations in total volumes of 1.0 ml contained in screw-capped tubes held at 36 C for 45 min. Cells were then infected with poliovirus (type 1, Mahoney) at a multiplicity of infection of 30 PFU per cell by adsorbing for 30 min followed by two washes in MEM (Joklik modified) medium containing 10% calf serum. After washing free of unadsorbed poliovirus, cells were resuspended in the initial volumes of AMD concentrations, and incubation continued at 36 C for 3.0 h PI. All cultures were washed free of AMD twice with MEM (Joklik; 10% calf serum) and diluted to contain 80 to 100 cells per 0.5 ml, with 0.5 ml added to each of five plastic tissue culture dishes (60 mm, Falcon Plastics no. 3002, Oxnard, Calif.) containing nearly confluent monolayers of HEp-2 cells. Cells were allowed to adsorb for 2.0 h before adding overlay medium. Plaques were stained with neutral red and counted 60 h after addition of infected cells to the monolayers. In determining the number of infectious centers produced under the influence of AMD, values were normalized to correspond to 100% efficiency in the untreated, infected cells.

Cell cloning experiments. The plating efficiency of HEp-2 cells treated with various concentrations of AMD was determined to establish levels of toxicity. Cells in suspension were treated with various concentrations of AMD as in the infectious center assay. After 3.75 h of exposure, a time comparable to that described in the previous experiment, cells were washed in MEM (Joklik) and resuspended in the same medium without AMD. Cells were diluted to 500 cells per 0.5 ml, and this amount was added to each of 5 60-mm Falcon tissue culture dishes with total volume adjusted to 5.0 ml with MEM (Joklik) medium containing 10% calf serum. After 8 days of incubation at 36 C in a mixture of 5% CO₂ and 95% air, dishes were stained with 0.05% crystal violet in formalin phosphate-buffered saline and clones were counted with the aid of a microscope.

Lysosomal enzyme redistribution in poliovirus-infected cells. Infection of cell suspensions, homogenization, and processing samples for determination of lysosomal enzyme release have been previously described (12). Briefly, 10^6 HEp-2 cells (pulsed for 45

min with 3 μ g of AMD per ml) were washed and $\frac{1}{2}$ of the cells were infected with poliovirus at a multiplicity of infection of 25 PFU/cell. The remaining $\frac{1}{2}$ of the cells were mock-infected and served as uninfected controls. After 30 min, cells were centrifuged and washed two times with MEM-spinner medium, then redistributed in 100-ml volumes into 10 flasks at a concentration of 10^6 cells per ml. At various times after infection portions were removed for determination of released and cellular-associated virus and for evaluation of CPE. The remaining portion of cells (95 ml) at each sample time was centrifuged at $16,300 \times g$ for 20 min at 4 C, then resuspended to 3 ml with 0.125 M sucrose. The relatively high g force used here insures the pelleting not only of whole cells but of lysosomes and cell debris, especially later in the infection period. After homogenization in a Dounce T homogenizer (Blaessig Glass Co., Rochester, N.Y.), the suspension was centrifuged at $20,000 \times g$ for 20 min, the supernatant fraction was removed, and the pellet (lysosomal fraction) was resuspended up to 3 ml with 0.2% Triton X-100. Both supernatant and lysosomal fractions were examined for beta-glucuronidase (EC 3.2.1.31) and beta-galactosidase (EC 3.2.1.23) activities as previously described (12). Identical procedures were followed for parallel infected cultures not exposed to AMD.

RESULTS

Inhibition of RNA synthesis by AMD. One formidable problem when using AMD in experiments involving cell cultures is the choice of a suitable AMD concentration that will not result in cell death within the viral replication period, and yet will accomplish the inhibition of cellular DNA-dependent RNA synthesis. Results of an experiment designed to determine what concentrations of AMD were necessary for the inhibition of RNA synthesis in suspended HEp-2 cell cultures are shown in Fig. 1. At 1 h after the addition of AMD, inhibition of [³H]uridine incorporation was virtually complete. A further 45-min exposure resulted in maximal inhibition levels of 97.5 and 99% (compared to uninhibited, untreated cultures) at 2.5 μ g of AMD per ml and 5 μ g of AMD per ml, respectively. The upward shift of the curve (Fig. 1) after removal of AMD suggests that inhibition of [³H]uridine incorporation was rapidly reversed at the lower concentration. Following a subsequent 6-h incubation in the presence of AMD, inhibition reached or approached levels acquired in the presence of AMD.

While examining the extent of AMD inhibition, we also observed that at concentrations of 2.5 μ g and 5 μ g/ml, cells treated with AMD for 1.25 h sustained less damage (as measured by erythrocin B exclusion) than those exposed to AMD for the entire period. This observation

FIG. 1. The effect of various AMD concentrations on HEP-2 cell DNA-dependent RNA synthesis. Samples were pulsed for a 35-min period with 2.5 μ Ci per ml of [3 H]uridine in the presence of the respective AMD concentrations. Each point represents [3 H]uridine incorporation at the end of the pulse. AMD was removed after 105 min (arrow). The percentage of inhibition was determined on the basis of [3 H]uridine uptake in untreated cultures pulsed for the same time periods.

supports evidence previously reported that the cytologic changes induced by AMD in primary dog kidney cells decreased in intensity with decreased exposure time (10). Our results are consistent with the interpretation that within the experimental period of 12 h, AMD at a concentration of 2.5 μ g/ml is adequate for the inhibition of cellular DNA-dependent RNA synthesis, with minimal direct cytotoxic effects.

Effects of AMD on poliovirus synthesis.

When poliovirus synthesis was examined under similar conditions, i.e., a 45-min exposure to 3 μ g of AMD per ml, released virus yields at 12 h PI were only 63% of the untreated, infected control (Fig. 2a).

Two experiments were designed to examine the basis of this inhibition, a cell cloning experiment and an infectious center experiment. The data shown in Table 1 demonstrate

that AMD at concentrations of 0.25, 1, 3, and 5 μ g/ml was capable of ultimately killing cells after an exposure period of only 3.75 h. At the 3 and 5 μ g/ml levels, 99% of the cells plated could not develop into colonies after an 8-day incubation period.

The results of an infectious center experiment using the same range of AMD concentrations are shown in Table 2. At the 3 μ g of AMD per ml level, 67% of the infected cells plated were capable of forming plaques. The other 33% were either killed or rendered nonproductive by AMD. When these results are interpreted together with the cell cloning experiment, it is apparent that most of those cells (66%) which ultimately die from exposure to a concentration of 3 μ g of AMD per ml were for a limited time still capable of supporting poliovirus replication, and that the apparent inhibition by AMD of poliovirus synthesis, i.e., 37% (Fig. 2a), was not due to a direct effect on the biosynthetic activity of the virus.

Role of the host-cell genome in initiating lysosomal enzyme release. The effects of AMD on lysosomal enzyme release in poliovirus-infected cells is shown in Fig. 2b. Cultures pretreated with 3 μ g of AMD per ml for 45 min and then infected with poliovirus at a multiplicity of infection of 25 PFU/cell were compared to untreated, infected cells. The kinetics of poliovirus production in AMD-treated and untreated HEP-2 cells were similar. Suppression of final virus yields at 12 h PI was not greater than 37% in the case of released virus, and 61% in the case of cell-associated virus (Fig. 2a). This inhibition was attributed to the toxic effects of AMD on the cell (see above). There was no inhibition of the onset or on the time-course of beta-glucuronidase and beta-galactosidase release from lysosomes into the cytoplasm of AMD-treated and untreated cultures (Fig. 2b). The CPE in AMD-treated cells, measured by erythrocin B uptake, preceded that detected in untreated, infected cells at 6 h PI and later. The more rapid onset of erythrocin B uptake in AMD-treated cells infected by poliovirus was the only dramatic difference detected that could be related to AMD toxicity during virus infection (Fig. 2c).

Phase contrast microscopy was also used to monitor morphological changes resulting from AMD, infection by poliovirus, or the coordinate action of both. The changes noted that were different from the poliovirus-induced CPE included swollen and distorted nuclei with granulation, and vacuolation of the cytoplasm. In contrast to CPE evaluation by erythrocin B

TABLE 1. Plating efficiency of AMD-treated HEp-2 cells

AMD concn (μg/ml) ^a	Ratio ^b	Cells forming clones (%) ^c
0	120/120	100
0.25	70/120	58.3
1.0	4.2/120	3.5
3.0	0.33/120	0.3
5.0	0.35/120	0.3

^a Cells exposed for 3.75 h.^b Number of clones to the number of cells plated.^c All values adjusted to 100% efficiency in untreated control.

TABLE 2. Poliovirus-infected HEp-2 cell infectious center information with various concentrations of AMD

AMD concn (μg/ml) ^a	Ratio ^b	Cells forming infectious centers (%) ^c
0	360/360	100
0.25	300/360	83.2
1.0	367/360	102
3.0	241/360	67
5.0	260/360	72.2

^a Cells exposed for 4.25 h.^b Number of infectious centers to the number of cells plated (10³ per ml in undiluted sample).^c All values adjusted to 100% efficiency in untreated control.

uptake, an equal level of CPE was detected at 6 h PI by means of phase microscopy in infected cells, whether AMD treated or not. Concurrent with the first detection of erythrocin B uptake at 6 h PI, over 50% of maximal release of beta-glucuronidase and beta-galactosidase took place from lysosomes into cell cytoplasm (Fig. 2b), thus showing that the enzyme redistribution process does not require new messenger RNA synthesis.

FIG. 2. The effect of AMD on poliovirus replication, CPE, and lysosomal enzyme redistribution in HEp-2 cells. Cells were treated with 3.0 μg of AMD per ml for 45 min prior to infection with 25 PFU of poliovirus per cell and compared to untreated cells. (a) Cell-associated poliovirus (cell v) and released poliovirus (rel v) were measured in the presence or absence of AMD. (b) The patterns of beta-glucuronidase (β glucr) and beta-galactosidase (β gal) redistribution in the presence or absence of AMD, with (PV) and without (K) poliovirus infection. (c) The onset and time-course of CPE as measured by erythrocin B uptake and by morphological changes observed in the phase contrast microscope.

DISCUSSION

AMD inhibition and its relation to poliovirus-induced CPE. In experimentation involving the study of CPE, interpretation is largely dependent upon the methods used to measure the CPE. Lake and Ludwig (15), for example, have emphasized that nuclear involvement precedes cytoplasmic changes including the modification of cytoplasmic membranes, commonly measured by the inability of infected cells to exclude dyes. We would also like to emphasize the existence of two phases of CPE: an intracellular phase easily detected by phase microscopy, characterized by juxtanuclear cytoplasmic granules, vacuolization, and enlargement and distortion of the nucleus, and preceeding loss of cell integrity as measured by erythrocin B uptake. The other is a phase of cytoplasmic membrane change measured by uptake of dyes such as trypan blue and erythrocin B.

The exact mechanism responsible for poliovirus-induced CPE is an elusive one. Three of the most probable explanations are listed below and discussed in the light of results presented in this paper.

The first possibility is that the CPE is mediated by the host-cell genome, a section of which is derepressed by the infecting virus. It is well known that under adverse conditions, apart from virus infection, cells can undergo progressive CPE (5) and so the cell carries some instruction on self destruction. The present investigation was focused on examining the role of host-cell mRNA synthesis on virus-induced CPE. Although CPE and lysosomal enzyme redistribution are not prerequisites for poliovirus (6) or mengovirus (15, 16) replication, enzyme release from lysosomes seems to always accompany poliovirus-induced CPE and thus serves as a marker. Results indicated that under the influence of 3 μ g of AMD per ml, host-cell RNA synthesis is inhibited by 99%, though during the course of infection CPE and accompanying lysosomal enzyme release progressed to the same extent as in infected cultures not treated with AMD. Thus, host-cell mRNA synthesis does not seem to be required at the time of CPE development or lysosomal enzyme release.

A second possibility is that the cut-off of host-cell macromolecular synthesis during virus infection leads to CPE (9). This proposal was deemphasized by its own authors (4) based on observations made while using AMD to simulate virus-induced inhibition of host-cell RNA synthesis. Our results also show that inhibition of host-cell RNA synthesis by AMD does not

bring about the same changes induced by poliovirus within the 12-h experimental period. In addition, release of lysosomal enzymes was not detected in AMD-treated, uninfected cells.

A third possibility is that the CPE results from synthesis of a structural or nonstructural viral coded protein(s) that can subsequently interact with cellular components (2, 3, 12). This is by far the most attractive hypothesis but must undergo some modification to account for recent evidence indicating that mitotically arrested cells are capable of supporting the synthesis of poliovirus without any demonstrable signs of CPE or lysosomal enzyme release (6). The expression of CPE is, therefore, dependent not only on the presence of a viral-induced substance (protein), but also on the state of the host-cell at the time of infection.

AMD and its effect on poliovirus replication. Separate controversies arise as to the ability of AMD to directly inhibit poliovirus synthesis and the basis of this inhibition (7, 11, 22, 24). It is now accepted that the primary mode of action of AMD inhibition is due to binding adjacent to a guanine-cytosine pair of a DNA molecule, resulting in the formation of a highly stable complex (17) and the inhibition of DNA-dependent RNA synthesis. It follows that inhibition of an RNA virus by AMD predicates an obligate requirement for host-cell DNA or newly synthesized transcript of DNA (11). The fact remains, however, that different strains of poliovirus are not inhibited to the same extent by AMD in the same host cell (22).

In this study, we have attempted to show the relationship of AMD activity to cytotoxicity and to viral CPE. In cases where the virus is not drastically inhibited (i.e., less than 90%), the observed inhibition could be a result of direct AMD toxicity on the same proportion of cells (i.e., less than 90%). Results presented here predict that in a population of 100 infected Hep-2 cells, treated with a concentration of AMD that inhibits 99% host-cell RNA synthesis, 99 cells will die due to the effects of AMD. Of these, 67 will produce poliovirus prior to their death. It is likely that at the time of AMD addition the 33 cells unable to produce poliovirus were in a physiologic or metabolic state different than the majority of cells. Thus, it can be inferred that the effects of AMD on poliovirus replication in Hep-2 cells are not directly related to inhibition of DNA-dependent RNA synthesis, but to the predisposition of the cells, which in a randomly growing population is a function of the cell cycle. Supporting this view are the data of Sawicki and Godman (21) and

Pederson and Robbins (18) which show that the cytotoxicity of AMD (21) and the DNA binding of AMD within whole cells (18) are related to the organization of the cell (i.e., the state of the cell within its cycle).

It is evident from the above discussion that the mechanism of viral-induced CPE and poliovirus inhibition by AMD may both be related to particular stages of cell growth.

LITERATURE CITED

- Allison, A. C., and K. Sandelin. 1963. Activation of lysosomal enzymes in virus-infected cells and its possible relationship to cytopathic effects. *J. Exp. Med.* **117**:879-887.
- Ameko, K., and S. Dales. 1967. Cytopathology of mengovirus infection. II. Proliferation of membranous cisternae. *Virology* **32**:201-215.
- Bablanian, R. 1972. Mechanisms of virus cytopathic effects. *Symp. Soc. Gen. Microbiol.* **22**:359-381.
- Baltimore, D., and R. M. Franklin. 1962. The effect of mengovirus infection on the activity of the DNA-dependent RNA polymerase of L-cells. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1383-1398.
- Bessis, M. 1964. Studies on cell agony and death: an attempt at classification, p. 287-316. *In* A. V. S. De Reuck and J. Knight (ed.), *Ciba Foundation Symposium: Cellular Injury*. Little Brown Co., Boston.
- Bienz, K., D. Egger, and D. A. Wolff. 1973. Virus replication, cytopathology, and lysosomal enzyme response of mitotic and interphase HEP-2 cells infected with poliovirus. *J. Virol.* **11**:565-574.
- Cooper, P. D. 1966. The inhibition of poliovirus growth by actinomycin D and the prevention of the inhibition by pretreatment of the cells with serum or insulin. *Virology* **28**:663-678.
- Flanagan, J. F. 1966. Hydrolytic enzymes in KB cells infected with poliovirus and herpes simplex virus. *J. Bacteriol.* **91**:789-797.
- Franklin, R. M., and D. Baltimore. 1962. Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* **27**:175-194.
- Garg, S. P., and J. E. Moulton. 1966. The effects of actinomycin D on cytopathological changes and production of infectious canine hepatitis virus *in vitro*. *Amer. J. Vet. Res.* **27**:1395-1403.
- Grado, C., S. Fischer, and G. Contreras. 1965. The inhibition by actinomycin D of poliovirus multiplication in HEP-2 cells. *Virology* **27**:623-625.
- Guskey, L. E., P. C. Smith, and D. A. Wolff. 1970. Patterns of cytopathology and lysosomal enzyme release in poliovirus-infected HEP-2 cells treated with either 2-(α -hydroxybenzyl)-benzimidazole or guanidine-HCl. *J. Gen. Virol.* **6**:151-161.
- Heding, L. D., and D. A. Wolff. 1973. The cytochemical examination of poliovirus-induced cell damage. *J. Cell. Biol.* **59**:530-536.
- Hotham-Iglewski, B., and E. H. Ludwig. 1966. Effect of cortisone on activation of lysosomal enzyme resulting from mengovirus infection of L-929 cells. *Biochem. Biophys. Res. Commun.* **22**:181-186.
- Lake, R. S., and E. H. Ludwig. 1971. Cellular changes attending mengovirus-induced cytolysis of mouse L-cells. *Biochim. Biophys. Acta* **244**:466-477.
- Lake, R. S., D. C. Winkler, and E. H. Ludwig. 1970. Delay of mengovirus-induced cytopathology in mitotic L-cells. *J. Virol.* **5**:262-263.
- Muller, W., and D. M. Crothers. 1968. Studies of the binding of actinomycin and related compounds to DNA. *J. Mol. Biol.* **35**:251-290.
- Pederson, T., and E. Robbins. 1972. Chromatin structure and the cell division cycle: actinomycin binding in synchronized HeLa cells. *J. Cell Biol.* **55**:322-327.
- Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. *Proc. Nat. Acad. Sci. U.S.A.* **49**:654-662.
- Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum. 1962. Action of actinomycin D on animal cells and viruses. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1238-1245.
- Sawicki, S. D., and G. C. Godman. 1971. On the differential cytotoxicity of actinomycin D. *J. Cell Biol.* **50**:746-761.
- Schaffer, F. L., and M. Gordon. 1966. Differential inhibitory effects of actinomycin D among strains of poliovirus. *J. Bacteriol.* **91**:2309-2316.
- Thacore, H., and D. A. Wolff. 1968. Activation of lysosomes by poliovirus-infected cell extracts. *Nature (London)* **218**:1063-1064.
- Tolskaya, E. A., G. Yu. Lipskaya, M. K. Voroshilova, and V. I. Agol. 1968. Study of temperature-dependent inhibition of reproduction of some picornaviruses by aurantine and mitomycin. *Arch. Gesamte Virusforsch.* **25**:160-176.
- Weissman, G., and P. Dukor. 1970. The role of lysosomes in immune responses. *Advan. Immunol.* **12**:283-322.
- Wolff, D. A., and H. C. Bubel. 1964. The disposition of lysosomal enzymes as related to specific viral cytopathic effects. *Virology* **24**:502-505.