Mechanism of Antiviral Action of Acetone on Rabbitpox Virus Replication

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Acetone added to the maintenance medium in a 1% concentration reduces the yield of infectious rabbitpox virus in L-cell monolayer cultures by 90 to 97%. This concentration of the inhibitor is not toxic for cells. It was established that there is an inhibitor-sensitive stage late in the infectious cycle. Acetone exerted no significant influence on production of early viral messenger ribonucleic acid, formation of polyribosomes, deoxyribonucleic acid (DNA) replication, or protein synthesis. In the presence of acetone, the assembly of so-called "acetone particles" occurred. These particles are similar to normal virions by morphological and sedimentation properties but are slightly different from them in buoyant density. The amount of virus-specific DNA and the optical density of the "acetone particles" are the same as those of normal virions despite a 10- to 25-fold difference in the infectivity of the preparations.

It has been shown previously (4) that acetone added to the maintenance medium in a final concentration of 1% significantly inhibits replication of poxviruses. The possibility of the selection of mutants resistant to the inhibitor has been demonstrated (4). The present work was devoted to the study of the mechanism of action of this inhibitor.

MATERIALS AND METHODS

Cells. An L-cell monolayer culture was grown in medium 199 supplemented with 10% bovine serum and antibiotics.

Virus. Rabbitpox virus, Utrecht strain, was propagated in chick embryos, concentrated, and purified by Joklik's method (7). Virus titration was performed on chorioallantoic membranes of 12-day-old chick embryos according to Westwood (23), with the use of five embryos per dilution. The infectious titer of the virus used in the experiments was 1.5×10^{10} to 2×10^{10} plaque-forming units (PFU)/ml.

Conditions of cell inoculation and maintenance of infected monolayers. Generally, the multiplicity of infection was 30 to 50 PFU/cell. In the case of the experiments on polyribosome analysis and determination of protein synthesis, the multiplicity of infection was 200 to 300 PFU/cell. The virus was sonically treated before inoculation. The virus was allowed to adsorb on the cells at 36°C for 20 min. The unadsorbed virus was removed, and the cells were washed with warm Earle's saline. The time postinfection was counted from the moment of the addition of warm medium 199. At certain intervals postinfection, the cells were disrupted by three cycles of freezing and thawing, and the virus yields were determined.

Acetone. In all experiments, unless indicated other-

wise, acetone (to a final concentration of 1%, v/v) was added together with medium 199 without serum immediately after adsorption.

Preparation of the cytoplasmic extract. The cells were washed with cold Earle's saline, scraped off with a rubber policeman, and sedimented by centrifugation at $1,000 \times g$ for 15 min. Then the cells were resuspended in hypotonic buffer. In the experiments on polyribosomes, the following buffer was used: 0.01 M triethanolamine-HCl, pH 7.8, 0.01 M NaCl, and 0.003 M Mg^{2+} (referred to as TNM). For the analysis of deoxyribonucleic acid (DNA)-containing material and preparation of labeled virions, phosphate-buffered saline (PBS), 0.001 M, pH 7.4, was used. The cells were allowed to swell for 10 min at 4°C and then disrupted with a Dounce homogenizer. Disruption of the cells was controlled microscopically. Nuclei were removed by centrifugation at $800 \times g$ for 5 min. In studies on polyribosomes, polyvinylsulfate (2 µg/ml) was added to the cytoplasmic extract. All cytoplasmic extracts, with the exception of the extract for polyribosome studies, were sonically treated (apparatus RFT, 800 kc, 10 w/cm², 60 sec) before layering on the top of a gradient.

Polyribosome analysis in sucrose gradient. The modified method of Moss and Filler (12) was used. At 2 hr postinfection, ³H-uridine (4 Ci/mmol) was added to the medium in a concentration of 10 µCi/ml. The cultures were incubated with the isotope for 10 min, and then were placed immediately into an ice bath. The cytoplasmic extract was layered onto a linear 5 to 20% (w/w) sucrose-TNM gradient with a cushion of 50% sucrose containing 4% formaldehyde (2) and were centrifuged in an MSE superspeed 65 centrifuge (3 × 23 bucket rotor, 90 min, 28,000 rev/min, 4°C). The gradient fractions were collected from the bottom of

the gradient through a needle inserted from above. The optical density and radioactivity of the fractions were determined. The acid-insoluble material was collected on membrane filters.

Determination of protein synthesis. At 5 hr postinfection, medium 199 containing one-fifth of the normal content of amino acids and ^{14}C -L-phenylalanine, ^{14}C -L-valine, and ^{14}C -L-leucine (100 mCi/mmol; 0.25 μCi of each amino acid per ml) was added to tube cultures (2×10^6 cells). At various intervals, the tubes were placed into an ice bath; the cells were washed with cold Earle's saline and disrupted by freezing and thawing in distilled water. Then acid-insoluble radioactivity was determined.

Analysis of DNA-containing material in sucrose gradient. The modified method of Joklik and Becker (8) was used. At 6 hr postinfection, the infected cells were labeled for 30 min with ^3H -thymidine (5 Ci/mmol, 3 $\mu\text{Ci}/\text{ml}$), and the medium was replaced with warm medium 199. At 24 hr postinfection, the cytoplasmic extract was prepared, layered onto a 25 to 40% (w/w) sucrose-PBS gradient, and centrifuged in an MSE superspeed 65 centrifuge (3×23 bucket rotor, 60 min, 15,000 rev/min, 4 C).

Preparation and purification of labeled virus. The infected cells were labeled with either ^{14}C - or ^3H -thymidine as described in the previous section. The cytoplasmic extract was prepared, layered onto a 25 to 40% (w/w) sucrose-PBS gradient, and centrifuged (60 min at 15,000 rev/min, 4 C, 3×23 bucket rotor). The fractions containing virions were pooled, diluted with PBS to 1:3 (v/v), and recentrifuged (28,000 rev/min, 90 min). The supernatant fluid was discarded, and the sediment was suspended in 2 ml of PBS.

Isopycnic centrifugation in sucrose and cesium chloride gradients. For analysis of buoyant density, a mixture of ^3H - and ^{14}C -thymidine-labeled virions was either layered on the top of the sucrose gradient (1.17 to 1.34 g/ml) or included in the CsCl (1.0 to 1.36 g/ml) gradient (2). Both gradients were prepared in PBS. The densities of the initial sucrose solution were determined pycnometrically with the refractometric control of the shape of the gradient. The densities of CsCl fractions were determined as described previously (2). The sucrose gradients were centrifuged in a 3×23 bucket rotor (16 hr, 28,000 rev/min, 4 C); the CsCl gradients, in a 3×5 bucket rotor (16 hr, 35,000 rev/min, 4 C).

Radioactivity determination. The radioactivity was counted in a liquid scintillation spectrometer (ABAC SL-40 Inter technique, France) with scintillation liquid of the following composition: 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxasoline) in 1 liter of toluene. When the determination of the samples containing both ^{14}C - and ^3H -labeled material was carried out, 20% of ^{14}C radioactivity was counted in the ^3H channel and was subtracted from the total of ^3H radioactivity determined in the channel; less than 1% ^3H radioactivity was counted in the ^{14}C channel.

Reagents. Acetone, chemically pure (VEB Laborchemic Apolda, DDR), triethanolamine (Chemapol, Czechoslovakia), and radiochemicals ("Izotop," USSR) were used.

RESULTS

Inhibitory effect of acetone on rabbitpox virus replication. Figure 1 shows the relationship between the inhibitory effect of acetone and the concentration of the inhibitor in the medium. A 1% concentration of the inhibitor was chosen for study because it inhibited the virus yield by 90 to 97%.

It should be mentioned that in special experiments we demonstrated that, when cells were grown in a medium containing 1% acetone, cell division and the formation of the monolayer proceeded within the same time as in the control cells. Synthesis of cellular macromolecules was not changed significantly in the presence of acetone (*to be published*). Thus, the inhibitory effect of acetone on replication of rabbitpox virus is not associated with a toxic effect on the cells. Furthermore, it was found that acetone, even in a concentration of 4%, did not exert any direct effect on rabbitpox virus during 24 hr of incubation at 37 C. No inactivating effect of the inhibitor on mature virus accumulated in infected cells up to 16 hr postinfection was found.

Acetone was reported to have a photodynamic effect and to inhibit template activity of DNA (22). As one can see from Table 1, the antiviral activity of acetone in our experiments was not associated with a photodynamic effect on the replicating virus.

A series of experiments was carried out with the aim of elucidating the stage of virus reproduction sensitive to the action of acetone. Acetone

FIG. 1. Effect of different concentrations of acetone on the virus yield. The cells (2×10^6) were inoculated with the virus as described in Materials and Methods, and were incubated at 36 C in medium containing different concentrations of acetone. The yield was determined at 24 hr postinfection.

TABLE 1. *Photodynamic effect of acetone*

Time postinfection (hr)	Virus yield (PFU/sample)
0	1.7×10^6
24 (without acetone)	2.5×10^6
24 (with acetone, 1%)	1.4×10^6
24 (with 1% acetone in the dark) ^a	1.8×10^6

^a The experiments and determination of infectivity were carried out under conditions excluding visible light.

either was added at various intervals postinfection or was added at zero time and removed at various intervals postinfection.

As can be seen from Fig. 2, the presence of acetone for only the first 9 hr postinfection had no influence upon the virus yield determined at 24 hr. At the same time, the presence of the inhibitor added at 2, 4, 6, or even 9 hr postinfection inhibited the production of infectious virus as effectively as when it was present through the entire reproduction cycle. Removal of the inhibitor after 24 hr and incubation of the infected cells for an additional 6 hr without the inhibitor did not result in any increase of the yield of infectious virus. Therefore, these experiments suggest that the late stage of virus reproduction should be sensitive to the effect of acetone.

Production of virus polyribosomes. The fact that the presence of acetone for at least the first 9 hr with subsequent removal did not affect the virus yield does not permit us, however, to exclude any effect of the inhibitor on virus-specific processes of this period. In this connection, the effect of the inhibitor on production of virus polyribosomes as well as on protein synthesis in the infected cell was studied.

Incorporation of labeled uridine into cytoplasmic structures of infected cells during a 10-min "pulse" reflects the production of viral messenger ribonucleic acid (mRNA) and virus-specific polyribosomes (12, 18). Under conditions of our experiments (multiplicity of infection, 200 PFU/cell), the maximal incorporation occurred between 1 and 2 hr postinfection. The incorporation of labeled uridine under these conditions in the presence of acetone was reduced by 20%, as a rule.

It can be seen from Fig. 3 that the production of polyribosomes in infected cells incubated with acetone (b) or without it (a) did not differ significantly. Some reduction in the amount of polyribosomal material in the presence of acetone par-

FIG. 2. *Effect of addition and removal of acetone at various intervals postinfection. Virus yield: (○) in the absence of the inhibitor; (●) after incubation with the inhibitor for 9 hr followed by washing of the cell monolayer; (▲) in the presence of the inhibitor from 0 to 24 hr postinfection; (■) inhibitor added at 9 hr and incubation with it continued until 24 hr postinfection; (■) incubation from 0 to 24 hr postinfection in the presence of the inhibitor followed by washing of the cell monolayer and additional incubation for 6 hr. Arrows indicate time of addition (↓) or removal (↑) of the inhibitor.*

alleled the reduction in the general incorporation of labeled uridine.

Protein synthesis in infected cells. In poxvirus-infected cells, the incorporation of amino acids beginning from 4 hr postinfection was shown to occur mainly into virus-specific proteins (6, 11, 19, 20). Therefore, we studied the effect of the inhibitor on accumulation of labeled proteins in infected cells at 6 to 10 hr postinfection (multiplicity of infection, 200 PFU/cell). As can be seen from Fig. 4, there occurred some reduction (30%) of protein synthesis in infected cells in the presence of acetone.

Production of "acetone particles." The results of the above-described experiments have demonstrated that acetone does not prevent the main virus-specific synthetic processes, including DNA replication (the latter not presented in this paper). Therefore, the purpose of the following experiments was to find out whether or not there was assembly of virus particles in the presence of the inhibitor. The results of a typical experiment are presented in Fig. 5. One can see that in the presence of acetone the formation of DNA-containing structures proceeds, and that structures appear similar to normal virions in their sedimentation properties. These particles were designated as "acetone particles." The radioactivity and optical density of "acetone particles" in the appropriate zone of the sucrose gradient were the same as the radioactivity and optical density of normal virions

FIG. 3. Production of virus polyribosomes. The cells (6×10^7) infected at a multiplicity of 200 PFU per cell and incubated with (b) or without (a) acetone were labeled 2 hr after inoculation for 10 min with ^3H -uridine; the cytoplasmic extracts were fractionated in 5 to 20% sucrose gradient overlaid onto a 50% sucrose cushion as described in Materials and Methods. (c) Control, uninfected cells.

FIG. 4. Effect of acetone on protein synthesis in infected cells. To the cells (2×10^8) infected at a multiplicity of 200 PFU/cell and incubated with or without acetone, ^{14}C -amino acids were added at 5 hr postinfection; incubation was continued until 10 hr postinfection. Samples were taken at the intervals indicated in the figure. Each dot represents the mean value from two parallel determinations.

in control samples; however, the infectivity of the former material was more than 25-fold lower. This result has been confirmed in five experiments. Electron microscopy of "acetone particles" showed them to be morphologically similar to normal virions.

Another noteworthy fact evident from Fig. 5 is that, although the amount of DNA incorporated into "acetone particles" is the same as that incorporated into normal virions, in the former case (Fig. 5b) a larger amount of DNA is found at the top of the gradient. This DNA appeared to be virus-specific, since the appropriate control (Fig. 5d) shows no additional release of cellular DNA from nuclei of the infected cells in the presence of acetone.

In a series of experiments, "acetone particles" and normal virions were analyzed in cesium chloride density gradients. As can be seen from Fig. 6a, the buoyant density of "acetone particles" does not differ from that of normal virions and is 1.28 g/ml. Besides, a certain heterogeneity in preparations of "acetone particles" as well as normal virions was found, namely, the presence of a more dense component (ρ , 1.31). Since in the presence of CsCl an aggregation of the particles may occur (7), and taking into account that formaldehyde has been used in isopycnic centrifugation analysis for preservation of some inherent properties of biological structures (2), we used this chemical in our experiments. The more dense component seemed to disappear in the presence of formaldehyde (Fig. 6b).

Since the CsCl gradient technique used in our experiments could not detect a density difference of more than 0.015 g/ml, we compared samples of acetone particles and normal virions using sucrose density gradient (50 to 80%) centrifugation. As can be seen from Fig. 7, the main component of "acetone particles" appeared to be localized in a zone of slightly lower density as compared with normal virions. Two components in both "acetone particles" and normal virion preparations noted in the CsCl gradients were also found in sucrose gradient experiments. The infectivity of both components was similar (correspondingly reduced by 95% for the preparation of "acetone particles"). We are inclined to believe that, since the more "dense" component found in samples of both "acetone particles" and normal virions

FIG. 5. Effect of acetone on formation of virus particles. The cells (3×10^7) were infected at a multiplicity of 30 to 50 PFU/cell and were maintained in the presence of acetone (b) or without it (a). They were incubated with ^3H -thymidine within the period from 6 to 6.5 hr postinfection at the period of maximal DNA synthesis, and the incubation was continued until 24 hr postinfection. The cytoplasmic extract was fractionated in a 25 to 40% (w/w) sucrose gradient as described in Materials and Methods. (c) Control, uninfected cells; (d) infected cells incubated with acetone and labeled for 30 min (from 1 to 1.5 hr postinfection before the onset of viral DNA synthesis). Both controls (c and d) were treated in the same way as variants a and b.

FIG. 6. Isopycnic centrifugation of "acetone particles" and normal virions in a preformed cesium chloride density gradient. Purified virus particles labeled with ^{14}C -thymidine (normal virions) and ^3H -thymidine ("acetone particles") were centrifuged in a CsCl gradient (a) and in a CsCl gradient containing 4% formaldehyde (b) for 16 hr at $90,000 \times g$ and 4°C as described in Materials and Methods.

disappears in the presence of formaldehyde, they may be aggregates of homogeneous particles.

DISCUSSION

The main experimental finding of these studies is that in rabbitpox virus-infected L cells there occurs, in the presence of acetone, the formation of DNA-containing structures designated as "acetone particles" which resemble normal vir-

ions by their sedimentation properties and morphological appearance, but differ slightly from them in buoyant density.

It seems likely that the DNA content in "acetone particles" does not differ significantly from that of normal virions. If this is the case, the fact that the radioactivity and the optical density of "acetone particles" are the same as those of normal virions indicates that the quantity of "acetone

FIG. 7. Isopycnic centrifugation of "acetone particles" and normal virions in sucrose density gradient. ^3H -acetone particles" and ^{14}C -normal virion-containing material purified in 25 to 40% (w/v) sucrose gradients were recentrifuged in 50 to 80% (w/v) sucrose gradients (16 hr at 80,000 $\times$ g at 4 $^{\circ}\text{C}$).

particles" is the same as that of normal virions, whereas their infectivity is reduced by 95%. It may be suggested, therefore, that in the presence of acetone there occurs the predominant production of a peculiar kind of noninfectious virions, "acetone particles."

The means by which acetone causes the formation of defective particles is still obscure. However, it is not associated with either its effect on mature particles or photodynamic effect on replicating virus. Obviously, the cause(s) of biological defectiveness may be connected with some alteration of either genetic material or the other components of viral particles. Some difference between the densities of "acetone particles" and normal virions may be a reflection of biological defectiveness of "acetone particles." It is possible that the mechanism of action of acetone involves its effect on several processes. Besides, the manifestation of the inhibiting action of acetone seems to depend on the host cell. Specifically, the inhibitory effect of acetone in the rabbitpox virus-chick embryo fibroblast system is as manifest as that in the L-cell system, but there are certain differences in the mode of inhibition (*in preparation*).

The mechanisms of action of inhibitors of replication of pox viruses that have been studied so far involve either the inhibition of some virus-specific synthetic processes (3, 10, 16, 24) or the impairment of virion assembly (5, 9, 13-15, 17, 21). Formation of noninfectious virions by a ts mutant of vaccinia virus has also been reported, but the defect was considered to involve an early function on the basis of temperature-shift experiments (1). Our data obtained in acetone-shift

experiments indicate the involvement of a late step of the infective cycle. This is compatible with the fact that we have not found significant abnormalities in some early virus-induced processes (synthesis of early mRNA, formation of viral polyribosomes, synthesis of viral DNA and protein). Moreover, formation of viruslike particles, that is, the assembly itself, may proceed. However, we have no data demonstrating the perfect similarity of the assembly processes in acetone-treated and normal cells. Therefore, it can be suggested that as a result of acetone treatment the assembly is somewhat modified, with noninfectious virions ("acetone particles") being produced. On the other hand, there is a possibility that in the normal course of virus reproduction the formation of noninfectious virions is a certain step preceding their complete maturation (acquisition on infectious properties). If that is the case, this very step may be affected by acetone.

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