ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1981, p. 213-217
0066-4804/81/020213-05$02.00/0

Vol. 19, No. 2

Inhibition of Vaccinia Virus Maturation by Zinc Chloride

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Zinc chloride (0.1 mM) inhibited by 96.4% the growth of vaccinia virus in HeLa cells. Approximately 50% inhibition in formation of particles that sedimented in sucrose gradients similarly to vaccinia virions occurred in the presence of zinc ions. Whereas the synthesis of the viral deoxyribonucleic acid was not affected by zinc chloride, a decrease in the overall synthesis of viral polypeptides and inhibition of the cleavage of precursors to the core polypeptides were observed.

Zinc ions have been previously shown to inhibit significantly the growth of rhino (11, 12); picorna (2); and togaviruses (1) by interference with post-translational processing of polypeptides. These ions also inhibit the growth of a few groups of deoxyribonucleic acid (DNA)-containing viruses; decreases in the synthesis of herpes simplex viral DNA (17) and polypeptides (4), and in the activity of the viral DNA polymerase (3), were observed. Recently, Zaslavsky (20) found that zinc ions also inhibit the growth of vaccinia virus. A slowdown in accumulation of thymidine kinase activity and a change in the profile of sedimentation of the viral ribonucleic acid were observed when zinc ions were added to the cultures within the first hour after infection (20). Since several polypeptides of vaccinia virus are also derived from high-molecular-weight polypeptides (8, 9, 13), we wished to find out whether zinc ions interfere with these processes and with the assembly of the virus.

MATERIALS AND METHODS

Cells. HeLa S3 and BSC1 cells were grown in monolayer cultures in M199 medium supplemented with 10% calf serum.

Virus. A stock of the WR strain of vaccinia virus was prepared in HeLa cells and titrated on BSC1 monolayers, as previously described (10).

Infection procedure. Monolayers of HeLa cells in 5-cm-diameter plastic petri dishes were washed with buffered saline, and 0.3 ml of virus dilution was added at a multiplicity of infection of 10 plaque-forming units per cell. After adsorption for 45 min at 37°C, the cells were washed, and medium supplemented with 2% calf serum was added.

Formation of particles. HeLa cells in 9-cm-diameter plastic petri dishes were infected with purified vaccinia virus at a multiplicity of infection of 20 plaque-forming units per cell. Five microcuries of [^{3}H]thymidine was added per ml at 1.5 h postinfection (p.i.). One and one-half hours later the cultures were washed, and Dulbecco-modified minimum essential medium supplemented with 2% calf serum and 0.001 mM thymidine was added. The cultures were harvested at 24 h p.i. The cells were suspended in reticulocyte standard buffer [10 mM tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$] and disrupted by Dounce homogenization. The cytoplasmic fraction obtained after low-speed centrifugation was subjected to sonic vibrations for 30 s in a Bransonic 12 sonicator and then layered on a 25 to 40% sucrose gradient in 1 mM tris(hydroxymethyl)-aminomethane-hydrochloride, pH 9.0, and sedimented in an SW50.1 rotor at 13,000 rpm for 35 min at 4°C. Fractions (10 drops) were collected from the bottom of the tube. One part (100 μl) of each fraction was directly precipitated with trichloroacetic acid, and another part was first digested with deoxyribonuclease (50 μg/ml; Worthington Diagnostics, Freehold, N.J.) in the presence of 10 mM $MgCl_2$ for 30 min at 37°C.

Polyacrylamide gel analysis. A discontinuous buffer system was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separating 13-cm-long gel contained 7.5% acrylamide-0.2% *N,N'*-methylene-bisacrylamide-0.1 M sodium phosphate (pH 7.1)-0.1% sodium dodecyl sulfate. The samples for electrophoresis were solubilized by incubation in 2% sodium dodecyl sulfate-1% mercaptoethanol at 100°C for 1 min. Electrophoresis took place at a constant current of 2 mA per gel overnight, until the dye front reached the bottom of the gel. After the gels were stained for 5 h with 0.1% Coomassie brilliant blue in 10% trichloroacetic acid, they were destained with 7.5% acetic acid. Gels were sliced longitudinally, dried, and placed in contact with X-ray film.

Chemicals and radioactive precursors. $ZnCl_2$ was purchased from Mallinckrodt Chemical Works, St. Louis, Mo.; [^{3}H]thymidine (21.6 Ci/mmol) was obtained from the Nuclear Research Centre, Negev, Israel; and [^{35}S]methionine (545 Ci/mmol) was from New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of zinc chloride on infectivity of vaccinia virus. The effect of different concentrations of $ZnCl_2$ on vaccinia virus-infected cultures was examined. HeLa cells were infected with the virus at a multiplicity of infection of 10 plaque-forming units per cell. After adsorption

for 45 min at 37°C, the cells were washed, and M199 medium supplemented with 2% calf serum and increasing concentrations of $ZnCl_2$ was added. The cultures were harvested at 24 h p.i., and virus infectivity was determined. The results shown in Fig. 1 indicate that a significant reduction (96.4%) in virus infectivity occurred with 0.1 mM $ZnCl_2$. The rate of inhibition was greatly reduced at lower concentrations and did not increase when $ZnCl_2$ at concentrations of 0.2 and 0.4 mM was added to the infected cultures (Fig. 1). Therefore, we used $ZnCl_2$ at a concentration of 0.1 mM in all subsequent experiments. No toxic effects of $ZnCl_2$ were visible at this concentration. The inhibition of the growth of vaccinia virus strain IHD in LM cells at 0.1 mM $ZnCl_2$, as reported by Zaslavsky (20), was somewhat lower than that observed in this study with the WR strain of the virus-infecting HeLa cells. However, when Zaslavsky was using higher concentrations of $ZnCl_2$ (0.2 to 0.3 mM), although the rate of inhibition increased, toxic effects on the cells were seen (20).

When $ZnCl_2$ was added to cells at times after infection, even if the addition occurred as late as 6 h p.i., a reduction of 97% in virus infectivity was observed. This inhibitory effect was reversible by early removal of $ZnCl_2$ (Table 1). Thus,

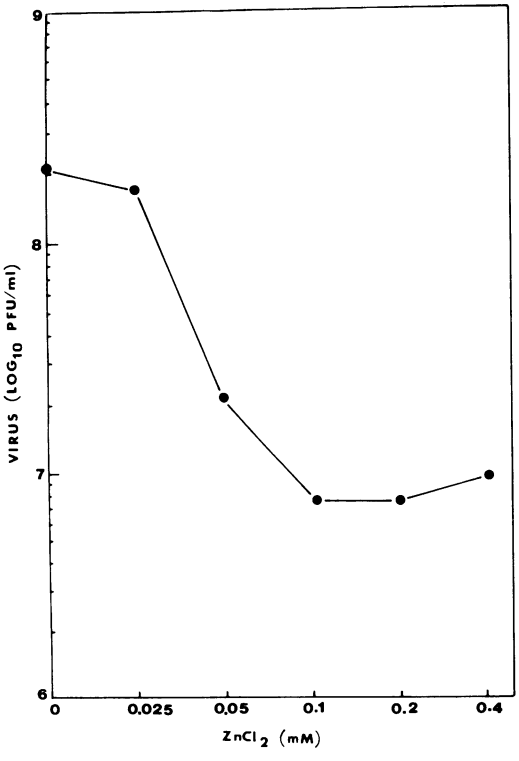

FIG. 1. *Growth of vaccinia virus in the presence of different concentrations of zinc chloride. PFU, Plaque-forming units.*

TABLE 1. *Removal of $ZnCl_2$ at times after infection with vaccinia virus*[a]

Time of removal (h)	Virus titer (PFU/ml)
2	6.0×10^7
4	4.7×10^6
6	5.0×10^5
Not removed	1.0×10^6
Not treated	5.0×10^7

[a] Zinc chloride (0.1 mM) was added to cells at 45 min p.i. The cultures were washed at time intervals thereafter, and fresh medium without $ZnCl_2$ was added. All cultures were harvested at 24 h p.i., and virus titer was determined. One infected culture was not treated with $ZnCl_2$. PFU, Plaque-forming units.

when $ZnCl_2$ was removed at 2 h p.i., the level of the infectious virus formed was similar to that of untreated cultures, but when the removal was further delayed, virus yield decreased (Table 1).

Formation of virus particles. The significant reduction in vaccinia virus infectivity caused by $ZnCl_2$ raised the question of whether noninfectious virus particles are formed. To investigate this question, vaccinia virus-infected HeLa cells were labeled with [^{3}H]thymidine and harvested, and virions were purified in sucrose gradients, as described in Materials and Methods. The profiles of the labeled virions, sedimenting into the middle of the sucrose gradients, are shown in Fig. 2. Most of the virus particles in this area of the gradient are resistant to deoxyribonuclease (Fig. 2). More than 50% of virus particles, compared with the untreated control, are formed in the presence of $ZnCl_2$ (Fig. 2). The proportion of particles resistant to deoxyribonuclease is similar for virions made in control cells and in the presence of $ZnCl_2$ (Fig. 2). Since the decrease in the yield of infectious virus in the presence of $ZnCl_2$ is significantly higher than that observed for the virions, it can be calculated that the relative amount of noninfectious virions is higher under these growth conditions. To further characterize this finding, the syntheses of the viral DNA and polypeptides were followed.

Viral DNA synthesis. Vaccinia virus DNA is synthesized in the cytoplasm of infected cells mainly between 2 and 5 h p.i. (6, 16). Infected cells were pulse-labeled with thymidine, the nuclei were removed by low-speed centrifugation after treatment of the cells with the nonionic detergent Nonidet P-40, and radioactivity was determined. The results presented in Fig. 3 show that $ZnCl_2$ did not affect significantly the synthesis of vaccinia virus DNA. Both the kinetics and the level of synthesis occurring in the presence of $ZnCl_2$ were similar to those found in untreated infected cells.

Viral polypeptide synthesis. The synthesis

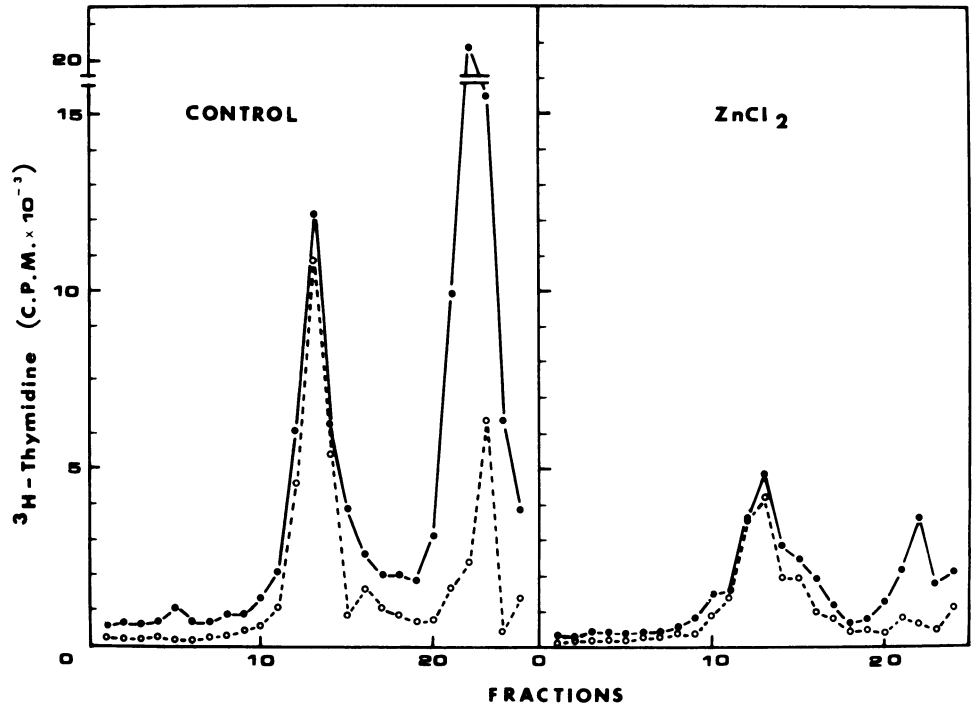

FIG. 2. *Formation of virus particles. Infected cells labeled with [^{3}H]thymidine were disrupted and run in sucrose gradients. Symbols: ●, total radioactivity; ○, deoxyribonuclease-resistant radioactivity.*

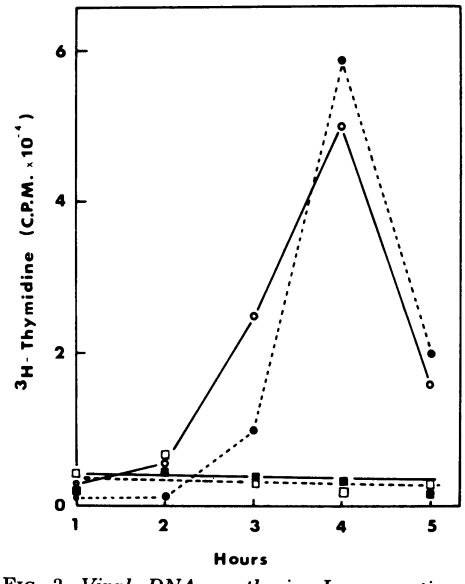

FIG. 3. *Viral DNA synthesis. Incorporation of [^{3}H]thymidine-labeled radioactivity into the cytoplasm of the cells. Symbols: ○, infected; ●, infected in the presence of $ZnCl_2$ (0.1 mM); ■, uninfected; □, uninfected in the presence of $ZnCl_2$ (0.1 mM).*

of vaccinia virus polypeptides can be followed by labeling infected cells with [^{35}S]methionine and analyzing the labeled polypeptides by sodium dodecyl sulfate-gel electrophoresis, as described in Materials and Methods. Infection with vaccinia virus causes inhibition of synthesis of host cell polypeptides soon after infection; this allows the identification of the newly synthesized viral polypeptides (15). The polypeptides are divided into two classes: "early" polypeptides, which are synthesized before initiating synthesis of the viral DNA; and "late" polypeptides, which are made thereafter. Since vaccinia viral DNA is synthesized normally in the presence of $ZnCl_2$, we followed the synthesis of late vaccinia polypeptides under these conditions. At least two of the late polypeptides, which are structural components of the core of the vaccinia virion, are made by a cleavage process from high-molecular-weight polypeptides during virus maturation (8, 9, 13). The cleavage is inhibited by the anti-poxvirus drug rifampin (5, 18). We followed the synthesis of the two structural polypeptides in the presence of $ZnCl_2$ by performing a pulse-chase experiment.

HeLa cells were infected with purified vaccinia virus at a multiplicity of infection of 10 plaque-forming units per cell. After an adsorption period of 30 min at 37°C, the cultures were washed with buffered saline and supplemented with minimum essential medium containing 2% calf serum, without or with $ZnCl_2$ (0.1 mM). At 6 h p.i., minimum essential medium containing

one-tenth the regular concentration of methionine ([^{35}S]methionine, 8 μCi/ml), with or without $ZnCl_2$, was added for 30 min. The cells were then washed, and medium without radioactive methionine but with a 10-fold excess of "cold" methionine was added. The cells were harvested at 9 h p.i. with the help of a rubber policeman, suspended in 1 ml of reticulocyte standard buffer, and Dounce homogenized, and the cytoplasmic fraction was collected after low-speed centrifugation. Incorporation of [^{35}S]methionine into acid-precipitable material during the labeling period was reduced by $ZnCl_2$ by 37.7% compared with infected untreated cultures. The partial inhibition of protein synthesis occurring in the presence of $ZnCl_2$ is also reflected in the intensity of the radioactivity of the polypeptides run in the polyacrylamide gels (Fig. 4). The main differences observed between the autoradiographs of the control and the $ZnCl_2$-treated cells (Fig. 4) are the absence of polypeptide 4a and a significant quantitative decrease of polypeptide 4b in the latter. These two polypeptides are the two prominent polypeptides comprising the high-molecular-weight polypeptides P4a and P4b. The inhibition of cleavage occurs in spite of the synthesis, although at reduced rates, of these two precursors in the $ZnCl_2$-treated cultures (Fig. 4).

DISCUSSION

Zinc chloride (0.1 mM) reduces by more than 95% the infectious yield of vaccinia virus, whereas rifampin (100 μg/ml) and isatin-β-thiosemicarbazone (14 μM) inhibit by more than 99% the growth of the same virus strain in HeLa cells (7, 14). Although the infectivity of the virus is significantly reduced by zinc chloride, virus particle formation is affected to a much lesser degree. Zinc chloride, which was previously found to significantly inhibit polypeptide cleavages occurring during the growth of several ribonucleic acid-containing viruses (1, 2, 11, 12), inhibits the cleavage of high-molecular-weight polypeptides, which give rise to two of the main polypeptides associated with the core of vaccinia virus. Although the identification of vaccinia virus thymidine kinase in the polypeptide profile obtained by polyacrylamide gel electrophoresis is not yet known, it is possible that the decrease in the activity of this enzyme, occurring in the presence of zinc chloride (20), resulted from interruption of a cleavage process.

Since the cleavage of the polypeptides of vaccinia virus has not yet been shown to occur in a cell-free system, many characteristics of this biological process are not clear to us. From studies carried out with infected cells, it was concluded that the cleavage is closely related to virus maturation and that product 4a is associated with particulate material and is hardly detectable in the soluble fraction of the infected cells (9). The two inhibitors of vaccinia virus growth, rifampin and isatin-β-thiosemicarbazone, which block vaccinia virus maturation (7, 14), also inhibit cleavage of P4a to 4a. It is not clear, however, whether this is a specific effect of the two compounds or an indirect effect resulting from inhibition of an earlier step that occurred during virus maturation. A similar conclusion can be drawn now with regard to the effect of zinc chloride on the cleavage process. The target of inhibition caused by zinc chloride during the vaccinia virus growth cycle may be later than that caused by rifampin and isatin-β-thiosemicarbazone, since deoxyribonuclease-resistant virus particles are formed in the presence of zinc chloride but are absent from infected cultures treated with the other two inhibitors of poxvirus growth.

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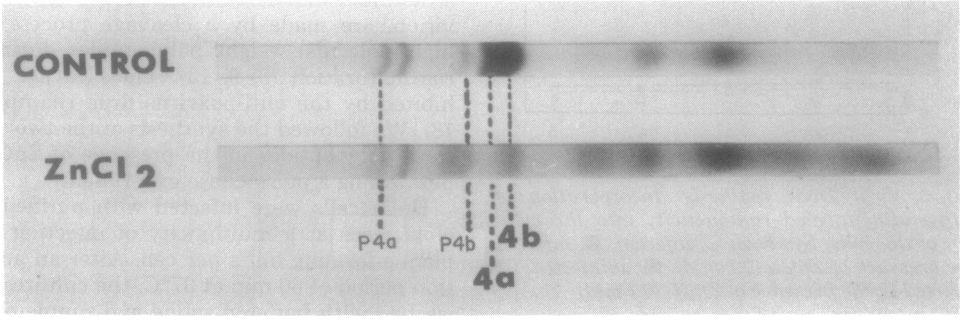

FIG. 4. *Autoradiographs of polyacrylamide gels of viral polypeptides, labeled in the absence and presence of $ZnCl_2$ (0.1 mM), after the chase period (9 h p.i.).*

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