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Synthesis of Vaccinia Virus Polypeptides in the Presence of Isatin-β-Thiosemicarbazone

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Isatin-β-thiosemicarbazone (IBT) at a concentration of 14 μM inhibited the multiplication of vaccinia virus in HeLa cells. For the first 3 h after infection, viral deoxyribonucleic acid (DNA) was synthesized in the presence of IBT at the same rate as in the control culture; the replication rate declined at a later stage. The DNA failed to be coated with proteins and to become resistant to deoxyribonuclease unless IBT was removed. "Early" and "late" viral polypeptides were formed in the presence of IBT, as revealed by polyacrylamide gel electrophoresis. The formation from precursor of a core polypeptide, a reaction blocked by rifampin, was not affected by IBT. Therefore, it is suggested that a maturation step later than the one blocked by rifampin is involved in the inhibition of vaccinia virus by IBT.

Isatin-β-thiosemicarbazone (IBT) is an effective inhibitor of poxviruses (16). The mechanism of action of this drug is not fully understood. It was found that IBT does not affect "early" events occuring during the multiplication cycle of the virus: uncoating is normal, early enzymes are synthesized (18), a number of early structural viral proteins are made (1), and viral deoxyribonucleic acid (DNA) replicates (3, 18). The rate of transcription of messenger ribonucleic acid (mRNA) is not affected, and the formed mRNA combines with ribosomes to form polyribosomes (18). However, polyribosomes which are formed by late mRNA are rapidly broken down (18). On the basis of this finding, Woodson and Joklik (18) suggested that a deficiency of late structural viral protein is the target of the inhibition of poxviruses by IBT.

In the present study, we followed the fate of viral DNA and the synthesis of early and late viral polypeptides in the presence of IBT, when more than 99% of infectious virus are inhibited (E. Katz et al., J. Gen. Virol., in press).

MATERIALS AND METHODS

Cell culture. HeLa S-3 cells were grown in monolayer cultures in Eagle medium (2) supplemented with 10% calf serum.

Virus. Stocks of WR strain (wild type, wt) and IBT-resistant mutant (IBT^R) of vaccinia virus, were prepared in HeLa cells and titrated on BSC1 monolayers, as described elsewhere (Katz et al., in press).

Infection procedure. Monolayers of HeLa S-3 cells in 60-mm plastic dishes (Nunc, Denmark) were washed with saline and infected with virus, at a multiplicity of infection of 5, in a volume of 0.2 to 0.3 ml. After incubation for 45 min at 37 C, the plates were washed, and Eagle medium containing 2% calf serum was added.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed essentially as described by Summers et al. (17). Gels (10 by 0.6 cm) were prepared of 7.5% acrylamide, 0.27% *N'*,*N*-methylenebisacrylamide in 0.1 M sodium phosphate (pH 7.1), and 0.1% sodium dodecyl sulfate (SDS). Prior to use, excess catalyst was removed from the gels by electrophoresis at 5 mA/gel for 1 h. Cytoplasmic extracts were prepared by Dounce homogenization and then reduced and dissociated with 2% SDS and 1% mercaptoethanol for 1 min at 100 C. Sucrose was added to the solubilized cytoplasm to 10%, and 200 μliters was applied to each gel. Electrophoresis was carried out at 3.5 mA/gel for 17 h. After electrophoresis, the gels were placed in 10% trichloroacetic acid, stained with 0.1% Coomassie blue in 10% trichloroacetic acid, and washed in 7.5% acetic acid. The gels of every experiment were sliced longitudinally, dried, and placed together in contact with X-ray film (4).

^{3}H-thymidine (18.9 mCi/mmol) and ^{35}S-methionine (130 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. 5-Iododeoxyuridine (IUdR) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Rifampin was a gift from Lapetit, Milan, Italy. IBT (Schwarz-Mann, Orangeburg, N.Y.) was freshly prepared before use by dissolving 5 mg in 1 ml of acetone and then 4 ml of 0.25 N NaOH was added.

RESULTS

Viral DNA synthesis. Vaccinia DNA is synthesized in HeLa cells mainly between 2 and 5 h after infection (7, 13). Since vaccinia virus multiplies in the cytoplasm of the cell, it is possible to follow viral DNA synthesis by determination of the incorporated thymidine into trichloroacetic acid-precipitable material after short pulses (7). Infected cells were pulsed with radioactive thymidine for 10 min. Cytoplasmic fractions were prepared by lysing the cells in RSB containing the nonionic detergent NP_{40} (0.5%), and nuclei were removed by centrifugation (19). The quantity of radioactively labeled DNA in the cytoplasmic fraction was measured after trichloroacetic acid precipitation. DNA synthesis in the infected cells started between 1 and 2 h after infection, reached the highest level at 4 h and then declined (Fig. 1). In IBT-treated infected cells, the synthesis rose until approximately 3 h and then remained at the same level (Fig. 1). Synthesis of DNA during infection with vaccinia or rabbit pox viruses, in the presence of IBT, has been observed previously (1, 3, 18).

Fate of viral DNA. Joklik and Becker (7) followed the fate of newly synthesized vaccinia DNA in infected cells. By analyzing viral DNA in the crude cytoplasmic fraction, it was observed that the DNA started to become resistant to deoxyribonuclease approximately 5 h after infection. Development of deoxyribonuclease resistance depended on protein synthesis and presumably resulted from "coating" of the DNA with proteins.

We studied the fate of the newly synthesized vaccinia virus DNA made in the presence of IBT, after sucrose gradient centrifugation, by using deoxyribonuclease digestion. In the absence of the drug, ^{3}H-thymidine-labeled DNA was found 18 h later both in a deoxyribonuclease-digestible form, at the top of the sucrose gradient, and in a resistant band, coincident with infectious virus (Fig. 2). In the presence of IBT, no virus band was detected; most of the radioactively labeled DNA was still deoxyribonuclease-sensitive and located at the top of the gradient (Fig. 2). However, this labeled DNA made in the presence of IBT was converted to a deoxyribonuclease-resistant form after the removal of IBT (Fig. 3).

Synthesis of viral proteins. The failure of vaccinia DNA, in the presence of IBT, to become resistant to deoxyribonuclease might result from the absence of structural proteins. Host protein synthesis is progressively inhibited after vaccinia virus infection, thus permitting the specific labeling of viral polypeptides (6, 10, 12, 14, 15). The viral proteins are divided into two classes: (i) "early" proteins, which are synthesized at the early period after infection, and which are produced even when the replication of the viral genome is inhibited; and (ii) "late" proteins, which are formed only after viral DNA synthesis. HeLa cells were labeled with ^{35}S-methionine between 1 and 2 h after infection with wt and IBT^R mutant of vaccinia virus. Similar amounts of radioactivity were incorporated into the cells. The proteins of the cytoplasm were solubilized with SDS and analyzed by polyacrylamide gel electrophoresis. The normal gradual transition from synthesis of host proteins to specific "early" viral proteins took place even in the presence of IBT (Fig. 4). The specific early viral proteins were also formed in the presence of IUdR, an inhibitor of DNA synthesis (Fig. 4). The effect of IBT on the formation of "late" proteins was studied as follows. ^{35}S-methionine was added to the cultures between 4 and 7 h after infection, and the

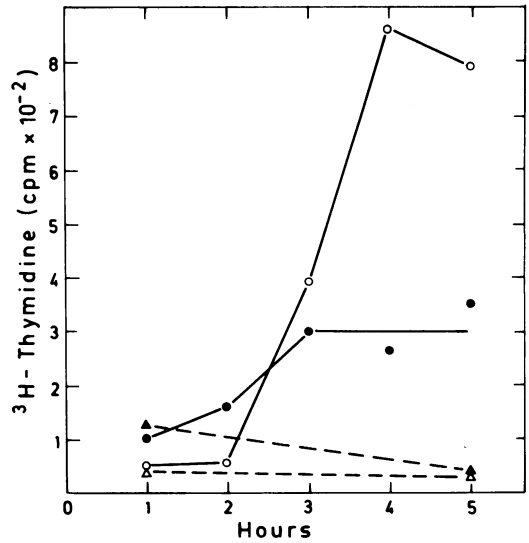

FIG. 1. *Effect of isatin-β-thiosemicarbazone (IBT) on viral DNA synthesis. At different times after infection, HeLa cells treated with IBT were incubated with 4 μCi of ^{3}H-thymidine/ml for 10 min. The pulses were terminated by cooling the cultures and washing the cells with cold saline. The cells were resuspended in 1 ml of 10 mM tris(hydroxymethyl)aminomethane, 10 mM NaCl, and 1.5 mM $MgCl_2$, pH 7.8 (RSB), containing 0.5% NP40. Nuclei were removed by centrifugation at 1,000 × g for 2 min. Radioactivity of trichloroacetic acid-precipitable material in the cytoplasm of the cells was measured by liquid scintillation counting. Symbols: ○, infected cells in the absence of IBT; ●, infected cells in the presence of IBT; △, uninfected cells in the absence of IBT; ▲, uninfected cells in the presence of IBT.*

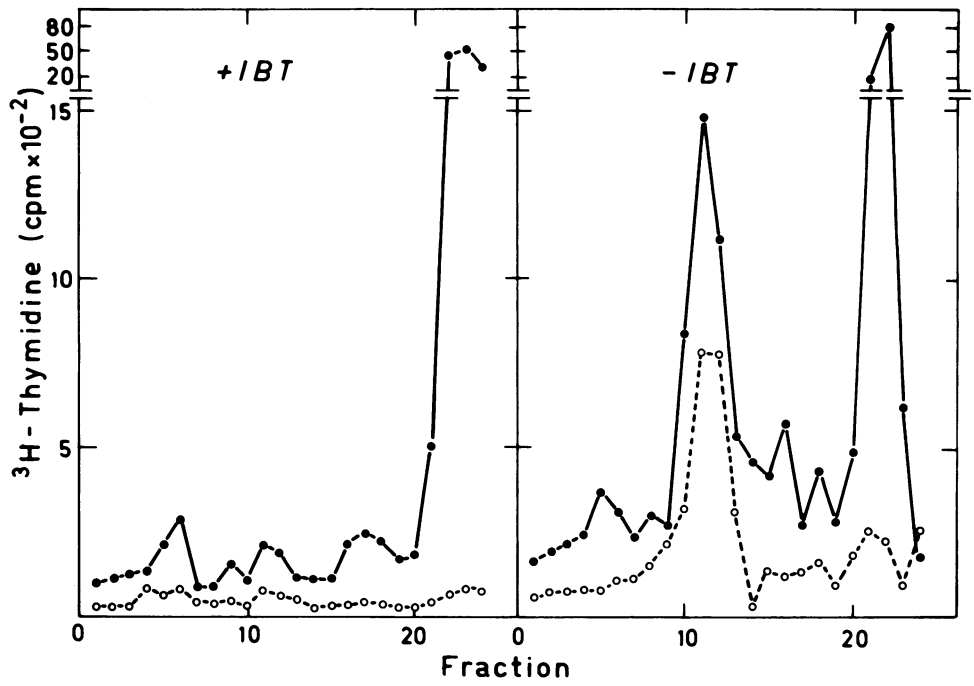

FIG. 2. *Fate of viral DNA in the presence of isatin-β-thiosemicarbazone (IBT). HeLa cells treated with IBT were incubated with ^{3}H-thymidine (8 μCi/ml) between 1.5 and 3 h after infection. The cells were then washed, and medium containing unlabeled thymidine (10^{-6} M) and IBT was added. At 21 h after infection, the cells were washed, resuspended in 1 mM sodium phosphate (pH 7.0), disrupted with a Dounce homogenizer, and layered directly on a 25 to 40% (wt/vol) sucrose gradient. After centrifugation in an SW 50.1 rotor at 13,000 rev/min for 35 min, fractions (0.3 ml) were collected from the bottom of the tube. One portion of each fraction was directly precipitated with trichloroacetic acid, and another was first adjusted to 10 mM $MgCl_2$ and incubated with deoxyribonuclease (50 μg/ml; Worthington Biochemical Corp.) for 30 min at 37 C. The fate of DNA in infected cells not treated with IBT was similarly determined. Symbols: ●, total; ○, deoxyribonuclease-resistant.*

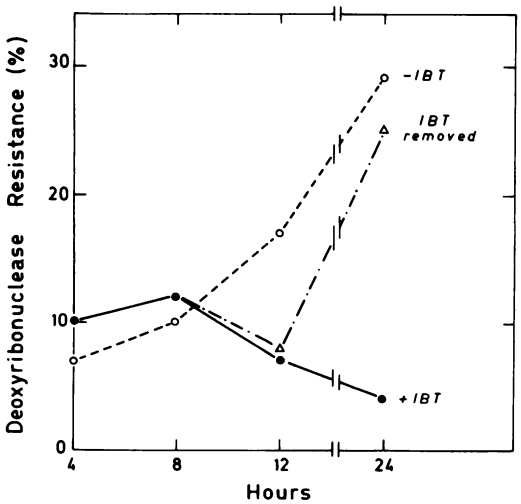

FIG. 3. *Resistance to deoxyribonuclease after removal of isatin-β-thiosemicarbazone (IBT). Infected HeLa cells were labeled with ^{3}H-thymidine as described in Fig. 2. Samples were removed at intervals starting at 4 h after infection, and cytoplasmic fractions were prepared, as in Fig. 2. One portion of the cytoplasm was directly precipitated with trichloroacetic acid and another was first digested with deoxyribonuclease, as in Fig. 2. The percentage of deoxyribonuclease-resistant DNA was calculated. Changes in deoxyribonuclease resistance were also followed in cultures from which IBT was removed at 8 h after infection and in cultures not treated at all with IBT.*

labeled polypeptides were then analyzed. Similar amounts of labeled methionine were incorporated to the cells. "Late" viral proteins are produced by wt and IBT^R strains of vaccinia virus, in both the absence and the presence of IBT (Fig. 5). In the presence of IUdR, a different polypeptide profile was obtained (Fig. 5), since IUdR prevents the formation of viral DNA and subsequently the appearance of "late" viral proteins.

Formation of the core polypeptide from its precursor. A major vaccinia virus structural polypeptide was shown by pulse-chase experiments to form from a higher-molecular-weight precursor (8, 9). This process appears to be a

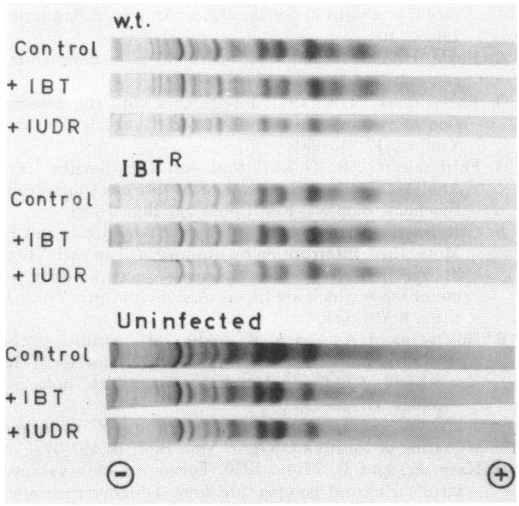

FIG. 4. *Synthesis of early viral polypeptides. HeLa cells were infected with purified wt strain of vaccinia virus or with the IBT^R mutant at a multiplicity of infection of 10. After 30 min of absorption at 37 C, Eagle medium containing one-twentieth of the regular concentration of methionine, and supplemented with IBT, was added. At 1 h after infection, ^{35}S-methionine (3 μCi/ml) was added, and 1 h later cytoplasmic extract was prepared and analyzed by polyacrylamide gel electrophoresis, as described in Materials and Methods. Additional cultures, to which IBT was not added, or to which IUdR (5 μg/ml) was added at the time of infection, were similarly examined. The effect of the drugs on polypeptide synthesis in uninfected cells was also studied.*

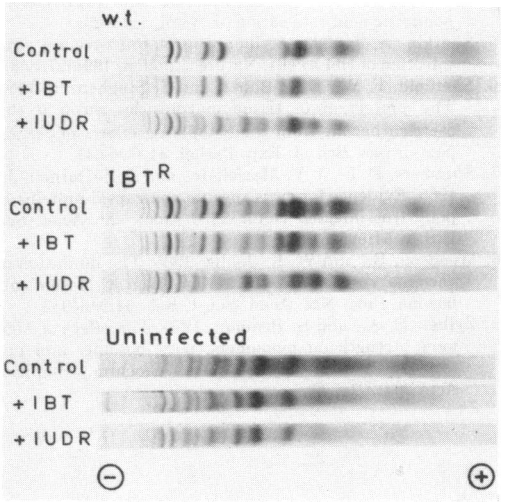

FIG. 5. *Synthesis of late viral polypeptides. HeLa cells were infected with wt and IBT^R as described in Fig. 4. ^{35}S-methionine (2 μCi/ml) was added to the cultures at 4 h after infection. Cytoplasmic extracts were prepared 3 h later and were analyzed by polyacrylamide gel electrophoresis, as described in Materials and Methods.*

late step associated with virus maturation and is completely prevented by rifampin (8, 9). We followed the formation of this structural polypeptide in the presence of IBT. At 7 h after infection, infected HeLa cells treated with IBT were labeled with ^{35}S-methionine for 30 min. The cells were then washed, and medium containing unlabeled methionine was added. The labeled polypeptides in the cytoplasm of the cells, at the end of the pulse period and after 3 h of chase, were examined by polyacrylamide gel electrophoresis. The precursor polypeptide (P4a) is formed in the presence of either IBT or rifampin (Fig. 6, pulse). However, the cleavage, which produces the structural polypeptide (4a), occurs in the presence of IBT but is prevented by rifampin (Fig. 6, chase). The amount of 4a formed in the control and in the presence of IBT was greater when a 4.5-h chase was performed. This suggests that the virus maturation event which is blocked by rifampin precedes the one which is inhibited by IBT.

DISCUSSION

Different observations concerning the formation of "late" proteins of poxviruses in the presence of IBT have been described. Appleyard et al. (1) studied rabbitpox infection in HeLa cells by immunoprecipitation and found that IBT had no effect on antigen production during the first 4 h of virus growth, but later prevented

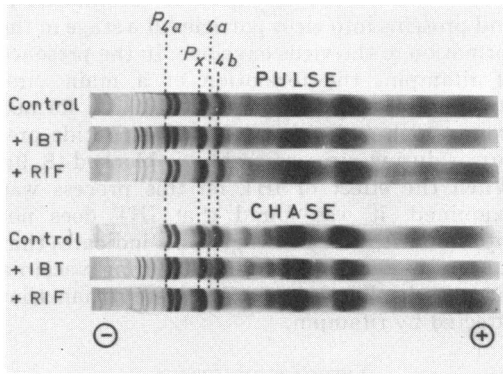

FIG. 6. *Formation of the core polypeptide (4a). HeLa cells infected with purified wt strain at a multiplicity of infection of 10 were treated with IBT. At 7 h after infection, the cells were labeled with ^{35}S-methionine (8 μCi/ml) for 30 min. The cultures were then washed, and medium containing a 10-fold excess of unlabeled methionine in Eagle medium and IBT were added. Cytoplasmic extracts were prepared at the end of the pulse and after a 3-h chase. The polypeptides were analyzed by polyacrylamide gel electrophoresis, as described in Materials and Methods. Additional cultures to which IBT was not added or to which rifampin (100 μg/ml) was added were similarly examined.*

the appearance of new viral antigens. Woodson and Joklik (18) observed a drastic decrease in incorporation of labeled amino acids into proteins later than 3 h postinfection in vaccinia-infected HeLa cells treated with IBT. On the other hand, Easterbrook (3), working with vaccinia virus in KB cells and using fluorescent-antibody staining and complement-fixation techniques, did not observe any decline in viral protein formation until later than 24 h after infection. Our study, based on analysis of vaccinia virus polypeptides in HeLa cells by polyacrylamide gel electrophoresis, demonstrated that a broad spectrum of both "early" and "late" viral proteins was synthesized in the presence of IBT. It is therefore possible to suggest that the mechanism of action of IBT on vaccinia virus multiplication does not involve an inhibition of all "late" viral proteins. Nevertheless, the vaccinia genome is large enough to code for hundreds of polypeptides and it is impossible to conclude that all necessary viral proteins are made. We suspect that the decrease in DNA replication, breakage of polyribosomes, and decline of protein synthesis occurring at late stages of virus multiplication in the presence of IBT are secondary effects of the drug, resulting from the accumulation of unassembled viral structural components.

From biochemical and electron microscopy studies, it was concluded (5, 11) that rifampin interferes with the assembly of vaccinia DNA and proteins into virus particles at a stage in the formation of the virus envelope. In the presence of rifampin, the formation of a main viral structural polypeptide (4a), which is formed from a high-molecular-weight polypeptide precursor during virus assembly, is blocked (8, 9). When the effect of IBT on this process was examined, it was found that IBT does not interfere with this cleavage. This indicates that the target for IBT inhibition during vaccinia virus multiplication is a later step than that affected by rifampin.

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