ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1972, p. 395-401

Vol. 2, No. 5
Printed in U.S.A.

Antiviral Action of Camptothecin[1]

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Received for publication 22 September 1972

At a concentration of 10 μM, camptothecin inhibited vaccinia deoxyribonucleic acid (DNA) synthesis in HeLa cells. Inhibition of viral DNA synthesis was observed when the drug was added before infection or at 1 or 2 hr after infection. Inhibitory effects of camptothecin on vaccinia DNA synthesis could be reversed, even after exposure to the alkaloid for 2 hr. Viral DNA, isolated from vaccinia-infected, camptothecin-treated cells, displayed an altered sedimentation constant after alkaline sucrose density gradient centrifugation. Incorporation of uridine into vaccinia messenger ribonucleic acid was inhibited by camptothecin, but the activity of ribonucleic acid polymerase, as tested in isolated vaccinia cores, was not affected by the drug. Camptothecin had essentially no effect on replication of poliovirus in HeLa cells.

Camptothecin is a cytotoxic plant alkaloid isolated from *Camptotheca acuminata* (family Nyssaceae; 25). We previously showed that camptothecin inhibits synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in HeLa cells and induces degradation of DNA (14). The drug inhibits the synthesis of nucleic acid in leukemia L1210 cells (21, 22) but has no effect on macromolecular synthesis in mitochondria prepared from various tissues in the rat (3). Camptothecin inhibits growth of experimental tumors in rodents (8, 10) and has been used for chemotherapy of certain neoplasms in man (9).

The present studies were undertaken to determine whether camptothecin would inhibit growth of viruses that replicate in the cytoplasm of the host cell. Vaccinia, a DNA virus whose nucleic acids are replicated on a DNA template, and poliovirus, a virus with an RNA template, were chosen for these experiments. Vaccinia virus cores were used to study the effects of camptothecin on cell-free synthesis of viral RNA.

Our results indicate that camptothecin inhibits synthesis of viral DNA and RNA in vaccinia-infected HeLa cells. Furthermore, the sedimentation characteristics of vaccinia DNA were altered when the drug was added to vaccinia-infected HeLa cells. The latter observation may underlie the antiviral action of camptothecin on DNA viruses. The alkaloid has essentially no effect on replication of poliovirus in HeLa cells. (A preliminary report of these studies was presented before the American Association of Immunologists, April 1972 [Fed. Proc. **31**:810, 1972].)

[1] Publication no. 266 from the Joan and Lester Avnet Institute of Molecular Biology.

MATERIALS AND METHODS

Chemicals. Camptothecin was kindly provided by M. E. Wall of the Research Triangle Institute, North Carolina, and by J. L. Hartwell and H. B. Wood, Jr., of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. The alkaloid, in the form of its sodium salt, was dissolved in water just prior to use. Actinomycin D was purchased from Mann Research Laboratories; fetal calf serum and tissue culture media, from Grand Island Biological Co.; and nonradioactive nucleotides, from P-L Biochemicals, Inc. ^{3}H-thymidine (6.7 Ci/mmole), ^{3}H-uridine (13.8 Ci/mmole), and ^{3}H-uridine triphosphate (UTP, 17.1 Ci/mmole) were obtained from New England Nuclear Corp.

Cells and viruses. HeLa S_3 cells were grown in suspension culture in Eagle minimal essential medium (MEM; 6) supplemented with 5% fetal calf serum. The WR strain of vaccinia virus, kindly provided by W. Joklik, was grown in HeLa cells and purified as described by Joklik (16). The number of vaccinia virions was determined by measuring the absorbance at 260 nm (17). Purified type 1 poliovirus was a gift from E. Ehrenfeld. Infection of actinomycin-treated HeLa cells with poliovirus at a multiplicity of 300 plaque-forming units/cell was performed as described by Summers et al. (24). Infectivity of poliovirus was determined by the plaque assay of Bishop and Koch (2).

Determination of vaccinia DNA synthesis. Virus-specific DNA synthesis was measured by determining the rate of incorporation of ^{3}H-thymidine into cold

trichloroacetic acid-insoluble material in the cytoplasm of infected HeLa cells. Cells (4×10^6/ml) were suspended in MEM supplemented with 5% fetal calf serum, and were then infected with purified vaccinia virus strain WR at a concentration of approximately 650 virions/cell. After incubating for 20 min at 37 C, the cell suspension was diluted to a concentration of 4×10^5 cells/ml; the time of dilution is represented as the initial point for all experiments shown. At the indicated times, samples (2 ml) of the cell suspension were removed, 5 μCi of ^{3}H-thymidine was added, and the incubation was continued for 30 min at 37 C. Cells were collected by centrifugation, washed twice with a cold buffered saline solution (7), and resuspended in 1.5 ml of buffer composed of 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, *p*H 7.5, 10 mM KCl, and 1.5 mM $MgCl_2$. The cells were allowed to swell in the buffer for 7 min at 4 C and then ruptured with a Dounce homogenizer calibrated to break more than 95% of the cells but less than 1% of the nuclei. Nuclei and unbroken cells were removed by centrifugation for 4 min at $800 \times g$. Trichloroacetic acid was added to the supernatant solution at a final concentration of 5%, and the acid-insoluble material was collected on 2.5-cm type HA membrane filters (Millipore Corp.). Radioactivity was determined by liquid scintillation counting after suspension of the dried filters in 10 ml of a toluene solution containing 0.5% 2,5-diphenyloxazole and 0.003% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene. The counting efficiency of this system for tritium was 14%.

Determination of vaccinia RNA synthesis. Viral RNA synthesis was measured by determining the rate of incorporation of ^{3}H-uridine into cold trichloroacetic acid-insoluble material in the cytoplasm of infected cells (1). Cells were infected, incubated for 20 min at 37 C, and diluted as described in the preceding section. Samples (4 ml) were removed from the suspension culture at the indicated times, and 2.5 μCi of ^{3}H-uridine was added. Cells were further incubated for 10 min at 37 C, washed, and ruptured with a Dounce homogenizer; the acid-insoluble material was measured as described above for the synthesis of DNA.

Assay for RNA polymerase activity in vaccinia cores. Vaccinia cores were prepared by treatment of purified virus with detergent as described by Kates and Beeson (20). The rate of RNA synthesis by this core-associated RNA polymerase was determined as follows. The reaction mixture contained, in a total volume of 0.4 ml: 2×10^{10} cores; adenosine triphosphate, 1 μmole; guanosine triphosphate, 0.5 μmole; cytidine triphosphate, 0.5 μmole; UTP, 0.02 μmole; ^{3}H-UTP, 1 μCi; $MgCl_2$, 2 μmoles; 2-mercaptoethanol, 4 μmoles; and Tris-HCl, *p*H 8.5, 20 μmoles. All components of the reaction mixture, except the four nucleotides, were preincubated for 5 min at 37 C in the presence or absence of inhibitor. The assay was initiated by addition of the nucleotides.

Alkaline sucrose density gradient analysis of the cytoplasmic fraction obtained from vaccinia-infected HeLa cells. Vaccinia virus-infected HeLa cells (4.5×10^6 cells) were collected by centrifugation, washed

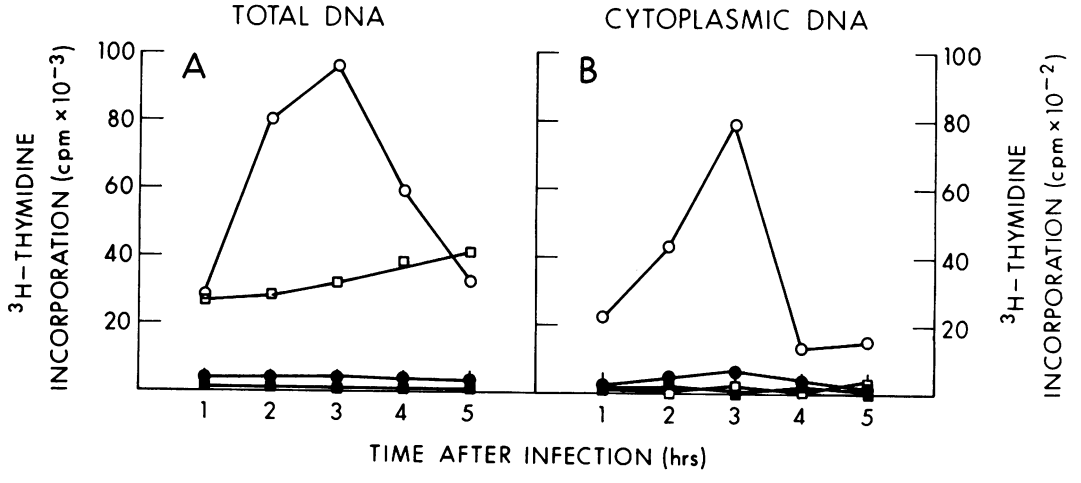

FIG. 1. *Effect of camptothecin on incorporation of thymidine into DNA in vaccinia-infected and uninfected HeLa cells. Cells were infected with vaccinia virus as described in Materials and Methods. Camptothecin was added 1 min prior to infection and maintained at a final concentration of 40 μM throughout the experiment. Cells (2.5 ml) were removed at intervals of 1 hr, and the rate of DNA synthesis was determined as described in Materials and Methods by adding ^{3}H-thymidine and continuing the incubation for 30 min. A sample (0.5 ml) of the suspension was removed, the cells were washed, 5% trichloroacetic acid was added, and the precipitates were collected on membrane filters. Radioactivity in the precipitate was taken as a measure of total DNA synthesis. Total DNA (panel A) represents the combined rate of host and viral DNA synthesis in infected cells. The remaining cells (2.0 ml) were ruptured mechanically, as described in Materials and Methods, and were centrifuged; the trichloroacetic acid-precipitable radioactivity in the cytoplasm was determined as a measurement of viral DNA synthesis (panel B). All data shown represent the average rate of DNA synthesis per 30 min per ml of cells. (□) Uninfected; (■) uninfected, camptothecin added; (○) infected; (●) infected, camptothecin added.*

twice with a buffer composed of 50 mM Tris-HCl, *p*H 8.0, 5 mM disodium ethylenediaminetetraacetate (EDTA), and 0.15 M NaCl, and then resuspended in 1.5 ml of a cold solution composed of 10 mM Tris-HCl, *p*H 8.0, 50 mM KCl, and 5 mM EDTA. These buffers were supplemented with 40 μM camptothecin for those experiments in which the drug was used as an inhibitor. Cells were allowed to swell for 7 min and then ruptured with a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation; 0.5 ml of the cytoplasmic supernatant solution was layered on a 5 to 20% alkaline sucrose density gradient and immediately centrifuged at 15,000 rev/min for 15 hr at 4 C in a Spinco SW27 rotor, as previously described (14).

RESULTS

Effect of camptothecin on the synthesis and properties of vaccinia DNA. At a concentration of 40 μM, camptothecin inhibited "total DNA" synthesis (Fig. 1A, representing the combined rate of DNA synthesis in host cell and virus) and prevented synthesis of DNA in the cytoplasm (Fig. 1B, representing synthesis of viral DNA). The effect of various concentrations of camptothecin on cytoplasmic DNA synthesis in infected cells is shown in Fig. 2. Camptothecin strongly inhibited synthesis of viral DNA at a concentration of 10 μM. At a camptothecin concentration of 1 μM, the apparent rate of DNA synthesis did not differ significantly from the control, and at 0.1 and 0.01 μM camptothecin the rate of thymidine incorporation into acid-insoluble material exceeded that of the control reaction.

The effect of camptothecin on viral DNA synthesis, when the drug was added at various times during the infectious cycle, is shown in Fig. 3. The results of this experiment indicated that camptothecin is an effective inhibitor either when added prior to the infection or at 1 or 2 hr after infection.

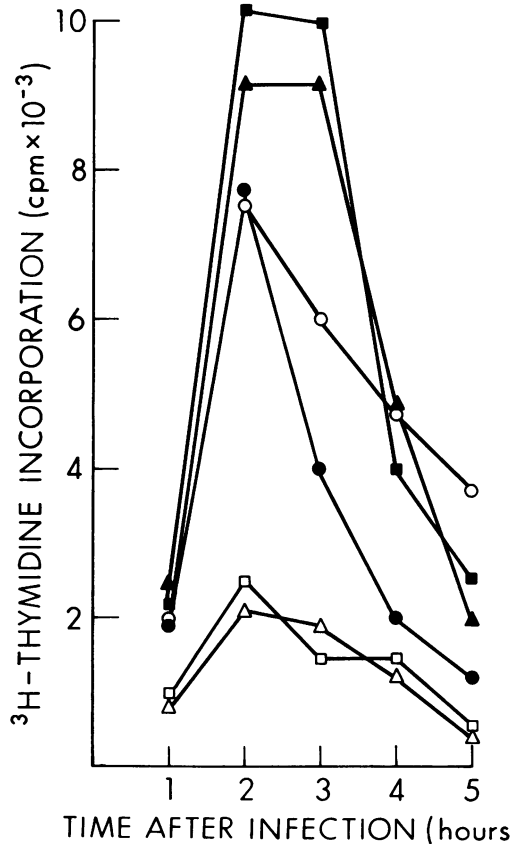

FIG. 2. *Effect of concentration of comptothecin on vaccinia DNA synthesis. Camptothecin was added 1 min prior to infection at the following concentrations:* △, *20 μM;* □, *10 μM;* ●, *1 μM;* ▲, *0.1 μM;* ■, *0.01 μM;* ○, *uninhibited control. Viral DNA synthesis was measured in the cytoplasmic fraction of vaccinia-infected HeLa cells, as described in Materials and Methods.*

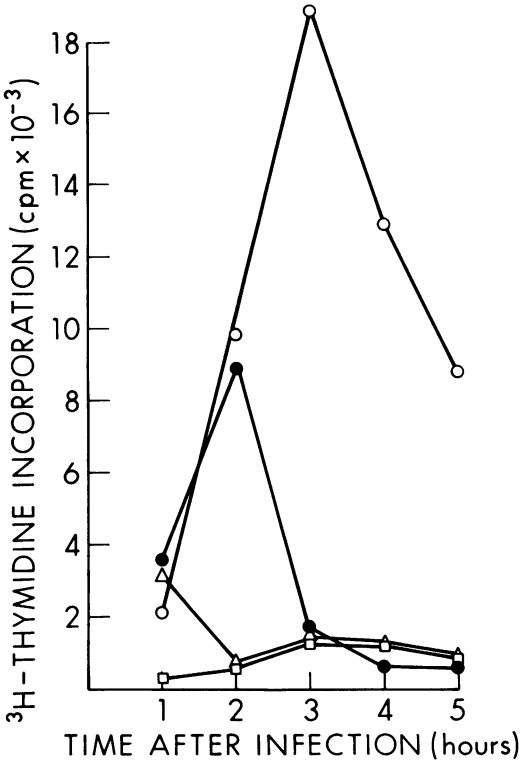

FIG. 3. *Inhibition of vaccinia DNA synthesis by camptothecin. Camptothecin was present at a final concentration of 40 μM.* (□) *Camptothecin added 1 min prior to infection;* (△) *camptothecin added 1 hr after infection;* (●) *camptothecin added 2 hr after infection;* (○) *uninhibited control. Viral DNA synthesis was measured as described in Materials and Methods.*

The inhibitory effect of camptothecin on viral DNA synthesis apparently can be reversed by washing the cells twice with warm MEM and resuspending the cells in MEM supplemented with 5% fetal calf serum (Fig. 4). In these experiments, the drug was added 1 min prior to infection. Full reversal of inhibition could still be achieved up to 2 hr after infection.

Viral DNA obtained from the cytoplasm of camptothecin-treated HeLa cells displayed a lower sedimentation constant than that obtained from untreated cells, as determined by alkaline sucrose density gradient centrifugation (Fig. 5). In these experiments, viral DNA was labeled by incubating infected cells with ^{14}C-thymidine for 60 min. Incorporation of radioactivity was stopped by washing the cells twice with ^{12}C-thymidine. Camptothecin was added, and the incubation was continued for 30 min. The pellet from the drug-treated preparation contained only about 30% as much viral DNA as the pellet from the untreated preparation. These alterations in sedimentation properties were observed only if camptothecin was present in the buffers used for the washing and swelling of drug-treated cultures.

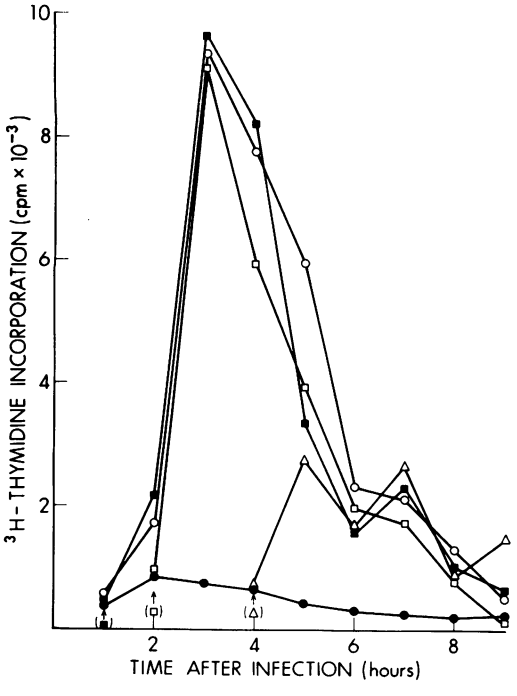

FIG. 4. *Reversal of inhibition of vaccinia DNA synthesis. Camptothecin was added 1 min prior to infection at a final concentration of 40 μM. At the times indicated by the arrow, cells (18 ml) were collected by centrifugation, washed twice with warm MEM, and resuspended in MEM containing 5% fetal calf serum. Samples (2 ml) were removed from the culture at 60-min intervals, and viral DNA synthesis was determined as described in Materials and Methods.* (●) *Camptothecin-treated;* (■) *exposed to camptothecin for 1 hr prior to reversal;* (□) *exposed to camptothecin for 2 hr prior to reversal;* (△) *exposed to camptothecin for 4 hr prior to reversal;* (○) *uninhibited control.*

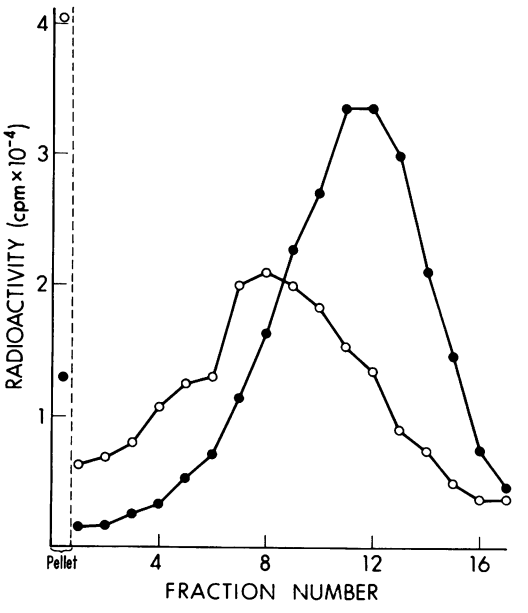

FIG. 5. *Alkaline sucrose density gradient analysis of vaccinia DNA. Cells (4.0 ml) were infected and diluted as described in Materials and Methods. At 60 min after infection, the cells were labeled for 60 min by adding ^{3}H-thymidine (1 μCi/ml). Cells were washed twice with cold MEM containing 20 μM ^{12}C-thymidine, resuspended in MEM containing 5% fetal calf serum, and incubated at 37 C for 15 min. The culture was divided; camptothecin, at a final concentration of 40 μM, was added to one half, and both cultures were incubated for an additional 30 min. Cytoplasmic fractions were prepared and analyzed on alkaline sucrose density gradients as described in Materials and Methods.* (●) *Camptothecin-treated;* (○) *uninhibited control.*

Effect of camptothecin on synthesis of vaccinia mRNA. Because camptothecin altered the sedimentation properties of DNA, the drug could inhibit synthesis of messenger RNA (mRNA) copied from the DNA template. Vaccinia virus mRNA is synthesized in the cytoplasm of infected cells and can be specifically labeled with a 10-min pulse of radioactive uridine. Significant amounts of host-cell RNA are not released from the nucleus into the cytoplasm during this period of time (1). The results shown in Fig. 6 indicate that camptothecin inhibits most of the synthesis of viral mRNA in infected cells.

The effect of camptothecin on synthesis of mRNA was also determined in isolated viral cores

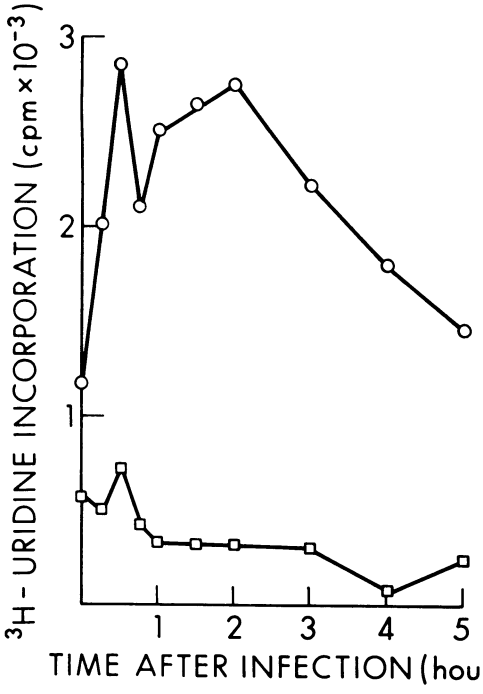

FIG. 6. *Effect of camptothecin on incorporation of uridine into the cytoplasmic fraction of vaccinia-infected HeLa cells. Camptothecin was added 1 min prior to infection at a final concentration of 40 μM. (□) Camptothecin-treated; (○) uninhibited control.*

(Fig. 7). Significant inhibition of mRNA synthesis did not occur when 0.1 mM camptothecin was preincubated with viral cores for 5 min prior to adding the four nucleotide triphosphate substrates. In contrast, actinomycin D, added to viral cores under similar conditions, totally inhibited synthesis of mRNA.

Effect of camptothecin on replication of poliovirus. The effect of camptothecin on RNA synthesis in a single-stranded RNA virus was determined by adding the drug to actinomycin-treated HeLa cells infected with type 1 poliovirus. Incubation was allowed to proceed for 30 min before the drug was added. Camptothecin had essentially no effect on incorporation of ^{3}H-uridine into viral RNA when tested at concentrations of 1, 10, or 100 μM (Fig. 8). Small variations in the amount of RNA synthesized were occasionally observed, but these differences were not dose-dependent. Guanidine, a compound known to inhibit poliovirus replication (5), served as a control for these experiments.

Cytoplasmic extracts of poliovirus-infected HeLa cells, labeled with radioactive uridine and exposed to 100 μM camptothecin throughout the infectious cycle, were treated with sodium dodecyl sulfate and analyzed on 15 to 30% neutral sucrose gradients by the method of Hecht and Summers (11). Under these conditions, a virus-specific 35*S* RNA peak was observed (data not shown); the sedimentation characteristics of this material were indistinguishable from those of the untreated control.

Camptothecin did not alter the yield of poliovirus when virus-infected HeLa cells were incubated with 4 × 10^{-5} M camptothecin for 6 hr (Table 1). Guanidine significantly inhibited infectivity of poliovirus when tested under similar conditions.

DISCUSSION

Camptothecin, at a concentration of 10 μM, promptly inhibited incorporation of thymidine into vaccinia DNA and was effective even when

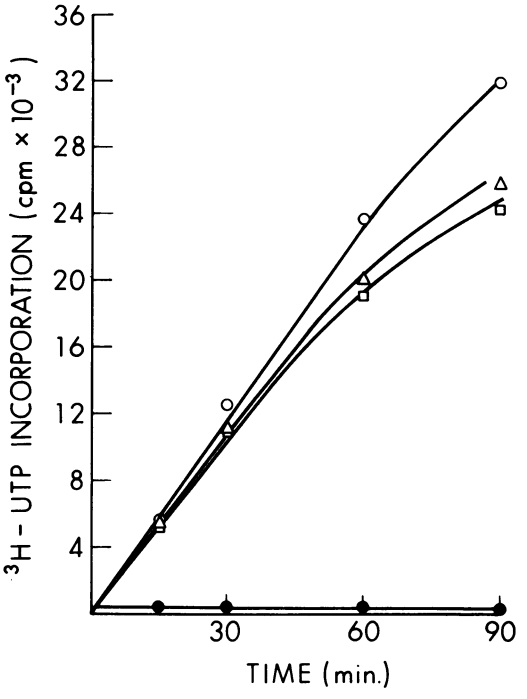

FIG. 7. *Effect of camptothecin on activity of RNA polymerase in vaccinia viral cores. Symbols: △, 10 μM camptothecin; □, 100 μM camptothecin; ●, 100 μM actinomycin; ○, uninhibited control. The assay was performed as described in Materials and Methods. The total volume of the reaction mixture was 3.0 ml. At the times indicated, samples (0.4 ml) were removed and added to 2.5 ml of cold 10% trichloroacetic acid. After 10 min at 4 C, 20 μmoles of sodium pyrophosphate were added, and the precipitates were collected on membrane filters. Filters were washed three times with 5% trichloroacetic acid, treated with 0.6 ml of NH$_4$OH for 15 min, and dissolved in 10 ml of the scintillation mixture described by Bray (4) for determination of radioactivity.*

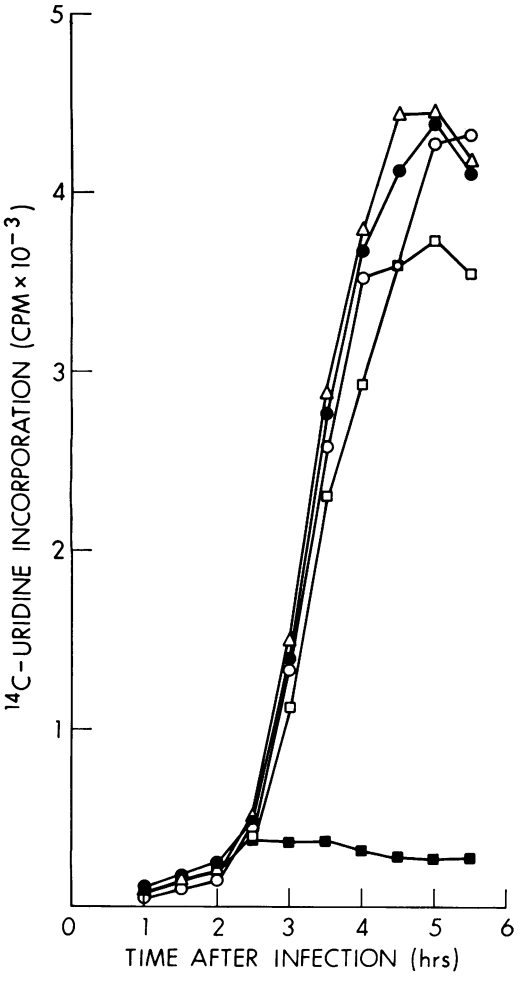

FIG. 8. *Effect of camptothecin on uridine incorporation into poliovirus RNA. HeLa cells were infected with poliovirus as described in Materials and Methods; camptothecin and ^{14}C-uridine (0.15 μCi/ml) were added 30 min after infection. At intervals of 30 min, samples (0.2 ml) of the cell suspension were removed and pipetted into 2.0 ml of cold Earle solution. Cells were centrifuged and lysed in 1.0 ml of water and precipitated by the addition of cold 5% trichloroacetic acid. Radioactivity in trichloroacetic acid-insoluble material was determined as described in Materials and Methods. Symbols: ●, 100 μM camptothecin; □, 10 μM camptothecin; △, 1 μM camptothecin; ■, 3 mM guanidine; ○, no additions.*

added 2 hr after infection. It would appear that camptothecin does not act by preventing adsorption or penetration of vaccinia virus, as these initial steps in the replicative cycle are completed within 1 hr after infection (19). Inhibition of viral DNA synthesis by camptothecin was reversed by washing the infected cells and resuspending them

TABLE 1. *Effect of camptothecin on yield of poliovirus*[a]

Addition	Concn (M)	Virus yield (PFU/ml)
None	—	1.1×10^9
Camptothecin	4×10^{-5}	1.0×10^9
Guanidine	3×10^{-3}	5.0×10^6

[a] Suspensions of HeLa cells (10^7 cells/ml) were inoculated with poliovirus at an input multiplicity of 10 plaque-forming units (PFU)/cell. After adsorption at 37 C for 60 min, the cells were centrifuged, washed, and resuspended at a concentration of 10^6 cells/ml in MEM containing the indicated additions. After incubation at 37 C for 6 hr, the samples were frozen and thawed three times and then centrifuged; the infectivity of the supernatant fluids was measured in the absence of camptothecin and guanidine.

in a drug-free medium. Even when this was carried out 2 hr after the cells were infected and treated with the drug, the subsequent rate of DNA synthesis was similar to that in uninhibited controls. This reversibility suggests the use of camptothecin for studies of macromolecular synthesis in virus-infected cells.

The molecular weight of vaccinia DNA (as calculated from the particle weight and DNA content) has been estimated at 160×10^6 (18). Processing of such high-molecular-weight DNA frequently results in partial degradation of the molecule, and isolation of vaccinia DNA from infected HeLa cells necessarily involves some mechanical shearing. The amount of high-molecular-weight viral DNA was further reduced in cells that were treated with camptothecin.

Although camptothecin inhibits synthesis of viral mRNA in infected cells, RNA polymerase activity in vaccinia cores was unaffected by the alkaloid. The enzyme contained in these cores is known to initiate, terminate, and release RNA chains (20). Failure of camptothecin to alter the activity of this enzyme suggests that the inhibitory activity of the drug requires additional factors present in the host cell or, possibly, that the drug is unable to penetrate viral cores.

The effects of camptothecin on viral DNA synthesis resembled some of the actions of the drug previously observed in HeLa cells (14). In those experiments, low concentrations of camptothecin were observed to inhibit synthesis of DNA and RNA, although they did not initially influence protein synthesis. Inhibition of RNA synthesis was reversible; furthermore, camptothecin did not significantly inhibit activity of isolated DNA and RNA polymerases. Incubation of HeLa cells with

camptothecin also resulted in partial degradation of DNA.

Camptothecin inhibits replication of adenovirus and alters the sedimentation properties of adenovirus DNA (12, 13). Like HeLa cells, adenovirus replicates its DNA in the nucleus. Since vaccinia virus replicates exclusively in the cytoplasm, the effects of camptothecin are confined neither to the nucleus nor to the cytoplasm.

Alterations in sedimentation of DNA observed after exposure of virus-infected cells to camptothecin could result from the action of endonucleases; such enzymes have been described in vaccinia virus (23). Degradation could also result from a chemical action of camptothecin on DNA. In either case, an altered template DNA could affect the synthesis of DNA and RNA. Alternatively, camptothecin may inhibit the activity of an enzyme involved in DNA repair, such as DNA ligase (cf. 15); degradation of DNA would, thereby, represent failure of DNA repair.

Camptothecin had essentially no effect on replication of poliovirus, a virus containing single-stranded RNA. Concentrations of camptothecin that did not influence synthesis of RNA in poliovirus completely inhibited synthesis of vaccinia virus DNA and mRNA. Apparently, camptothecin can inhibit synthesis of mRNA from a DNA template in the cytoplasm but not mRNA made from an RNA template. Since infectivity of poliovirus was not altered after replication in the presence of camptothecin, the drug does not seem to alter synthesis or assembly of poliovirus proteins. Actinomycin was not present during the assay for poliovirus replication, eliminating the possibility that camptothecin and actinomycin might interact. Poliovirus synthesized RNA from exogenously administered nucleosides in the presence of camptothecin; therefore, it is unlikely that the drug acted on vaccinia virus by preventing uptake of nucleosides required for nucleic acid synthesis or the conversion of these nucleosides to their respective phosphorylated forms.

ACKNOWLEDGMENTS

We are grateful to M. Wall for providing us with our original samples of camptothecin and to E. Ehrenfeld and D. Summers for reviewing this manuscript.

These investigations were supported by Public Health Service grant CA-10666 from the National Cancer Institute and by American Cancer Society grant IC-61D. The senior author is a Career Development Awardee (K4-GM-11,147) of the U.S. Public Health Service.

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