Inhibition by Trichothecene Mycotoxins of Replication of Herpes Simplex Virus Type 2

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The effects of the trichothecene mycotoxins diacetoxyscirpenol, neosolaniol and T-2 toxin on the replication of herpes simplex virus type 2 (HSV-2) in HEp-2 cells were examined. The 50% inhibitory concentrations for HSV-2 replication were 2.3 ng/ml for diacetoxyscirpenol, 52.0 ng/ml for neosolaniol, and 1.6 ng/ml for T-2 toxin. The addition of these toxins to the cells within 4 hr after HSV-2 infection was necessary for the inhibition of the virus replication. Viral polypeptides synthesized in HSV-2-infected cells treated with the toxins were analyzed by immunoblotting using rabbit antiserum to HSV-2. The syntheses of early viral proteins were greatly inhibited when the toxins were added 1 hr after infection, and those of late viral proteins were also inhibited by toxin exposures 4 hr through 6 hr after infection. The toxins added after the completion of the latter protein syntheses affected the HSV-2 replication insignificantly. However, viral RNA synthesis was not inhibited when the toxins were added 1 hr after infection. These results indicate that trichothecene mycotoxins inhibit HSV-2 replication by blocking viral protein syntheses.

Trichothecene mycotoxins such as diacetoxyscirpenol (DAS), neosolaniol (NEOS), and T-2 toxin (T-2) produced by Fusarium species of fungi are a family of sesquiterpenoid compounds and have a double bond at C9, 10 and an epoxy ring at C12, 13 of the molecules.\(^1\) They belong to the A group of trichothecenes characterized by the presence of either a hydrogen atom or a hydroxyl group (or the esters of the hydroxyl group) at C8 of the molecules without a macrocyclic ring between C4 and C15.\(^1\) These trichothecenes have various biological properties including suppression of antibody production,\(^2\) depression of lymphocyte responses of T- and B-cells to mitogens,\(^3\) prolongation of skin graft rejection time\(^2\) and inhibition of cell proliferation.\(^4\) In mammalian cell cultures, trichothecenes first inhibit protein synthesis and then DNA synthesis, but synthesis of RNA is not severely affected.\(^4\) Furthermore, T-2 induces DNA single-strand breakage in lymphoid cells\(^5,6\) and chromosome aberrations in both lymphoid cells and Chinese hamster fibroblast cells.\(^7\)

Although these biological properties of trichothecenes have been studied in detail, the action of these toxins on virus growth is not well understood. In this study, therefore, we attempted to examine the effects of these toxins on virus replication and syntheses of viral macromolecules. Herpes simplex virus type 2 (HSV-2) is a common human herpesvirus that causes genital infection and encephalitis.\(^8\) HSV-2 has a linear double-stranded DNA genome of about 150 kilobase pairs, an icosahedral nucleocapsid, and an envelope acquired by budding of the nucleocapsid through the infected cell nuclear membrane.\(^9\) Because the replication cycle of HSV-2 is relatively short (<24 hr) in cultured mammalian cells, HSV-2 was used as the target of trichothecene mycotoxins in this study. In our previous paper, we examined the cytotoxicity of trichothecenes by a colony-forming inhibition test on HEp-2 cells used as host cells for HSV-
This paper reports that the trichothecenes inhibit HSV-2 replication by blocking viral protein synthesis and that the virus-growth inhibition test will be useful for analysis of the structure–activity relationship in terms of the inhibition of viral protein synthesis by trichothecenes.

**MATERIALS AND METHODS**

**Cell and virus.** HEp-2 cells were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Strain 186 of HSV-2 was used in this study. Virus stock was prepared in human embryonic lung cells with Eagle’s MEM containing 5% FCS. Virus infectivity was measured by plaque formation in HEp-2 cells overlaid with Eagle’s MEM containing 1.5% methylcellulose and 5% FCS. The infectivity titer of the virus stock was 2 x 10^6 plaque-forming units (PFU)/ml in HEp-2 cells at 37°C.

**Trichothecenes and drugs.** DAS, NEOs, and T-2 were used in this study and their chemical structures are shown in Fig. 1. Acyclovir (ACV), an inhibitor of HSV DNA synthesis, was obtained from Wellcome Research Laboratories, Buckinghamshire, England. Actinomycin D (Act D) and cycloheximide (CHX) were purchased from Boehringer-Mannheim, Mannheim, West Germany. All toxins and drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml. Stock solutions (200 μg/ml) were prepared, passed through 450-nm membrane filter (Millipore Corp., Bedford, MA, U.S.A.), and stored at -20°C before use. The maximum concentration of DMSO used was 0.1%, and this concentration had no observable effect either on the viability of HEp-2 cells or on HSV-2 replication.

**Virus-growth inhibition test.** Confluent monolayers of HEp-2 cells in 30-mm plastic dishes were infected with HSV-2 at a multiplicity of 0.2 PFU per cell. After 1 hr of adsorption, the cells were incubated at 37°C in Eagle’s MEM containing 5% FCS with or without trichothecenes. Fifteen hours after infection, the cells were washed three times with Eagle’s MEM to remove the toxins and then scraped into fresh medium supplemented with 30% FCS. After freezing and thawing the infected cells three times, the infectivity of cell-associated virus was measured by plaque assay.

**Immunoblotting analysis.** HSV-2-infected HEp-2 cells were washed with CaCl2- and MgCl2-free phosphate-buffered saline (PBS), harvested, and then solubilized for 1 hr with ice-cooled PBS containing 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM phenyl methylsulfonyl fluoride, and 1 mM ethylenediaminetetraacetate (EDTA). The resulting lysates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were electrophoresed on a 7.5% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (SDS) as described previously.13 Proteins from the gel were electrophoretically transferred to a nitrocellulose sheet (TM-2, Toyo Roshi Co., Ltd., Tokyo) at 50 V (0.2–0.4 A) for 3 hr in a blotting buffer [25 mM tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine, and 10% methanol]. Viral antigens on the sheet were detected by a biotin-streptavidin system (Amersham, Buckinghamshire, England). The sheet was incubated with a 1:60 dilution of anti-HSV-2 rabbit serum and then treated with biotinylated protein A, peroxidase-conjugated streptavidin, and 3,3’-diaminobenzidine tetrahydrochloride (DAB) substrate solution (PBS containing 0.05% DAB, 0.01% H2O2, and 0.075% cobalt chloride) as recommended by the supplier. The following molecular weight markers from Boehringer-Mannheim were used in this study: β-galactosidase (116,000 daltons, 116K), phosphorylase a (94K), bovine serum albumin (68K), and glutamate dehydrogenase (53K).

**Incorporation of [3H] uridine.** [5-3H]Uridine (>25 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. HEp-2 cells in 16-mm wells (2.4 x 10^5 cells/well) of a 24-well plate were infected with HSV-2. After a 1-hr adsorption period, cells were labeled for 5 hr with [3H]Uridine (0.1 µCi/ml) containing 0.1 µg/ml of nonradioactive uridine in the presence or absence of trichothecenes and Act D. Thereafter, the cells were washed twice with PBS containing 0.02% EDTA and resuspended in 1 ml of cold PBS. The cell suspensions were sonicated and treated with 1 ml of 20% trichloroacetic acid (TCA) for 20 min at 0°C. Incorporation of [3H]Uridine into TCA-insoluble materials of the cells was measured as described previously.15

**DNA hybridization analysis.** Virions were purified from extracellular fluid (3 l) of HSV-2-infected HEp-2 cells through dextran gradient centrifugation by the method of Spear and Roizman. HSV-2 DNA was isolated from the purified virions by treatment with SDS (1%) and pronase (1 mg/ml) for 5 hr at 37°C, followed by extraction once with phenol and twice with phenol–chloroform–isoamyl
alcohol (25:24:1), and collected by ethanol precipitation. The preparation was treated with RNase A (20 μg/ml), extracted with phenol and dialized against 0.1 x SSC (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The purified HSV-2 DNA was labeled with biotin by nick translation in the presence of the dTTP analog biotin-11-dUTP (Bethesda Research Laboratories, Inc., Gaithersburg, MD, U.S.A.).

DNA was hybridized by the method of Southern with minor modifications. The nucleic acids of HSV-2-infected HEp-2 cells treated with or without trichothecenes were prepared by lysis in SDS, followed by pronase digestion, phenol extraction, and ethanol precipitation as described above. The nucleic acids (about 20 μg) were digested with 50 units of restriction endonuclease HindIII (Takara Shuzo Co., Ltd., Kyoto) for 5 hr at 37°C. The digestion products were electrophoresed on a 0.8% agarose gel in TAE buffer (10 mM Tris–5 mM sodium acetate buffer (pH 7.8) containing 0.5 mM EDTA), denatured by soaking the gel in 0.2 M NaOH and 0.5 M NaCl, neutralized with TAE buffer, and then electrophoretically transferred to a nylon sheet (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA, U.S.A.) at 40 V (0.4–0.8 A) for 4 hr in TAE buffer. The sheet was prehybridized overnight at 42°C in nonradioactive Northern blotting solution containing 45% formamide. The blot was washed twice with 2 x SSC–0.1% SDS at 25°C, twice with 0.2 x SSC–0.1% SDS at 25°C, and twice with 0.16 x SSC–0.1% SDS at 50°C. The biotinylated DNA on the sheet was detected using peroxidase-conjugated streptavidin and DAB substrate as described in the previous section (immunoblotting analysis).

RESULTS

Effects of trichothecenes on HSV-2 replication

Because none of the three toxins (DAS, NEOS and T-2) decreased the viability of monolayers of HEp-2 cells at a concentration below 200 ng/ml for 3 days at 37°C, the antiviral activities of the toxins were compared at concentrations from 1 ng to 200 ng/ml using HEp-2 cells as host cells for HSV-2 (Fig. 2).

HSV-2 replication was inhibited by the toxins in a dose-dependent manner, and the concentrations required for the 50% inhibition of HSV-2 replication (IC50) were 2.3 ng/ml for DAS, 52.0 ng/ml for NEOS, and 1.6 ng/ml for T-2. As shown in Fig. 2, the virus yield decreased to less than 0.5% of that of control cells (toxin-untreated infected cells) by treatment with 10 ng/ml of DAS or T-2, or 200 ng/ml of NEOS. Therefore, these concentrations of the toxins were used in the experiments described below.

Effects of removal and addition of trichothecenes on HSV-2-infected cells at different intervals after infection

HEp-2 cells infected with HSV-2 were treated with individual toxins after a 1-hr adsorption of the virus and incubated for various times, thereafter their culture media containing toxins were replaced with fresh media without toxins followed by reincubation. The cultures were harvested 15 hr after infection, and the virus titers were measured (Fig. 3A). For almost complete inhibition of HSV-2 replication, the treatment with DAS or T-2 from 1 hr to 4 hr (for 3 hr) or with NEOS from 1 hr to 6 hr (for 5 hr) after virus infection was required. The effects of toxin
exposure at different times after infection on HSV-2 replication were also examined. Figure 3B shows that the addition of each toxin within 4 hr after infection was necessary for complete inhibition of HSV-2 replication. Thereafter, virus yields were increased by delaying the addition of each toxin after infection. These results clearly indicate that the toxins can affect HSV-2 at an early stage of the replication cycle and that their action on the virus is irreversible.

Fig. 3. Effects of Removal and Addition of Trichothecenes on HSV-2-Infected Cells at Different Intervals after Infection.

HEp-2 cells were infected with HSV-2 at a multiplicity of infection of 0.2 PFU per cell. Each medium containing DAS (10 ng/ml), NEOS (200 ng/ml) or T-2 (10 ng/ml) was removed from (A) or added to (B) the infected cell cultures at different time intervals after infection. The cells were harvested 15 hr after infection, and virus titers were measured. The virus yield in toxin-untreated infected cultures was $6.2 \times 10^6$ PFU/ml. •, DAS; △, NEOS; ▲, T-2.

Fig. 4. Immunoblotting Analysis of HSV-2-polypeptides from HSV-2-Infected Cells Treated with Trichothecenes at Different Intervals after Infection.

HEp-2 cells were infected with HSV-2 at a multiplicity of infection of 0.2 PFU per cell. Medium containing ACV (5 μg/ml), Act D (1 μg/ml), CHX (20 μg/ml), DAS (10 ng/ml), NEOS (200 ng/ml), or T-2 (10 ng/ml) was added to the infected cells at different times after infection. Fifteen hours after infection, detergent-solubilized extracts of infected cells were electrophoresed on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. The sheets were immunostained with a 1:60 dilution of anti-HSV-2 rabbit serum, as described in the text. Mock (uninfected cells) and none (untreated infected cells) served as a negative control and as a positive control, respectively. H.A.I., hours of addition of trichothecenes or drugs after infection.

Thereafter, virus yields were increased by detection.
Mechanism of inhibition of HSV-2 replication by trichothecenes

Effects of the toxins on viral protein, RNA, and DNA syntheses in HSV-2-infected HEP-2 cells were examined. Extracts of HSV-2-infected cells treated with individual trichothecenes or with antimetabolites (ACV, Act D, and CHX) from 1 hr to 15 hr (for 14 hr) after virus infection were analyzed by immunoblotting using rabbit antiserum to HSV-2 (Fig. 4). Both early (130K, 84K, 58K, and 56K) and late (135K, 116K, 110K, 105K, 43K, and 40K) viral polypeptides were detected in untreated cells infected with HSV-2 (Fig. 4, lane 2), and the former viral polypeptides were detected in HSV-2-infected cells treated with ACV which inhibited viral RNA synthesis (Fig. 4, lane 3). No HSV-2-specific polypeptide was detected in HSV-2-infected cells treated with trichothecenes (DAS, NEOS and T-2), Act D or CHX, although major two nonspecific polypeptides (120K and 78K) were detected in these treated cells (Fig. 4, lanes 4, 5, 6, 11, and 16) as well as in mock-infected cells (Fig. 4, lane 1).

The three toxins did not inhibit the incorporation of \(^{3}H\)uridine into HSV-2-infected cells (Table I), indicating that these toxins did not affect viral RNA synthesis. In addition, no specific DNA fragments were observed in HSV-2-infected cells treated with the toxins or ACV (Fig. 5), indicating that viral progeny DNA was not synthesized.

We also found whether the toxins affected the syntheses of viral proteins and viral DNA when the toxins were added to the HSV-2-infected cell cultures at various times (4 hr to 10 hr) after infection (Figs. 4 and 6). When the toxins were added to the HSV-2-infected cells 4 hr through 6 hr after infection, early viral proteins and viral DNA fragments were detected, while late viral proteins were inhibited by the toxins (lanes 7, 8, 12, 13, 17, and 18 in Fig.

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**Table I. Effects of Trichothecenes on Incorporation of \(^{3}H\)Uridine into HEP-2 Cells Infected with HSV-2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation of (^{3}H)uridine (cpm x 10^5/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.04 (1.00)*</td>
</tr>
<tr>
<td>DAS (10 ng/ml)</td>
<td>2.13 (1.04)</td>
</tr>
<tr>
<td>NEOS (200 ng/ml)</td>
<td>2.28 (1.11)</td>
</tr>
<tr>
<td>T-2 (10 ng/ml)</td>
<td>2.56 (1.24)</td>
</tr>
<tr>
<td>Act D (1 µg/ml)</td>
<td>0.26 (0.13)</td>
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* Values in parentheses represent the ratios of counts per minute incorporated into infected cells treated with trichothecenes or with Act D to those incorporated into untreated infected cells.

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**Fig. 5. DNA Hybridization Analysis of Viral DNA from HSV-2-Infected Cells Treated with Trichothecenes.**

HEP-2 cells were infected with HSV-2 at a multiplicity of infection of 0.2 PFU per cell. Medium containing DAS (10 ng/ml), NEOS (200 ng/ml), T-2 (10 ng/ml), or ACV (5 µg/ml) was added to the infected cells 1 hr after infection. Fifteen hours after infection, the nucleic acids were prepared from the infected cells, digested with HindIII, electrophoresed on a 0.8% agarose gel, and then electrophoretically transferred to a nylon sheet. The nucleic acids on the sheet were hybridized with biotinylated HSV-2 DNA and stained with peroxidase-conjugated streptavidin, as described in the text. None (untreated infected cells) and ACV-treated infected cells served as a positive control and as a negative control, respectively. The molecular weight markers indicated at the left side of gel were fragments of phage lambda DNA digested with HindIII.
4, and lanes 2, 3, 6, 7, 10, and 11 in Fig. 6). The addition of the toxins 10 hr after infection did not severely affect the syntheses of early and late viral proteins and viral DNA (lanes 10, 15, and 20 in Fig. 4, and lanes 5, 9, and 13 in Fig. 6).

DISCUSSION

Normal cellular DNA and protein syntheses rapidly decline after HSV-2 entry into the cells. The virus is uncoated, and the viral DNA moves into the nucleus, where it is first transcribed to viral mRNAs. The early viral proteins including viral DNA polymerase are synthesized on polyribosomes of the cytoplasm within a few hours after infection and some of them are transported to the nucleus. The viral progeny DNAs are replicated in the nucleus of the infected cell. The late viral proteins, the viral capsid and envelope, are synthesized during the late stage of infection in the cytoplasm and most are transported to the nucleus. After assembly of the nucleocapsid (the capsid together with the enclosed viral DNA) in the nucleus, the envelope is acquired by budding of the nucleocapsid through the altered nuclear membrane. Enveloped virions are moved to the cell surface by the transportation system of the cell and released from the cell.

This study demonstrated that the trichothecenes inhibited the syntheses of HSV-2 early proteins and viral DNA but not viral RNA synthesis, when the toxins were added to the cell cultures 1 hr after infection. These results suggest that the inhibition of early viral polypeptides by the toxins resulted in the decrease of virus infectivity and that the inhibition of virus progeny DNA synthesis was a secondary effect of the toxins because of the reduced synthesis of one early polypeptide (130K), probably HSV-2 DNA polymerase. In this study, we also demonstrated that the toxins inhibited the virus replication by blocking late viral protein syntheses in the stage after syntheses of early viral proteins but did not significantly affect the virus replication after the completion of late viral protein syntheses. These results suggest that viral protein

Fig. 6. DNA Hybridization Analysis of Viral DNA from HSV-2-Infected Cells Treated with Trichothecenes at Different Intervals after Infection.

HEp-2 cells were infected with HSV-2 at a multiplicity of infection of 0.2 PFU per cell. Medium containing DAS (10 ng/ml), NEOS (200 ng/ml), or T-2 (10 ng/ml) was added to the infected cells at different time intervals after infection. Fifteen hours after infection, the nucleic acids were prepared from the infected cells, digested, electrophoresed on a 0.8% agarose gel, and then electrophoretically transferred to a nylon sheet. The nucleic acids on the sheet were hybridized with biotinylated HSV-2 DNA and stained with peroxidase-conjugated streptavidin, as described in the text. H.A.I., hours of addition of trichothecenes after infection.
synthesis is the only target of trichothecene mycotoxins.

As for the structure-antiviral activity relationship of trichothecenes, the activity of DAS was equivalent to that of T-2 but about 23-fold higher than that of NEOS, indicating that both hydroxylation and hydrolysis at the C8 position of DAS and T-2, respectively, resulted in a great decrease in antiviral activity. These results suggest that the virus-growth inhibition test will be useful for analysis of the relationship between structure and antiviral activity of the trichothecenes, because it is a reliable system that directly reflects inhibition of viral early proteins in the first stage of syntheses of viral macromolecules.

Previous studies have shown that T-2 causes single-strand breakage of cellular DNA resulting in radiomimetic lesions in lymphoid cells. On the other hand, our study demonstrated by Southern's blotting analysis that restriction endonuclease cleavage patterns of virus DNAs from infected cells treated with trichothecenes in the stage after the syntheses of early viral proteins were similar to that from toxin-untreated infected cells (Fig. 6), suggesting that the toxins did not induce breakage in HSV-2 DNA.

Further experiments on the antiviral activities of the other trichothecene mycotoxins including deepoxytrichothecenes are now in progress.

REFERENCES