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Irreversible inhibition of human cytomegalovirus replication by topoisomerase II inhibitor, etoposide: a new strategy for the treatment of human cytomegalovirus infection

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Summary

We demonstrated previously that human cytomegalovirus (CMV) infections could enhance the expression of cellular topoisomerase II and this enzyme activity is essential for CMV to replicate in vitro (Benson and Huang, 1988; Benson and Huang, 1990). In this study, we further show that in addition to m-AMSA and VM26 which we had previously reported, a widely used and clinically available drug, etoposide (VP-16 or VePesid) can irreversibly inhibit CMV replication at the drug concentration (2.5 μg/ml) greatly below toxic levels to stationary phase cells. Growing cells were more sensitive to etoposide than stationary phase cells and slight growth inhibition occurred at 2.5 μg/ml level. This inhibitor does not prevent the expression of CMV immediate-early and early genes, but can inhibit viral DNA and late viral-proteins synthesis. Because of their irreversible inhibitory effects and approval usage in clinical oncology, it is suggested that this group of compounds, particularly etoposide (VP-16), can be used to control life-threatening CMV infections, such as CMV pneumonitis and CMV retinitis, in cancer and immunocompromised patients or patients with AIDS.

Human cytomegalovirus; Etoposide; Infection; Topoisomerase II

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Introduction

In addition to its association with developmental abnormalities and mental retardation, human cytomegalovirus (CMV) has become one of the most common causes of opportunistic infections in organ transplant recipients and patients with acquired immunodeficiency syndrome (AIDS) (Meyers et al., 1982; Macher et al., 1983). The search for and the development of effective anti-CMV compounds has become one of the important goals of present antiviral research. Due to the lack of virus-specific thymidine kinase (TK), several potent anti-herpes compounds, such as acyclovir and BVdU which are very potent against herpes simplex virus, are ineffective against human CMV replication (Estes and Huang, 1977). In view of the fact that human CMV encodes a virus-specific DNA polymerase (Huang, 1975), many anti-CMV compounds have been developed with viral DNA polymerase as a target. Among them the nucleoside analogs such as the 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and 1-(2′-deoxy-2′-fluoro-beta-D-arabinofuranosyl)-5-iodocytosine (FIAC) and pyrophosphate analogs including phosphonoacetate and phosphonoformate selectively inhibit the CMV DNA polymerase (Huang, 1975; Mao et al., 1975; Mar et al., 1982, 1983, 1984, 1985). However, the inhibitory effects of these compounds are reversible. Viral DNA synthesis and virus replication resume upon the removal of these drugs (Mar et al., 1982, 1983, 1984, 1985). Because of the reversible nature of these anti-CMV compounds, we have searched for other possible targets with the hope to develop potent anti-CMV compounds which do not have such reversible anti-CMV effects.

Topoisomerase II is an enzyme that has a unique ability to catalytically unknot, relax and decatenate covalently closed DNA molecules via a strand-crossing reaction involving a double-strand break (Wang, 1985). This enzyme has been shown to play an essential role in the replication of SV40 (Yang et al., 1987) as well as yeast (DiNardo et al., 1984; Holm et al., 1985). Recently, we found that CMV infection greatly induced cellular topoisomerase II synthesis and demonstrated that the topoisomerase II activity was essential to CMV replication in CMV-infected human fibroblasts (Benson and Huang, 1988, 1990). A DNA-intercalative acridine derivative, 4′-9′-(Acridinylamino) methanesulfon-m-anisidide (m-AMSA), was able to inhibit CMV DNA replication in vitro by inducing the double- and single-stranded-DNA breaks, via the mechanism of which the ends of DNA molecules are covalently bound to topoidomerase II in the presence of drug (Zwelling et al., 1981; Nelson et al., 1984). In this communication, we further demonstrate that an FDA approved and clinically available anti-tumor compound, etoposide (VP-16), a phyllotoxin derivative without DNA-intercalative properties, can irreversibly inhibit CMV replication in CMV-infected human fibroblasts. The application of this compound alone or in combination with DHPG (Ganciclovir) might be the better choice to control fatal CMV infection in patients with cancer or AIDS.

Materials and Methods

Cells and virus

Human embryonic lung (HEL 229, ATCC 137-CLL) fibroblasts (passage 16–24) and Towne strain CMV (passage 33–39) were used for infection and drug inhibition assays. HEL cells were subcultured in MEM supplement with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human CMV infected cells were maintained in MEM medium supplement with 6% fetal calf serum, antibiotics and various concentrations of topoisomerase II inhibitors at the indicated concentration.

Anti-CMV compounds studied

Etoposide or VP16, {4′-demethylepipodophyllotoxin 9-[4,6-*O*-(R)-ethylidene-*B*-D-glucopyranoside]}, manufactured by Bristol in the form of aqueous solution at 100 mg/5 ml was obtained from the Pharmacy of North Carolina Memorial Hospital. m-AMSA, 4 (9-acridinylamino) methylsulfonanisidate, was obtained as crystal from the Drug Synthesis Branch, National Cancer Institute, NIH, Bethesda, MD. This crystal compound was first dissolved in dimethyl sulfoxide (DMSO) at the concentration of 4 mM and sterilized by filter filtration as stock solution. DHPG, 9-(1,3-dihydroxyl-2-propoxymethyl)guanine, was manufactured by Syntex and obtained from the Pharmacy of the North Carolina Memorial Hospital with prescription name as Ganciclovir.

DNA-DNA dot hybridization

Viral DNA synthesis was monitored by DNA-DNA dot hybridization using nick-translated ^{32}P labeled CMV DNA as the probe. Confluent HEL cells, on 24-well culture plates, were infected with Towne strain CMV at the multiplicity of 1–2 PFU per cell. After 2 h of absorption, MEM medium (supplement with 6% fetal calf serum) and topoisomerase II inhibitors at various concentrations were added to CMV-infected cultures. At various times after infection, CMV-infected and mock-infected HEL cells, with or without drug treatment, were lysed with 0.2 ml of lysis buffer solution, [0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA, 1% sodium dodecyl sulfate (SDS) and 0.1 mM $CaCl_2$], and digested with proteinase K at 100 μg/ml for 1 h at 37°C. The lysate was extracted with phenol-chloroform (1:1) once, chloroform once and then precipitated with 2.5 vol. of alcohol at −20°C overnight. The nucleic acid precipitate was dissolved in 0.2 ml of TE buffer (0.01 M Tris-HCl, pH 7.4, and 0.001 M EDTA). For dot hybridization, 0.05 ml of the infected- or mock-infected DNA solution from each experimental sample was first denatured in 0.5 N NaOH with 1.5 M NaCl for 1 h at room temperature and then neutralized on ice with 1.1 N HCl in 0.2 M Tris to final pH around 7.4. The mixture was then adjusted to 6 × SSC (SSC = 0.15 M NaCl and 0.015 sodium citrate), and single-stranded DNA was

immobilized on nitrocellulose membrane using Minifold apparatus (Schleicher and Schuell, Keene, NH 03431). Membrane baking and nucleic acid hybridization were performed following standard protocols as described previously (Mar et al., 1983). The ^{32}P labeled nick-translated Towne strain human CMV DNA (specific-activity, 5×10^7 to 1×10^8 cpm/μg) at the level of 1×10^6/ml of hybridization solution was used. Hybridization was carried out at 66°C for 18 h. Kodak RP/R2 X-ray film was used for radioautography to trace the radioactive labeled ^{32}P DNA hybridized. After autoradiography, individual dots from samples on the hybridized membrane were cut for measuring the radio-activity by liquid scintillation counter.

Effect of topoisomerase II inhibitors on viral protein synthesis

The effect of various topoisomerase II inhibitors on virus-specific protein synthesis was studied by ^{35}S methionine protein labeling and polyacrylamide gel electrophoresis (PAGE). CMV-infected (multiplicity of infection, 1–2 pfu per cell) or mock-infected cell cultures in 75 cm^2 culture flasks were pulse-labeled, at various times after infection, for 2 h with ^{35}S methionine (50 μCi/ml) in minimal essential medium containing 4% of dialyzed fetal calf serum and 1/20 the strength of MEM amino acid. Cells were scraped into TBS (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl), washed 3 times and dissolved in SDS-PAGE sample buffer [0.05 M Tris-HCl, pH 6.8, 1% SDS, 10 mM beta-mercaptoethanol and 1 mM *p*-methylphenylsulfonyl fluoride (PMSF)]. After boiling for 5 min in water bath, proteins were resolved on a 4% stacking and 10% running SDS-PAGE gel as described by Laemmli (1970). Autoradiography was done by exposing the dried gel to Kodak X-ray film at -70°C for 3 days.

Isolation of viral DNA from CMV-infected cells for the analysis of drug-induced DNA cleavage

The detection of drug-induced DNA cleavage and the detection of covalent topoisomerase II-DNA complexes were done following the method of Rowe et al. (1986) with minor modifications. Drug treated or untreated CMV-infected cells in 75 cm^2 flask were lysed with 3 ml of lysis buffer containing 0.02 M Tris-HCl, pH 8.0, 0.02 M EDTA and 1% SDS. The proteases inhibitor phenylmethylsulfonyl fluoride was then added to the lysate at the final concentration of 1 mM. An equal vol of phenol was added, the sample was gently mixed by inversion, and phases were separated by centrifugation at 2000 $\times$ *g* for 5 min at room temperature. The aqueous phase containing protein-free DNA was carefully removed to preserve the integrity of DNA molecules and precipitated with 2.5 vols. of cold alcohol. The gelatinous and proteinaceous interphase was carefully collected and precipitated with 2.5 vols. of cold alcohol. Both precipitates were collected by centrifugation and redissolved in TE (0.01 M Tris-HCl, pH 7.4, and 1 mM EDTA) buffer with 100 μg per ml of

proteinase K. The samples were incubated at 37°C for 2 h and phenol/chloroform extracted twice, alcohol precipitated, and then dissolved in TE buffer with 40 μg/ml of RNase A. After 1 h incubation at 37°C, the samples were deproteinized and redissolved in TE (with 0.1 mM EDTA) before the analysis with restriction enzyme or sucrose gradient centrifugation.

Sucrose gradient analysis of CMV DNA in infected-cells treated with etoposide

DNA from etoposide-treated cells was loaded on a 12 ml linear 10–40% sucrose gradient in neutral TE buffer. Samples were preheated to 65°C for 5 min before loading to dissociate aggregates. After centrifugation in an SW-41 rotor at 25 000 rpm for 24 h at 20°C, 0.6 ml fractions were collected from the top of the gradient and stored at −20°C until analysis. DNA from each fraction was alcohol precipitated and resuspended in 0.2 ml alkaline buffer (0.5 M NaOH and 1.0 M NaCl). The samples were heated for 5 min at 80°C, then neutralized with an equal vol of neutralization buffer (1 M Tris-HCl, pH 7.4 and 1 M NaCl) and subsequently immobilized on nitrocellulose paper using Minifold Apparatus. Hybridization to radioactive CMV DNA probe was done as described above.

Results

Effect of etoposide on HEL cells viability and growth

HEL cells were grown to confluence in 24-well Corning petri-dishes. Etopside was then added to the media to final concentrations as indicated in Table I. No obvious cell death was found at etopside concentration of 25 μg/ml (41.25 μM) during 8 days of treatment. Cell toxicity, as determined by the appearance of cells, such as rounding up, and cellular inclusion, was observed on the 4th day as etoposide concentration reached 50 μg/ml (82.5 μM) or above. At this drug concentration, 20% of cells were morphologically rounded up and 6% of cells were not able to exclude the Trypan blue vital dye. By the 8th day the cytopathic- and dead-cells were increased to 50% and 10%, respectively. Severe cytotoxicity was observed when etoposide concentration reached 100 μg/ml (165 μm). Half of cells were dead after 6 days of 100 μg/ml etoposide treatment and by 8th day only 20% of cells were viable.

The cytotoxicity of etoposide on growing cells was measured by the comparison of cell growth (based on the viable cell count) in the presence of various concentrations of etoposide. As shown in Fig. 1, approximately 34% and 50% growth retardations occurred at etoposide concentrations of 2.5 and 5 μg/ml, respectively. Complete growth inhibition was observed when etoposide concentration reached 40 μg/ml. The ED_{50} for growth retardation of the dividing HEL cells was estimated to be around 5 μg/ml (Fig. 1). Obvious morphological change with cell flattening and intracellular granulation was found when etoposide concentration reached 80 μg/ml.

TABLE 1

Effect of etoposide on the morphology and viability of stationary phase HEL cells[a]

Treatment time and toxicity	Drug concentration (μg/ml)					
	0	25	50	100	150	200
1 day						
round up	<0.1%	3%	22%	32%	42%	58%
viable	100%	100%	100%	100%	100%	100%
2 days						
round up	<0.1%	1%	20%	100%	100%	100%
viable	>99.9%	>99%	96%	99%	99%	99%
4 days						
round up	<0.1%	2%	20%	100%	100%	100%
viable	>99.9%	98%	94%	89%	60%	50%
6 days						
round up	<0.1%	3%	45%	100%	100%	100%
viable	>99.9%	97%	92%	51%	15%	4%
8 days						
round up	<0.1%	3%	50%	100%	100%	100%
viable	>99.9%	97%	92%	20%	5%	0

[a]HEL cells were grown to confluence in 24-well petri-dishes. Cultures were changed with MEM maintenance media containing various concentrations of etoposide, as indicated. The viability of cells in presence of etoposide was measured by Trypan blue exclusion assay.

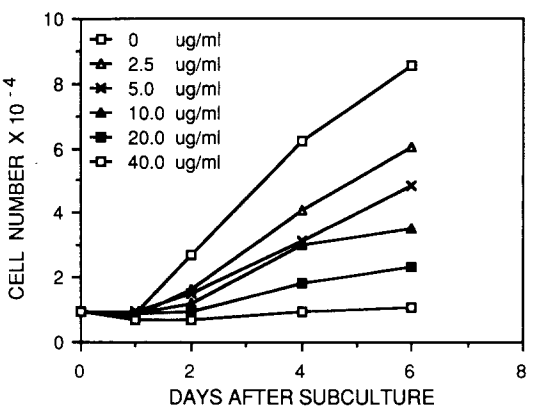

Fig. 1. Effect of etoposide on the growth of HEL cells. HEL cells were subcultured into 24-well petri-dishes in MEM medium containing 10% fetal calf serum and various concentrations of etoposide. Each culture was originally seeded with 1 ml of 0.95×10^4/ml HEL cells per well. On the indicated day, cells were trypsinized and counted for viable cell number. Media, with or without etoposide, were changed on the 4th day after subculture. Cell number indicated was the average value from 4 culture samples per drug concentration.

Inhibitory effects of etoposide on human CMV DNA replication

Fig. 2 shows human CMV DNA synthesis levels at various times postinfection in cultures treated with etoposide at concentrations ranging from 0.625 μg/ml (1.03 μM) to 20 μg/ml (66 μM). The multiplicity of infection used at this experiment was 0.1 PFU per cell. The 85% effective dose (ED_{85}) of etoposide, as measured and determined from 3 separate experiments, was approximately 0.625 μg/ml, while ED_{99} was about 1.25 μg/ml (2.06 μM). No significant viral DNA synthesis was detected at drug concentrations of greater than 2.5 μg/ml, even up to 9 days after infection.

Reversibility of etoposide treatment on human CMV DNA replication

To examine the reversibility of the inhibitory effects of etoposide on human CMV DNA replication, confluent HEL cells were simultaneously infected with CMV (at the MOI of 0.1 PFU per cell) and treated with various concentrations of etoposide for 9 days with media changed on day 4. Drug was removed on the fourth day and replaced with drug-free medium after 3 30-min washings. On day 3 and day 5 after the removal of etoposide, CMV DNA synthesis was detected in the cultures previously treated with etoposide at 0.625 μg/ml (at the level of 30% of control) and 1.25 μg/ml (5.8% of control), but was not detected in cultures treated with 2.5 μg/ml of etopside (Fig. 3). Fig. 4 shows the reversibility of m-AMSA and DHPG suppressive effect on CMV DNA synthesis for the purpose of comparison. CMV DNA synthesis readily resumed upon the removal of DHPG (removed on fourth day after infection), even at the DHPG concentration as high as 4 μM. With m-AMSA, or the combination of m-AMSA and DHPG, the reversibility of CMV DNA synthesis was prevented after the removal of drugs.

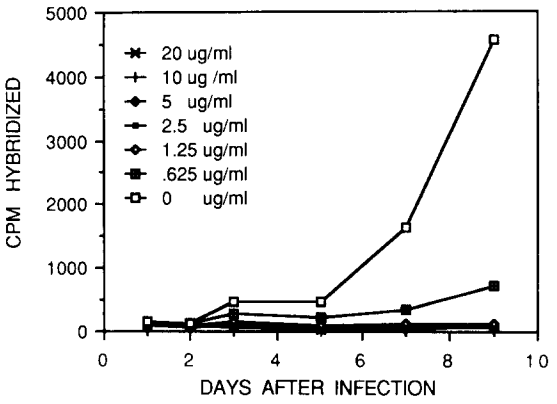

Fig. 2. Inhibition of human CMV DNA synthesis by etoposide. Confluent HEL cells were infected with CMV at M.O.I of 0.1 PFU per cell and treated with etoposide at the concentrations as indicated. Cells were lysed at indicated times. DNA extraction, dot blots and nucleic acid hybridizations were performed as described in the text.

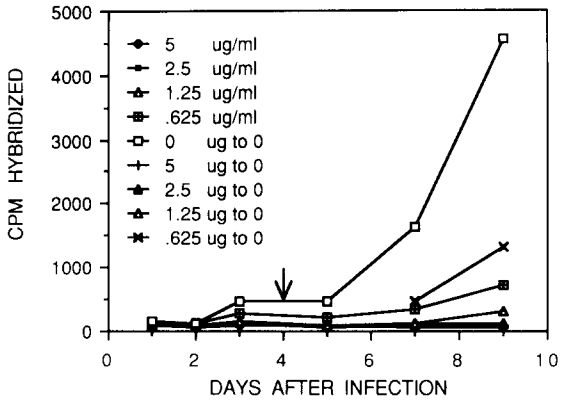

Fig. 3. Reversibility of anti-CMV effect of etoposide. Infected confluent HEL cells were treated with etoposide at the concentrations as indicated for 4 days. The drug was then removed and drug free medium was added to the cultures. On the 3rd and 5th day after the removal of drug, the cultures were harvested. DNA extraction and dot blot hybridization to monitor viral DNA synthesis were performed as described in the text. The value of '5 μg–0' indicates the data for the cultures which were treated with 5 μg/ml of drug for 4 days, the drug was then removed by replacing with drug free medium. The arrow indicates the day the etoposide was removed from the culture. The data of cultures of continuous treatment without removal of drug were included for comparison.

Fig. 5 shows the cytopathic effects of CMV-infected cultures 5 days after removal of DNA synthesis inhibitors. Extensive cytopathic effect and detachment of infected-cells were found in CMV-infected cultures without inhibitor on 9th day after infection (Fig. 5B). Severe cytopathic effect was also found in infected-culture treated with 2 μM of DHPG for 4 days and then with the drug removed for 5 days (Fig. 5C), and same phenomenon was also found when low concentration of etoposide, at concentration lower than 0.655 μg/ml, was used (Fig. 5D). No obvious spreading of typical CMV-CPE was found in higher concentration of etoposide-treated cultures (Fig. 5E,F).

Comparison of polypeptide synthesis in PAGE between etoposide- and m-AMSA-treated and nontreated CMV-infected cells

CMV-infected cultures were treated with 5 μg/ml of etoposide or 2 μM of m-AMSA as indicated. Cells were pulse-labeled for 4 h at various times post-infection with ^{35}S methionine and low methionine medium (Gibco). Fig. 6 reveals that CMV immediate-early and early protein (arrow heads; 71 kDa, 47 kDa, 38 and 31 kDa) synthesis was not significantly inhibited by etoposide(VP) and m-AMSA(mA) treatment at the drug concentrations of 5 μg/ml (8.25 μM) and 2 μM, respectively, while CMV late protein synthesis (as indicated by triangle; 140 kDa, 120 kDa, 60 kDa, 32 kDa and 25 kDa) was inhibited by etoposide and m-AMSA at the same drug concentrations as indicated in the text. The PAGE polypeptide patterns do not show notable differences between etoposide-treated and m-AMSA-treated cultures (Fig. 6).

CMV DNA fragmentation induced by etoposide treatment of CMV-infected cells

Replication-dependent fragmentation of human CMV genome was observed upon the treatment of CMV-infected cells with etoposide after CMV DNA replication (J. Benson's PhD dissertation, 1989). These fragments appear to be heterogeneous in size. As a separate means of determining the size of fragmentation products, aqueous and phenol interphase samples of etoposide

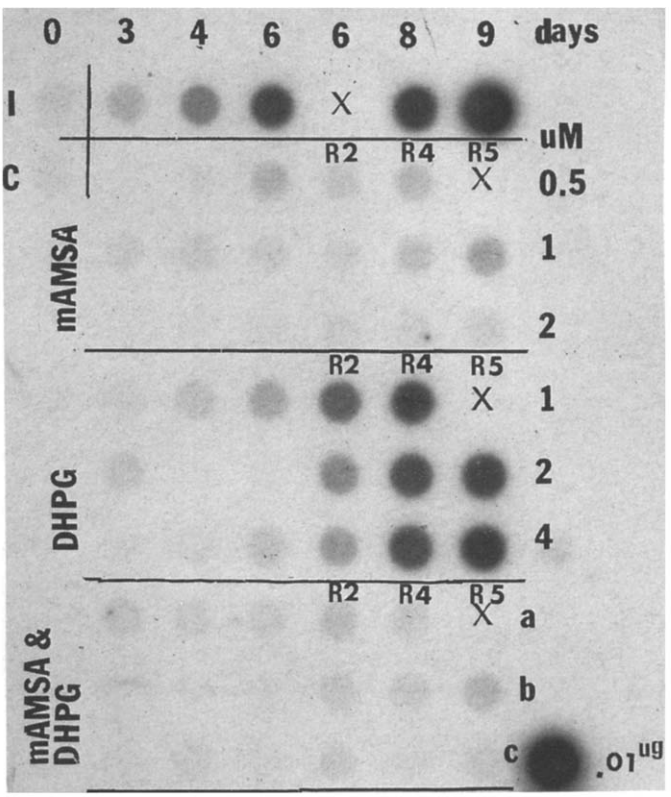

Fig. 4. Inhibition and reversibility of m-AMSA and DHPG on CMV DNA replication. CMV-infected HEL cells were treated with indicated concentrations of m-AMSA, DHPG or both compounds right after absorption. On 4th day after drug treatment, the drug was removed from a subset of cultures by replacing the medium with drug free medium. In the control experiment, the fresh medium with drug or without drug was supplied at the same time. Dot hybridization was performed as described in text. (I) and (C) dots on upper left corner indicate the hybridization control of infected and noninfected cell cultures, respectively, harvested right after virus absorption. The dots of the first horizontal row indicate the DNA synthesis in the absence of anti-CMV compound on various days after infection. The drug concentrations of m-AMSA and DHPG in μM are indicated on the right side edge of the figure. In 'm-AMSA & DHPG' co-treatment section, the drug concentrations are indicated as (a) 0.5 μm of m-AMSA and 1 μM of DHPG; (b) for 1 μM of m-AMSA and 2 μM of DHPG; and (c) 2 μM of m-AMSA and 4 μM of DHPG. The R2, R4, R5 on the top of each section of figure indicate the 2nd, 4th and 5th day, respectively, after the removal of drugs, for hybridization dots in vertical direction. The hybridization dot on the right bottom corner is 0.1 μg CMV DNA positive control. (×) indicates no sample was tested.

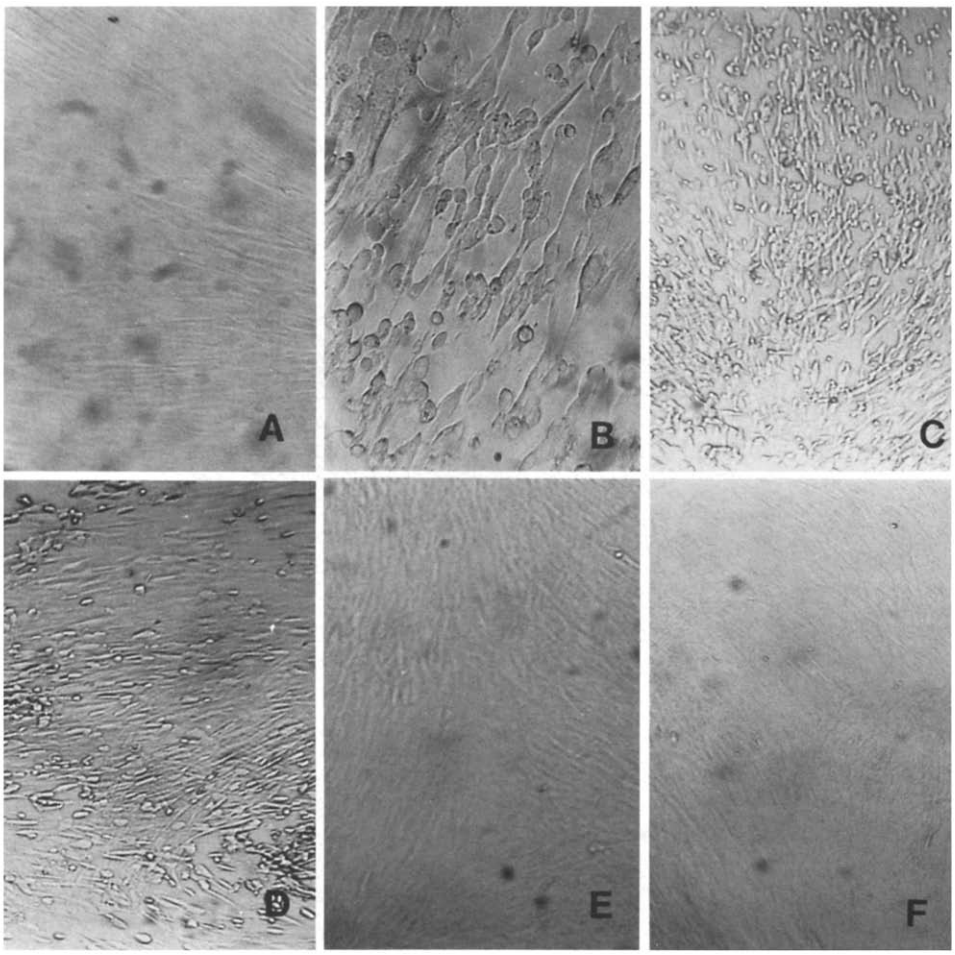

Fig. 5. The cytopathic effects in CMV-infected cultures 5 days after the release of DNA synthesis inhibitors. CMV-infected HEL cells were treated with indicated concentrations of etoposide, DHPG or m-AMSA for 4 days. The drug was then removed by replacing with drug free medium. The photographs show the CMV cytopathic effects of various CMV- infected cultures with or without drug treatment on day 9 (5th day after the removal of drug) after infection. (A) non-infected HEL; (B) infected control, no drug; (C) infected HEL with 2 μM DHPG; (D) infected HEL with 0.625 μM etoposide; (E) infected HEL with 2.5 μM etoposide; and (F) infected HEL with 5 μM etoposide.

treated, with 10 μg/ml for 30 min at 72 h after infection, and untreated CMV-infected cultures were analyzed by sucrose gradient sedimentation. The rationale of this experiment was based on the observation that topoisomerase II poison induced double- and single-stranded-DNA breaks, the ends of which were covalently bound to topoisomerase II. Fig. 7 shows the sedimentation profiles of CMV DNA fragments isolated from aqueous and phenol phases, the relative quantity of viral DNA from each fraction was monitored by dot blot hybridization. The CMV DNA molecules purified from the interphase fraction,

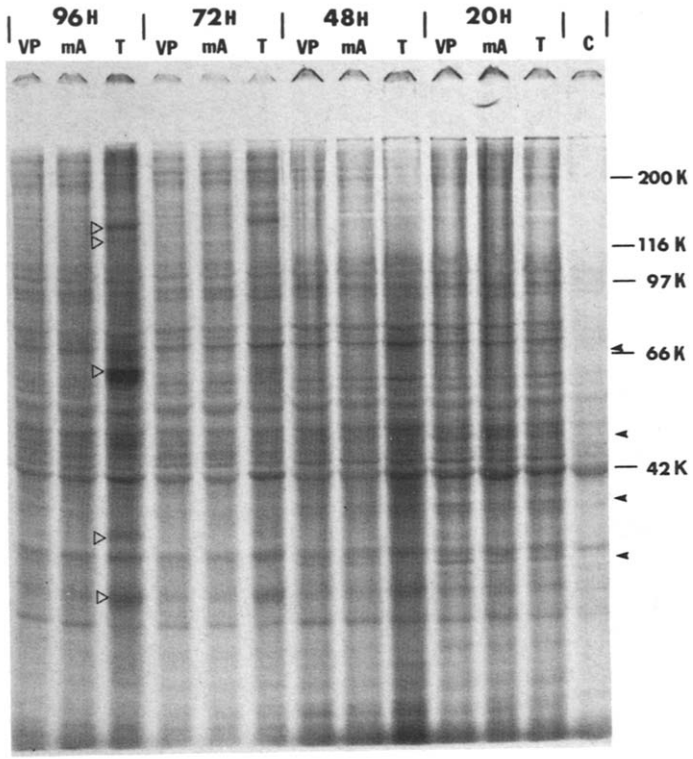

Fig. 6. Comparison of the effect of topoisomerase II poisons on polypeptides synthesis in CMV-infected HEL cells by SDS-PAGE. Polypeptide synthesis was investigated by the incorporation of ^{35}S methionine. Infected cells were pulse-labeled with 50 μCi/ml of ^{35}S methionine for 4 h at indicated times after infection. The polyacrylamide gel was subjected to autoradiography for 3 days with Kodak X-ray film. Molecular weight markers are indicated on the right. (C) indicates the HEL cells control; (T) Towne strain CMV infected cells without inhibitor; (mA) CMV-infected HEL cells with m-AMSA at 2 μM; and (VP) CMV-infected HEL cells with etoposide at 5 μg/ml (8.25 μM). Arrow-heads indicate the peptides which had appeared at earlier stage of infection. Open-triangles show the peptides which appeared after viral DNA replication.

where the covalent topoisomerase II-DNA complexes were trapped, were extensively fragmentated with size predominantly of approximately 500 bp. The size of DNA was determined by the comparison with *Hin*dIII digested lambda DNA fragments in 1% agarose gel electrophoresis (J. Benson, PhD Dissertation, 1989). The DNA obtained from the aqueous phase, where the free viral DNA located, is larger than that of interphase and is predominantly greater than 7 kb. There is little CMV DNA in the interphase of virus-infected cells without etoposide treatment, because of the lack of covalent topoisomerase II-DNA complexes. The DNA from aqueous phase of drug-free control was larger in size than that of etoposide-treated cultures (Fig. 7).

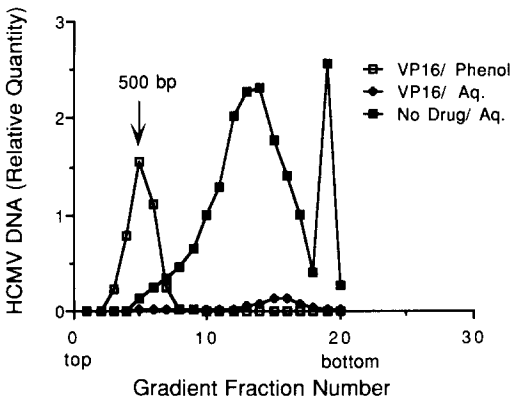

Fig. 7. Sucrose gradient analysis of CMV DNA in etoposide-treated late stage CMV-infected HEL cells. DNA isolated from the interphase or aqueous phase of etoposide-treated (10 μg/ml for 30 min) or non-treated CMV infected HEL cells 72 h after infection was subjected to a 12 ml 10–40% neutral sucrose gradient centrifugation. Fractions with the vol of 0.6 ml each were collected from the top of the gradient. DNA from each fraction was dot blotted onto nitrocellulose and probed with nick-translated human CMV DNA. After autoradiography, the amount of CMV DNA in each fraction was monitored and compared using the intensity of the autoradiogram from each sample. The intensity, from densitometer, of fraction No. 10 of the aqueous phase/no drug sample was used as 1 for plotting the figure.

Discussion

In the past two decades, substantial effort has been devoted to the search for potent and selective compounds for the treatment of herpes virus infections. As the result of this effort, a large number of nucleoside and pyrophosphate analogs have been developed and applied to clinical use with significant efficacy. Among these compounds, the nucleoside analogs, such as acyclovir (ACV) and bromovinyl deoxyuridine(BVdU), require virus-specific thymidine kinase to phosphorylate them into active monophosphate and diphosphate derivatives, respectively, which are further phosphorylated by host enzyme into triphosphate derivatives, by which the virus-specific DNA polymerase is preferentially inhibited (Elion et al., 1977; Cheng et al., 1981; De Clercq, 1984). Unlike HSV type 1 and 2, human CMV does not encode its own thymidine kinase (Estes and Huang, 1977). Therefore, human CMV is not sensitive to ACV and BVdU but, rather, is sensitive to several other nucleoside analogs, such as DHPG and FIAU derivatives, which can be phosphorylated to mono- and subsequently to di- and triphosphate derivatives by cellular kinase (Elion, 1985; Fox et al., 1985; Mar et al., 1982–1985). Clinical studies of FIAC by the Memorial Sloan-Kettering Clinical Center Group showed that after oral or i.v. adminstration of FIAC, FIAC was rapidly and almost totally deaminated and converted into FIAU. Therefore, the active FIAC derivative that inhibit herpes group virus replication is FIAU triphosphate rather than FIAC triphosphate (Fox et al., 1985; McLaren et al., 1985). The pyrophosphate derivatives, such as phosphonoacetate (PAA) and phosphonoformate (PFA), can also preferen-

tially interact with almost all herpes group virus-specific DNA polymerases (Huang, 1975; Mao et al., 1975). Therefore, PAA and its derivatives exhibit their inhibitory effect on herpes virus DNA synthesis using virus-specific DNA polymerase as the target. Unfortunately, these nucleoside and pyrophosphate analogues inhibit herpes virus DNA replication only in a virostatic reversible fashion. Viral DNA synthesis resumes immediately after the removal of these inhibitors. In addition, drug resistant mutants are frequently encountered after a long course of treatment both in vitro and in vivo. Therefore, our discovery of the irreversible inhibition of human CMV replication by etoposide at a concentration of 2.5 μg/ml may offer an alternative and complementary approach to control severe life threatening CMV infection in cancer patients or patients with AIDS.

Although human CMV does not encode its own topoisomerase II, this virus not only induces cellular topo-II synthesis but also appears to require this enzyme activity for its own genomic replication (Benson and Huang, 1988, 1990). Substantial viral DNA fragmentation in the presence of topoisomerase II inhibitors during replication may be the major explanation of the irreversibility of this inhibitory effect. It is clear that since etoposide can cause fragmentation both at early and late stages of viral replication that topoisomerase II might be used for immediate early gene transcription and CMV genome replication (Benson's PhD dissertation, 1989). After the onset of viral DNA replication, etoposide induced fragmentation of viral DNA was observed (Fig. 7). Sucrose gradient sedimentation analysis revealed that etoposide caused accumulation of protein-DNA complexes with an average length of 0.6–0.8 kb. One explanation for such an abundance in topoisomerase II cleavage might be the increased intracellular topoisomerase II level at late times of infection.

A number of groups have shown that as a general characteristic, transformed cells have higher steady state levels of topoisomerase II enzyme than primary cells (Deffie et al., 1989; Heck et al., 1988; Potmesil et al., 1989). This may be a reflection of altered regulation of topoisomerase II expression in these cells. This is in contrast to normal cells, in which levels of topoisomerase II are tightly linked to the mitotic status of cell (Heck and Earnshow, 1987; Heck et al., 1988; Hsiang et al., 1988). This intrinsically high level of topoisomerase II in transformed cells, along with the possibility of the selective sensitivity of transformed cells to topoisomerase II poisons, has led to the idea that the presence of topoisomerase II in the cell dictates the sensitivity of that cell to compounds such as etoposide (Deffie et al., 1989). In other words, since topoisomerase II poisons are thought to prevent reversal of the cleavage intermediate, exposure of cells to these drugs simply leads to an accumulation of such intermediates, which are then detected as covalently associated DNA-topoisomerase II complexes upon lysis of the cells with SDS. This type of DNA damage represents a novel DNA lesion that has become the postulated explanation for the antiproliferative effects of topoisomerase poisons. Similar postulation should be applicable to the inhibitory effect of topoisomerase II poisons on CMV replication.

Etoposide has been clinically available since 1983 as an anticancer agent (Bristol NDC 0015-3091-45). Unlike DHPG (gancyclovir) or phosphonoformate (foscarnet), the current anti-CMV agent used, it is well absorbed orally. Etoposide-associated toxicity at a dose daily exceeding the ED_{99} for CMV when given to cancer patients as a single agent resulted in leukopenia (Einhorn et al., 1990). This toxicity is comparable to the toxicity of ganciclovir where a granulocyte count less than 500/μl develops in 30–50% of HIV patients. Based on our in vitro inhibition study and previously available clinical data, it is conceivable that this compound can be an effective anti-CMV agent and can lead to lengthy suppression after it is removed. Since suppression of CMV by etoposide is irreversible the prolonged therapy which is necessary with ganciclovir would not be needed.

One important issue we need to investigate is the carcinogenicity of etoposide at the lowest dose which can effectively control human CMV infection. In tissue culture system, the mutagenic and genotoxic potential of etoposide have been demonstrated in mammalian cells. It caused chromosome aberration, sister chromatid exchanges and DNA strand breakages (Bristol Laboratories Product Information, 30950DIM-18). In addition, carcinogenicity tests with etoposide have not been conducted in the laboratory animal. The incidence of secondary tumors in cancer patients treated with etoposide is also unclear. Therefore, an animal model such as a mouse model system using mouse CMV infection may be of great value to establish the potential and the effectiveness of etoposide in control of severe CMV infection in humans.

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