Repair of DNA following Incorporation of 1-ß-d-Arabinofuranosylcytosine into Herpes Simplex Virus Type I

Glenn J. Bubley, Clyde S. Crumpacker, and Lowell E. Schnipper

ABSTRACT

The nucleoside analogue 1-ß-d-arabinofuranosylcytosine (ara-C) is incorporated into herpes simplex virus type 1 (HSV-1) DNA, and this correlates with inhibition of virus replication. The technique of Weigle-type reactivation (WR) was used to compare the ability of induced cellular DNA repair pathways to recognize or repair ara-C incorporated into HSV-1 DNA and ultraviolet (UV)-irradiated virus DNA (254 nm). Pretreatment of monkey cells with low-fluence UV irradiation, growth with cis-dichlorodiammineplatinum(II), or growth in ara-C followed by infection after a 24-hr incubation period resulted in enhanced survival of UV-irradiated HSV-1. Under the same experimental conditions, no reactivation of HSV-1 inactivated by growth in ara-C is observed.

Comparisons between uninfected Vero cells exposed to UV irradiation (30 J/m²) or grown in 10⁻⁶ M ara-C demonstrated repair replication in irradiated cells, whereas there was no evidence for DNA repair at various time intervals following removal of the nucleoside analogue. These observations suggest that, once ara-C is incorporated into HSV-1 or eukaryotic DNA, it is not recognized as a repairable lesion within the limits of the DNA repair assays used in these studies.

INTRODUCTION

ara-C³ is an effective agent in the treatment of acute nonlymphocytic leukemia (14) and is a potent inhibitor of HSV replication in vitro (6). ara-CTP is incorporated into internucleotide linkage in cell and viral DNA (12, 16), and inhibition of clongenic survival of leukemia cells and inhibition of HSV replication correlate with the formation of ara-C-incorporated DNA (11, 12). Although 3'-5' exonucleases activities capable of excising incorrect nucleotides or nucleotide analogues have been described in uninfected cells and in association with the HSV-encoded DNA polymerase (7, 11), it is not known whether ara-C that has been incorporated into internucleotide linkage is recognized as a lesion that can be repaired.

A useful biological system with which to study DNA repair activity when the precise biochemical pathways are not known is WR. This phenomenon was first observed when enhanced survival of UV-irradiated bacteriophage was demonstrated following growth in Escherichia coli that were irradiated prior to infection (23). WR is a manifestation of induction of the SOS functions in bacteria and is error prone, such that the progeny phage contain a high frequency of mutants (5, 24). WR in eukaryotes is analogous to the system defined in bacteria that repair activity has been induced in mammalian cells by pretreatment with UV, γ-irradiation, alkylating agents, antimetabolites, or nucleoside analogues, followed by infection with irradiated SV40, adenovirus, or HSV-1 (2, 3, 9, 15, 19, 20).

In the current study, WR has been used to compare the reactivation of HSV inactivated by either growth in ara-C or UV irradiation and then grown in cells in which DNA repair activity has been induced using specific physical or chemical stimuli. These stimuli are exposure to UV irradiation, growth in ara-C, or cis-DDP. cis-DDP results in DNA-DNA and DNA-protein cross-links, which are believed to be related to its cytotoxic effects (27). In a parallel series of biochemical studies, the ability of DNA that has incorporated ara-C into internucleotide linkage in uninfected cells is compared as a substrate for repair replication to DNA after exposure to UV irradiation. Repair of ara-C-incorporated DNA is not observed in either assay system.

MATERIALS AND METHODS

Cells and Virus. The established Vero line of African green monkey kidney cells was obtained from Microbiological Associates (Walkerville, MD) and maintained at 37° and 5% CO₂ in minimal essential medium containing 2% newborn calf serum and antibiotics (penicillin, 250 units/ml, and streptomycin, 250 μg/ml).

HSV-1 (strain KOS) was propagated by infection of plaque-purified virus on Vero cell monolayers at low multiplicity of infection (0.01 PFU/cell), and virus was harvested at maximal cytopathic effect. Infected cells were frozen and thawed, clarified by low-speed centrifugation, and stored at -80°. Infectious virus was quantitated by plaque titration on monolayers of Vero cells (1).

Infectious HSV-1 containing ara-C in viral DNA was prepared by virus infection of Vero cells at 1 PFU/cell, with ara-C added to the medium after a 1-hr adsorption period at 37°. At 3+ cytopathic effect, infected cells were harvested by scraping, pooled with the supernatant, frozen, thawed, and clarified. Virus was pelleted by centrifugation at 40,000 × g for 1 hr and resuspended in minimal essential medium.

UV Irradiation. HSV-1 (strain KOS) was irradiated in 35-mm Petri dishes with constant stirring, using a UV germicidal lamp at 254 nm (Model G8T5; General Electric) at an incident dose rate of 250 J/m². The dose rate was determined by using a Blak-Ray UV meter (UV Products, Inc., San Gabriel, CA).

Vero cells for repair replication were irradiated by removing the medium and exposing the monolayers to UV light at a dose rate of 30 J/m² for the measurement of repair replication.

Drugs. ara-C and cis-DDP were obtained from Sigma Chemical Co.
Results

Enhanced Survival of UV-irradiated HSV-1 by ara-C, cis-DDP, and UV Irradiation. Enhanced survival of partially inactivated HSV was measured as the ratio of virus survival (titer of inactivated HSV to titer of unactivated HSV) in cells exposed to an appropriate physical or chemical stimulus to virus survival in untreated cells and can be referred to as the RF (20). To determine and compare the effects of ara-C or cis-DDP on induction of enhanced virus survival, Vero cells were exposed to increasing concentrations of these compounds (0.125 to 0.5 μg of ara-C/ml; 0.25 to 1.0 μg of cis-DDP/ml) for approximately 48 hr. The subconfluent cells were incubated for 24 hr in the absence of drug, at which time they were infected with HSV-1 or HSV-1 partially inactivated by exposure to UV irradiation (250 J/m²) in a plaque titration assay. The duration of the induction period (the interval between the removal of the DNA-damaging agent and infection) was held constant in these studies at 24 hr, although virus reactivation has been observed between 16 and 48 hr following removal of the inducing stimulus (4).

ara-C pretreatment and UV irradiation of Vero cells resulted in marked reactivation of UV-inactivated HSV-1 (Chart 1). This effect was dependent upon concentrations of the analogue or UV fluence, up to 0.25 μg/ml and 8 J/m², respectively. Above these levels, the RF falls, presumably because of residual toxic effects on the cells that interfere with virus replication. cis-DDP pretreatment also induced virus reactivation, but under these experimental conditions, the RF was smaller in magnitude than that observed with ara-C and UV light (Chart 1). At higher

<table>
<thead>
<tr>
<th>UV J/m²</th>
<th>ara-C μg/ml</th>
<th>Cis-DDP μg/ml</th>
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<tbody>
<tr>
<td>4</td>
<td>.125</td>
<td>.25</td>
</tr>
<tr>
<td>8</td>
<td>.25</td>
<td>.5</td>
</tr>
<tr>
<td>12</td>
<td>.25</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Cell pretreatment

Chart 1. Enhanced survival of HSV-1 in cells pretreated with physical or chemical agents. Enhanced survival of HSV-1 inactivated with UV (250 J/m²) was determined in cells pretreated with increasing fluences of UV light (254 nm), ara-C, or cis-DDP as described in “Materials and Methods.” RF is the ratio of the survival of UV-inactivated HSV-1 in treated cells to that of untreated cells. UV pretreatment (---), ara-C pretreatment (-----), cis-DDP pretreatment (---).
concentrations of cis-DDP, sufficient cellular cytotoxicity was observed to preclude determination of virus survival.

Enhanced Survival of HSV-1 Containing ara-C Incorporated in Viral DNA. Before establishing whether HSV-1 grown in ara-C was a substrate for induced virus reactivation, we sought to determine whether these virions contained ara-C-incorporated DNA, were infectious, and whether their DNA contained alkali-labile sites. Viral DNA was purified from virions grown in 5 × 10^{-6} M (1.25 μg/ml) [3H]ara-C and subjected to isopyknic centrifugation in CsCl. A sharp peak of radioactivity was observed at the density of HSV-1 DNA (1.726 g/ml) (Chart 2) corresponding to ara-C-incorporated HSV-1 DNA. Following growth in 5 × 10^{-6} M (1.25 μg/ml) or 5 × 10^{-7} M (0.125 μg/ml) ara-C, partially purified virions are infectious, although their titer is lower than that observed with HSV-1 (strain KOS) grown in the absence of this analogue (10^{-6} and 10^{-7} PFU/ml versus 10^{-8} PFU/ml, respectively).

To determine if incorporation of ara-C into HSV-1 DNA alters its structural properties, virus was grown in the presence of ara-C (5 × 10^{-7} M, 10^{-6} M) or in its absence, and purified HSV-1 DNA was analyzed by sedimentation in alkaline sucrose gradients. A concentration-dependent shift to slower sedimenting species of viral DNA was observed (Chart 3), and this indicates that incorporation of ara-C is associated with the introduction of alkaline-labile sites in HSV-1 DNA.

### Table 1

<table>
<thead>
<tr>
<th>Cell pretreatment</th>
<th>UV-irradiated HSV</th>
<th>5 × 10^{-6} M ara-C-containing HSV (1.25 μg/ml)</th>
<th>5 × 10^{-7} M ara-C-containing HSV (0.125 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV (J/m²)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>3.8 ± 1.2</td>
<td>0.7 ± 0.05</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>5.6 ± 1.5</td>
<td>0.9 ± 0.07</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>4.0 ± 1.0</td>
<td>0.7 ± 0.05</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>cis-DDP (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>2.4 ± 0.3</td>
<td>0.7 ± 0.07</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>2.9 ± 0.8</td>
<td>0.8 ± 0.08</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>2.6 ± 0.7</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>ara-C (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>4.7 ± 1.3</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>0.25</td>
<td>6.9 ± 1.6</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>3.0 ± 1.0</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of at least 4 experiments.

Enhanced survival of ara-C-containing HSV-1 was determined in Vero cells exposed previously to 4 to 12 J of UV irradiation/m², 0.125 to 0.5 μg of cis-DDP/ml, or 0.125 to 0.5 μg of ara-C/ml. These physical and chemical stimuli were capable of inducing enhanced survival of UV-irradiated HSV-1 (Table 1). Reactivation of HSV-1 containing ara-C-incorporated DNA was not observed, however, under the same experimental conditions. Inability to reactivate ara-C-incorporated HSV-1 did not appear to be related to the extent of formation of ara-C-incorporated DNA, since neither HSV-1 grown in 5 × 10^{-7} M nor 5 × 10^{-6} M ara-C demonstrated enhanced survival (RF ≤ 1.2; Table 1).

### Repair Replication in Uninfected Cells Exposed to ara-C.

When mammalian cells are grown in ara-C, there is a concentration-dependent incorporation of the analogue into DNA (13, 16, 26), and pretreatment of cells with ara-C served as a strong inducing stimulus for enhanced survival of UV-irradiated virus but not ara-C-incorporated HSV-1 (Chart 4). Since ara-C induces a cellular pathway that repairs UV-induced dimers in HSV DNA, as
manifest by enhanced virus survival, we attempted to extend these observations to studies in uninfected cells. Vero cells were grown in $10^{-6}$ M ara-C for 24 hr, and repair replication was determined at 2, 6, 8, and 24 hr following removal of this nucleoside analogue using $[^{3}H]$BrdUrd as a density label. Repair replication is quantitated in parental density DNA following its separation by isopyknic centrifugation from high-density semi-conservatively replicated DNA. The ratio of $[^{3}H]$BrdUrd to $^{32}$P incorporated into parental density-prelabeled DNA serves as a measure of repair synthesis. No evidence of repair replication was observed at any time point up to 24 hr post-ara-C, and the ratio of $[^{3}H]$BrdUrd to $^{32}$P was not significantly changed from that of untreated cells (Chart 5). The ratios of $[^{3}H]$BrdUrd to $^{32}$P are 0.014 for no treatment; 0.015 for 2 hr post-removal of ara-C, 0.014 for 6 and 8 hr post-removal of ara-C, and 0.016 for 24 hr post-removal of ara-C. In contrast, cells exposed to 30 J/m² demonstrated repair synthesis, with a ratio of $[^{3}H]$BrdUrd to $^{32}$P of 0.22 (Chart 5).

**DISCUSSION**

Enhanced survival of UV-irradiated HSV-1 was observed in monkey kidney cells exposed to ara-C, UV irradiation, or cis-DDP prior to infection. This is consistent with observations from our laboratory and others, that physical or chemical agents (the effects of which are mediated through structural alterations in DNA or that affect DNA synthesis) are able to induce enhanced survival of animal viruses inactivated by UV light (15, 19, 20). These stimuli are of unequal potency, since cis-DDP results in the least virus reactivation of the 3, and attempts at increasing reactivation by increasing drug concentration resulted only in Vero cell cytocytotoxicity. The potent stimulus provided by ara-C for enhanced survival of UV-irradiated HSV-1 did not appear to recognize or repair ara-C moieties contained in internucleotide linkage in HSV-1 DNA. The inability to demonstrate enhanced survival of ara-C-incorporated HSV-1 was also observed following UV irradiation of Vero cells. This suggested that infectious viral DNA containing incorporated ara-C residues was not a good substrate for DNA repair induced in a WR system, and this inability was not related to the inducing stimulus. One possibility to explain this finding is that ara-C in internucleotide linkage is associated with a minimal effect on the structural integrity of the DNA duplex, such that it is not recognized by and does not serve as a substrate for DNA repair enzymes. We have established that incorporation of ara-C residues in DNA is associated with production of alkaline-labile sites in the duplex. The number of these lesions varies with the concentration of ara-C and the amount of ara-C-incorporated DNA formed (12). They may relate either to distortion of the double helix by the arabinosyl analogue and/or ara-C acting as a chain terminator (12, 25). In any case, these alterations in DNA structure are insufficient to be recognized by the repair pathways studied in these investigations. Failure to reactivate ara-C-incorporated HSV-1 following pretreatment of cells with ara-C is not likely to be a result of residual ara-CTP persisting throughout the 24-hr induction period. ara-CTP pools have been shown to rapidly dissipate following the removal of this compound (21), and a similar induction stimulus (e.g., ara-C) results in marked enhancement in survival of UV-irradiated HSV-1 (Table 1).

The observation that cell exposure to ara-C prior to infection induces repair pathways that recognize UV-irradiated HSV-1 DNA suggests that repair pathways that recognize pyrimidine dimers are involved. It is not known whether ara-C incorporated into internucleotide linkage in eukaryotic cell DNA is a substrate for this type of repair activity. Repair replication has been used to measure excision repair and involves a complex series of molecular events which result in cleavage of a pyrimidine dimer, or bulky chemical adduct, excision of adjacent nucleotides, and DNA synthesis followed by ligation of the patch (8). This activity is readily demonstrated following exposure of cells to UV light, but it is not observed at any interval during the 24-hr period following growth of uninfected cells in $10^{-6}$ M ara-C. This concentration is sufficient to result in incorporation of 11.2 pmol of ara-C over 12 hr in human leukemia cells in vitro (13).

Inability to demonstrate enhanced reactivation of ara-C-incorporated HSV-1 or repair replication in uninfected cells following growth in ara-C suggests that, once incorporated into internucleotide linkage, this analogue is not recognized by the repair pathways measured by these techniques. Confirmational stress on the double helix has been associated with incorporated arabinosyl derivatives (25), and failure to repair ara-C-incorporated DNA may be the result of insufficient distortion of the DNA molecule, despite evidence that incorporation of ara-C into HSV-1 DNA is associated with the production of alkaline-labile lesions. In contrast, cyclobutane dimers produced by UV irradiation are
bulky photoproducts that appear to cause sufficient distortion of the double helix to be recognized by specific DNA repair pathways (2, 8).

Precedent for the repair of ara-C-induced lesions in DNA may be found in prokaryotes. Mutants of Bacillus subtilis expressing an altered DNA polymerase activity or defects in genetically encoded DNA repair functions (e.g., Rec B or Rec D genes) are very sensitive to growth inhibition by ara-C, whereas the parent strains are not (18). This has led to the inference that ara-C sensitivity can be correlated with a DNA repair defect in these organisms. In the eukaryotic systems studied in these investigations, ara-C-containing DNA is not repaired, although the DNA has been shown to be structurally abnormal. These findings do not exclude the possibility that cells contain an ara-C repair pathway not detected by the techniques used. To explore this question further, direct measurements of the removal of radiolabeled ara-C in internucleotide linkage will be required.

REFERENCES
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