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EFFECT OF INCORPORATION OF 5-IODO-2′-DEOXYURIDINE INTO HSV-1 DNA ON VIRION SENSITIVITY TO ULTRAVIOLET LIGHT

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Abstract—First generation progeny herpes simplex type 1 (HSV-1) virions grown in the presence of 5-iodo-2′-deoxyuridine (IdUrd) were irradiated with either 254 or 302 nm ultraviolet (u.v.) light. The kinetics of virus inactivation revealed decreased sensitivity of IdUrd-substituted virions to irradiation with 302 nm light under all conditions examined, and with 254 nm u.v. light when substituted and control virions were irradiated at equal particle concentrations. Comparison of virus survival after irradiation measured in Vero or Xeroderma Pigmentosum (complementation group A) cells indicated that cellular repair of ultraviolet-induced lesions was not a significant factor in the observed decrease in u.v. sensitivity. IdUrd substitution altered neither the formation of ultraviolet-induced thymidine photoproducts nor the ability of irradiated virions to express delayed early viral enzymes (thymidine kinase, DNA polymerase). It is suggested that nucleocapsid proteins or the highly ordered structure of IdUrd-substituted virions play a key role in u.v. desensitization, either by the formation of non-lethal photoproducts or by the prevention of the formation of DNA-uracilyl free radicals.

The incorporation of halogenated pyrimidines into the DNA of mammalian cells, bacteria or viruses generally causes an increased sensitivity to ultraviolet (u.v.) irradiation [reviewed in Ref. 1]. The dose–response of this inactivation is highly dependent upon which pyrimidine analog is under study as well as the sample environment and wavelength employed during irradiation [2–4]. Early studies by Wacker and colleagues [5] reported that herpes simplex virus substituted with 5-bromo-2′-deoxyuridine (BrdUrd) was sensitized to the lethal effects of ultraviolet irradiations, whereas substitution with 5-iodo-2′-deoxyuridine (IdUrd) afforded protection.

Treatment of herpes simplex virus type 1 (HSV-1) infected cells with IdUrd leads to the substitution of IdUrd for thymidine in the viral DNA at levels which parallel the inhibition of virus replication [6]. The present study examines the kinetics of u.v. inactivation of IdUrd-substituted HSV-1 and explores some of the biochemical consequences of u.v. irradiation.

MATERIALS AND METHODS

Cells and virus. The CL-101 strain of HSV-1 was propagated in Vero cells at a multiplicity of infection (MOI) of 0.01 plaque-forming units (pfu) per cell. The virus yield was determined by plaque assay as described [7]. Vero cells were grown as monolayer cultures in Dulbecco's minimum essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% newborn calf serum (GIBCO). XPA cells (Xeroderma Pigmentosum complementation group A, NIGMS Human Genetic Mutant Cell Repository No. GM4312A, supplied by P-F. Lin and F. H. Ruddle) were grown as monolayer cultures in DMEM supplemented with 20% FBS.

Propagation and purification of IdUrd-substituted HSV-1 virions. For large scale virus growth, Vero cells were grown on Cytodex 1 beads (Pharmacia) in 850 cm^2 plastic roller bottles (Falcon) (1 g beads per roller bottle). Swelling of the Cytodex beads and the attachment and growth of the Vero cells were performed as per the manufacturer's instructions. When Vero cells reached confluence (approx. 200 cells/bead) they were infected with HSV-1 at a multiplicity of infection of 10 pfu/cell. Following a 1-hr adsorption period, the virus was removed, the cells were washed with PBS, DMEM plus 2% FBS with or without 20 μM IdUrd was added, and the cultures were incubated at 37°.

Eighteen to twenty hours post-infection (p.i.), cells, beads and supernatant medium from each culture were harvested. Cells were lysed by five cycles of freeze–thaw in a dry-ice/ethanol bath, 1/7 vol. of 2 M sucrose in Tris/NaCl (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) was added, and nuclei and beads were pelleted by centrifugation (10 min, 800 *g*, 4°). The supernatant fraction was adjusted to 15% sucrose by the addition of 1/10 vol. of 2 M sucrose in Tris/NaCl, and "crude virus" was pelleted for 1.5 hr at 15,000 rpm in a SW27 rotor at 4°.

The crude virus was resuspended in 2 ml Tris/NaCl and layered onto a 10-ml 25–55% linear sucrose gradient. Virus was separated from cell debris by centrifugation for 1 hr at 30,000 rpm, 4°, in an SW40 rotor. The visible HSV-1 band was removed with a syringe fitted with an 18-gauge needle, diluted 1:3

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with Tris/NaCl, and concentrated by a 1.5-hr centrifugation at 15,000 rpm in an SW40 rotor.

This "purified virion" pellet was resuspended in 1 ml Tris/NaCl with 30 mM $MgCl_2$, and residual sucrose was removed by dialysis against this buffer. Infectious virus titer was determined by plaque assay; virion particle number was determined spectrophotometrically. We have determined previously a linear correlation of optical density at 280 nm of a virion solution in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) to virion particle number as determined by electron microscopy (data not shown).

Irradiation. Virion preparations were diluted in phosphate-buffered saline (PBS) to either an equal pfu or equal particle concentration. Samples were kept cold and were stirred during irradiation. The ultraviolet light source was either a GE G-15T8 bulb (254 nm peak) or a UV Products 34-000-3901 bulb (302 nm peak; 285–400 nm distribution) with incident doses of 0.36 J/m^2/sec and 0.45 J/m^2/sec respectively.

Surviving virus fraction was determined by plaque assay on Vero or XPA cells.

Thymidine photoproduct determination. Virus was grown as described above in the presence of 5 μCi/ml [*methyl*-^{3}H]dThd (40 Ci/mmol, Moravek Biochemicals), purified, and irradiated in PBS at equal particle concentrations with 0, 10 or 200 J/m^2 254 nm u.v. light.

Viral DNA was isolated by digestion of irradiated virions with 0.1 mg/ml pronase in the presence of 0.1% SDS for 2 hr at 37°. Peptides were removed by extraction with chloroform/isoamyl alcohol (24:1, v/v), and the DNA was precipitated with ethanol in the presence of 100 μg/ml carrier salmon sperm DNA.

Nuclease digestion was performed as described by Farland and Sutherland [8]. Briefly, the DNA in 200 μl of 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, was treated with 10 μg DNase I for 5 min at room temp., 60 μl of freshly prepared digestion mix (2 mg/ml snake venom, 20 mg/ml bacterial alkaline phosphatase, 10 mM $MgCl_2$ and 0.2 M Tris–HCl, pH 8.0) was added, and digestion was allowed to proceed at 37° for 1 hr.

Nuclease-resistant photoproducts were quantitated by spotting on Whatman DE81 filter discs that were washed twice in 1 mM ammonium formate, and once in water. Counts were released from the filters by 15 min of agitation in 1 ml of 1 M NaCl and counted in Optifluor (United Technologies, Packard) in a Beckman liquid scintillation counter.

Enzyme assays. Vero cell monolayers were infected with ultraviolet-irradiated IdUrd-substituted or control virus at a pre-irradiation MOI of 5 pfu/cell. At various times post-infection cells were harvested by scraping and were resuspended in extraction buffer for the appropriate enzyme assay to be performed.

The viral thymidine kinase activity was determined by a modification [9] of the method of Summers and Summers [10] which utilizes [^{125}I]IdCyd as the substrate. For the DNA polymerase assay, cells were suspended in an extraction buffer [20 mM Tris–HCl, pH 7.8, 0.3 M KCl, 5 mM EDTA, 1 mM dithiothreitol (DTT)], and were lysed by 3 cycles of freeze-thaw in dry-ice/ethanol followed by two 7-sec sonication pulses. Crude lysates were adjusted to 20% glycerol, and DNA polymerase activity was determined in a reaction volume of 60 μl after 30 min of incubation at 37°. The reaction mixture contained, in addition to the cell lysate, 50 mM Tris–HCl, pH 8.5, 1 mM DTT, 200 μg/ml BSA, 5 mM $MgCl_2$, 50 μM each dGTP, dCTP and dTTP, 250 μg/ml activated calf thymus DNA [11], 100 μCi/ml [α-^{35}S]-thio-dATP (Amersham), with and without 150 mM KCl. HSV-1 DNA polymerase activity is evident in the presence of KCl, while under this condition the cellular polymerase is inactive [12].

Protein concentration of lysates was determined by the method of Lowry *et al.* [13].

RESULTS

Inactivation kinetics after irradiation at equal pfu concentrations. Studies of u.v. inactivation of HSV-1 reported in the literature define only pre-irradiation infectious virus (pfu) concentrations [e.g. Refs. 14–17]. Therefore, our initial comparisons were made on wild type and IdUrd-substituted virus under conditions of equal pfu concentration, employing 254 nm u.v. light.

The results of this comparison are shown in Fig. 1A. It is clear that any difference in u.v. sensitivity between control and IdUrd-substituted virions that did exist was significantly less than the 10-fold sensitization reported in other systems [see Ref. 1]. Linearization using a probit transformation [18] indicated only a 12% increase in the u.v. sensitivity of the IdUrd-substituted virus with respect to the control.

Similar results have been reported by Rahn *et al.* [4] for IdUrd-substituted *Escherichia coli* after irradiation with 254 nm light. These workers also examined bacterial survival after exposure to 313 nm light and observed a 50–90% increase in u.v. sensitivity in IdUrd-treated bacteria. For this reason, we investigated the effect of 302 nm incident light on virion sensitivity to u.v. light. This wavelength has also been shown to increase the u.v. sensitivity of halouracil-substituted DNA over that observed after 254 nm irradiation [reviewed in Ref. 19]. Surprisingly, under conditions of equal pfu concentration, we observed approximately a 25% decrease in u.v. sensitivity in the IdUrd-substituted virus population (Fig. 1B).

Inactivation kinetics after irradiation at equal particle concentration. While treatment of HSV-1 infected Vero cells with 20 μM IdUrd leads to a 100-fold decrease in infectious virus production, there is not a concomitant decrease in virus particle number [6]. For this reason, we felt that irradiation at equal particle concentration would more closely reflect *in vivo* conditions.

The results of irradiation under conditions of equal particle concentration are presented in Fig. 2. Under these conditions, there was again an apparent decrease in u.v. sensitivity of the IdUrd-substituted virions (IdUrd-HSV-1 virions). The IdUrd sample exhibited only 60% of the control sensitivity after 254 nm irradiation and 72% that of control virions

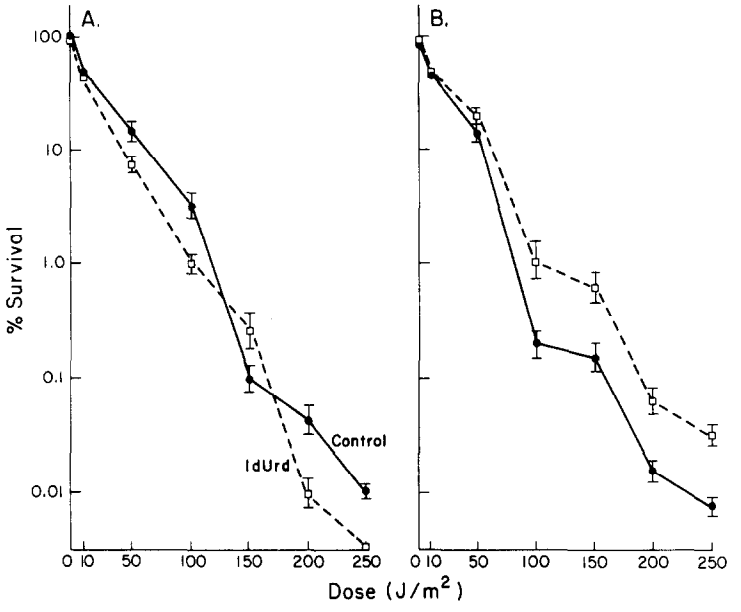

Fig. 1. Equal pfu irradiation. Purified control and IdUrd-substituted HSV-1 virions were irradiated at equal infectious virus concentrations (10^7 pfu/ml). Virus survival was determined by plaque assay in Vero cells. Each point is the average of at least six determinations; error bars represent a range of ±2 standard deviations. Panel A: 254 nm incident light; panel B: 302 nm. Key: (●) control, and (□) IdUrd-HSV-1.

in response to 302 nm fluence. It is interesting to note that, when the u.v. source had a 302 nm peak, irradiation at equal particle or pfu concentrations yielded similar relative sensitivities (Figs. 1B, 2B).

Effect of host cell repair mechanisms on u.v. sensitivity. One explanation for the observed decrease in u.v. sensitivity of the IdUrd-HSV-1 cultures could be an increase in the efficiency of host cell repair enzymes in excising lesions from IdUrd containing DNA. Rapid excision of photochemically produced uracil from BrdUrd-DNA irradiated in mammalian cells has been reported [20]. Such excision might

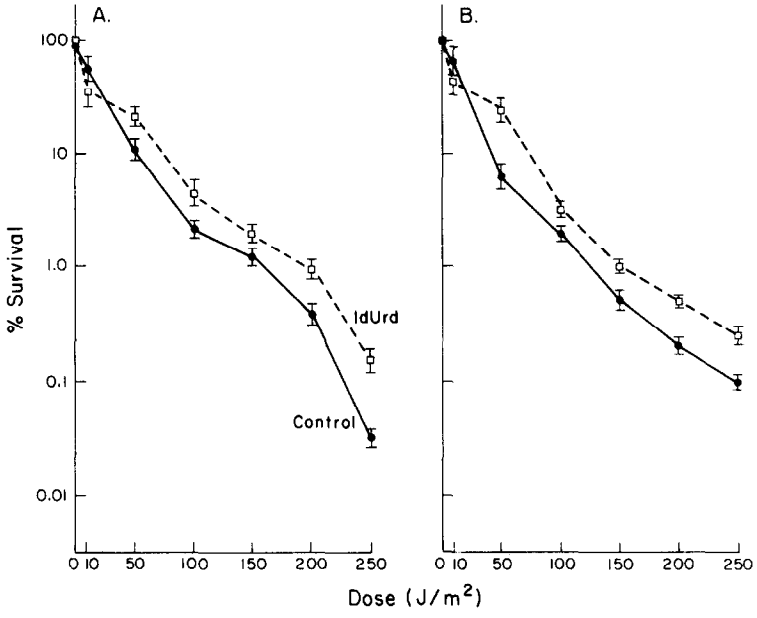

Fig. 2. Equal particle irradiation. Purified control and IdUrd-substituted HSV-1 virions were irradiated at equal virus particle concentrations (10^9 particles/ml). Virus survival was determined by plaque assay in Vero cells. Each point is the average of at least six determinations; error bars represent a range of ±2 standard deviations. Panel A: 254 nm incident light; panel B: 302 nm. Key: (●) control, and (□) IdUrd-HSV-1.

enhance IdUrd-HSV-1 survival by more efficient repair of irradiated IdUrd-substituted sites than non-substituted sites. The relative sensitivity of halouracil substituted and wild type bacteriophage to ultraviolet irradiation has been shown to depend on the ability of the host cell to repair ultraviolet-induced lesions [4, 21].

To preclude host cell effects, u.v. survival studies were performed in Xeroderma Pigmentosum, complementation group A (XPA) cells. These cells exhibit less than 2% of normal ultraviolet-induced unscheduled DNA synthesis due to their lack of excision repair mechanisms (NIGMS Human Genetic Mutant Cell Repository). When titration of virion survival was performed in XPA cells, the relative u.v. sensitivities observed were very similar to those seen in Vero cells. When irradiated with 254 nm incident light, IdUrd-HSV-1 cultures were equally sensitive to u.v. inactivation as control virions at equal pfu concentrations, but only 65% as sensitive at equal particle concentrations.

Ultraviolet-induced thymine photoproduct formation. No firm evidence exists for the formation of BrdUrd-containing photoproducts in irradiated DNA [reviewed in Ref. 1]; however, the presence of halogenated uracils in DNA might be expected to alter the amounts of thymine-containing photoproducts formed upon u.v. irradiation. This is possible through a variety of mechanisms, the simplest of which would be decreases in such photoproduct formation due to the decreased amount of thymidine incorporated into the DNA.

Under the conditions used in this study, there is a 40% substitution of IdUrd for thymidine in the HSV-1 genome [6]. If this significant decrease in thymidine incorporation were to lead to a concomitant decrease in thymine photoproduct formation upon irradiation, one might expect IdUrd-HSV-1 virions to exhibit the observed decrease in u.v. sensitivity.

The nuclease digestion assay described by Farland and Sutherland [8] is a quick, reproducible method for the determination of the relative formation of photoproducts in irradiated DNA that are not susceptible to enzymic digestion to the nucleoside level. In this assay, filter bound radioactivity was reported to be a linear function of the thymine photoproduct content of DNA [8]. Application of this technique to irradiated IdUrd-HSV-1 and control virus revealed no significant difference in the amounts of photoproducts formed in these two cultures that were not converted enzymically into nucleosides (Fig. 3).

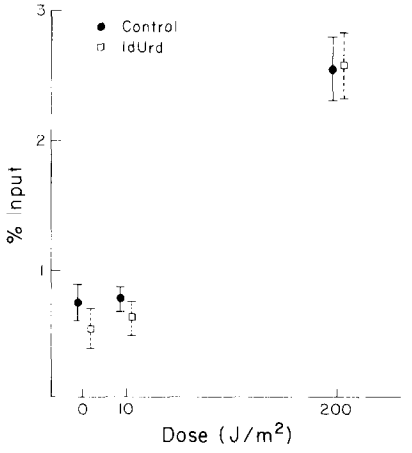

Fig. 3. Thymidine photoproduct formation. The relative formation of [*methyl*-^{3}H]thymidine-containing photoproducts was determined as described. The percent input retained on filters is proportional to photoproduct formation [8]. One hundred percent = 10^5 cpm. Key: (●) control, and (□) IdUrd-HSV-1. Points and error bars are as in Fig. 1.

Delayed early enzyme production. If the observed decrease in u.v. sensitivity of IdUrd-HSV-1 virions were due to alterations in the ability of these virus particles to penetrate the cell membrane or uncoat within the cell, one might see this alteration reflected in the production of viral enzymes. We analyzed the levels of the HSV-1 encoded thymidine kinase and DNA polymerase enzymes at various times post-infection in Vero cells infected with irradiated virus. The results (shown for 15 hr p.i. in Fig. 4) revealed no difference between IdUrd and control cultures. Indeed, when the infections were performed at equal post-irradiation multiplicities, these enzyme activities were unaffected by IdUrd substitution or dose (data not shown).

DISCUSSION

In contrast to observations in other systems, but in agreement with the findings of Wacker *et al.* [5], the incorporation of IdUrd into HSV-1 DNA did not lead to increased virion sensitivity to ultraviolet irradiation. Instead, under most conditions examined, we observed decreased kinetics of inactivation in IdUrd-HSV-1 cultures.

A trivial explanation of this observation would suggest that the extent of incorporation of IdUrd into HSV-1 DNA is heterogeneous and that the virus infectivity being measured in this study is that of minimally or unsubstituted virions in the IdUrd-HSV-1 cultures. The DNA of such a heterogeneous virus population would be expected to sediment in a neutral sucrose density gradient as a broad band spanning densities corresponding to unsubstituted and fully substituted HSV-1 DNA. Fischer and colleagues [6] in our laboratory have shown that this is not the case. IdUrd-HSV-1 DNA sediments as a tight band, similar in band width to unsubstituted HSV-1 DNA, at a density (higher than that of control DNA) determined by the concentration of IdUrd in the original preparation.

To examine further the mechanism by which IdUrd incorporation apparently protects HSV-1 cultures from u.v. inactivation, we explored some of the biochemical consequences of u.v. irradiation.

The strong correlation between u.v. sensitivity of bacteriophage and host cell repair processes [2, 4, 21] led us to the examination of host cell effects on ultraviolet-irradiated IdUrd-HSV-1 survival. XPA cells were chosen for this study since the decreased ability of these cells to repair ultraviolet-induced lesions is well documented. As expected, survival of both ultraviolet-irradiated control HSV-1 and IdUrd-HSV-1 was significantly less in XPA cells than in Vero cells; however, the relative sensitivity of

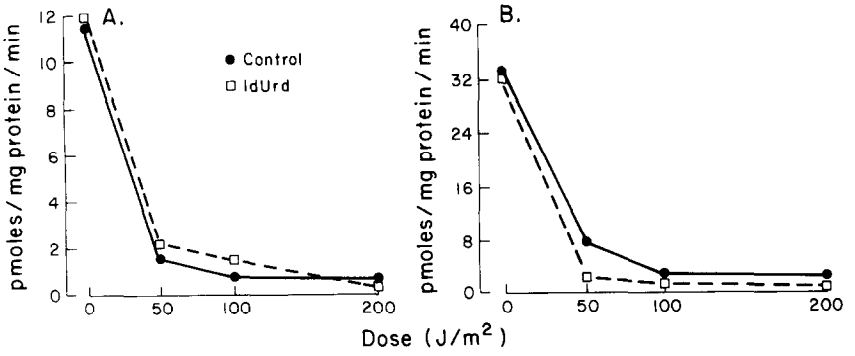

Fig. 4. Delayed early enzyme production. Vero cells were infected with ultraviolet-irradiated virus at a pre-irradiation MOI of 5 pfu/cell. Enzyme activities were determined in crude lysates 15 hr p.i. Key: (●) control, and (□) IdUrd-HSV-1 infected cultures. Panel A: thymidine kinase activity expressed as amount of [^{125}I]dCMP formed. Panel B: DNA polymerase activity expressed as [α-^{35}S]-thio-dATP incorporated in the presence of 150 mM KCl.

IdUrd-HSV-1 to control cultures was the same in both cell lines.

The formation of cyclobutyl pyrimidine dimers in ultraviolet-irradiated DNA leads to a resistance of such DNA to complete nuclease digestion [8]. Using this as the basis for assay, we found no alteration in the formation of thymidine-containing photoproducts after substitution of IdUrd for thymidine in HSV-1 DNA. Although it has been reported that there is little or no involvement of BrdUrd in dinucleotide formation upon u.v. irradiation of highly substituted DNAs [reviewed in Ref. 1], studies by Wacker *et al.* [5] indicate that HSV substituted with BrdUrd is markedly sensitized to u.v. inactivation.

Earlier studies in this laboratory have shown alterations in the amounts and distribution of virion polypeptides in IdUrd-HSV-1 virus preparations [22]. Becker *et al.* [23] found that u.v.-irradiation of unsubstituted HSV (Kos Strain) completely suppressed expression of viral DNA polymerase and viral thymidine kinase activity.

Similarly, we found that u.v. irradiation of unsubstituted HSV produced a decrease in the expression of viral DNA polymerase and viral thymidine kinase; however, virus substituted with IdUrd did not affect the amount of decreased enzymic activity. Rupp and Prusoff [2] have demonstrated the protective effect of hydrogen donor molecules to ultraviolet-irradiated IdUrd-substituted bacteriophage [see also Ref. 1]. HSV-1 virions are composed of an electron-opaque core, an icosahedral capsid, an electron-dense tegument, and a spiked envelope [reviewed in Ref. 24]. At least six virion polypeptides are closely associated with the viral DNA and may affect, either directly or by an alteration in their configuration, the nature of the photoproducts formed. Thus, for example, cysteamine has been shown to protect IdUrd-substituted bacteriophage from u.v. inactivation [2]. Hence, an altered virion structure may play a direct role in the decreased u.v. sensitivity of IdUrd-substituted HSV-1.

Another possibility, however, is derived from the photochemical studies by Rahn and Sellin [25] of the photochemical cross-sections of IdUrd, poly(IUra) and IdUrd-substituted DNA. They suggested "that incorporation of IdUrd into an ordered structure restricts the freedom with which the iodine atom is ejected from the DNA following splitting of the C—I bond and formation of the C.–I. intermediate complex. Depending upon the surroundings either C. and I. can recombine, or I can leave". Thus, the microenvironment in the virion may favor recombination.

Support for one of these hypotheses may come from studies on the u.v. sensitivity of naked DNA from control and IdUrd-substituted virus preparations in the presence and absence of added virion polypeptides. Such studies will define whether the virus coat plays a crucial role in defining u.v. sensitivity, indicated by either equal or increased sensitivity upon irradiation of naked IdUrd-HSV-1 DNA as compared to control DNA. The addition, pre-irradiation, of virion polypeptides from either control or IdUrd-HSV-1 cultures may define a role of individual proteins in radiation sensitization. Such studies are required for further elucidation of the biochemical basis of the effects of 5-iodo-2′-deoxyuridine incorporation into HSV-1 DNA on the sensitivity of the substituted virion to u.v. radiation.

A review of the literature on ultraviolet radiation in a wide variety of systems leads one to anticipate that exposure to 302 nm radiation requires 50–100 times the dose needed at 254 nm to yield the same inactivation [e.g. see Refs. 4 and 26]. In the present study, we observe only a 2- to 4-fold difference in the doses of 254 nm and 302 nm radiation required to inactivate a similar proportion of control virions (Figs. 1 and 2). This apparent discrepancy can be resolved by examining the light sources employed in these studies. In the present study, we employed a 302 nm peak 15 W bulb, not a monochromatic source. This bulb has a spectral distribution of 285–400 nm with significant emission between 295 and 350 nm. A comparison of the efficacy of inactivation by 295 and 254 nm incident light reveals only a 4- to 8-fold difference in dosage required for similar effects [26, 27]. It is probable that these lower wavelengths are a significant factor in the observed inactivation of virus at 302 nm. This does not, however, in any

way affect the results or hypotheses discussed in this paper.

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