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Mechanisms of photodynamic inactivation of herpes simplex viruses: comparison between methylene blue, light plus electricity, and hematoporhyrin plus light.

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Research Article

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Mechanisms of Photodynamic Inactivation of Herpes Simplex Viruses

COMPARISON BETWEEN METHYLENE BLUE, LIGHT PLUS ELECTRICITY, AND HEMATOPORPHYRIN PLUS LIGHT

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ABSTRACT Herpes simplex virus (HSV) types 1 and 2 have been inactivated in vitro using low concentrations of methylene blue (MB), light (λ) plus electricity (E), or hematoporphyrin derivative (HPD) plus λ. Both techniques introduce single strand interruptions into viral DNA, but do not make double strand breaks. MB, λ plus E-treated virions adsorb normally to and penetrate susceptible cells, whereas HSV inactivated with HPD and light does not. This difference is emphasized by the induction of new viral and cell DNA synthesis after infection with MB, λ plus E-treated virions, whereas only cell, DNA but no HSV DNA, is made subsequent to HPD and λ exposure. These observations reflect disparate mechanisms of viral inactivation. A block(s) in viral maturation, subsequent to viral DNA synthesis, occurs as a result of treatment with MB, λ, and E, whereas HPD plus λ-treated particles fail to enter a susceptible cell, and therefore do not initiate an infection.

INTRODUCTION

Herpes simplex viruses (HSV)[1] are a frequent cause of uncomfortable primary or recurrent mucocutaneous infections (1, 2), and may cause protracted painful illnesses in immunocompromised hosts (3). Although a number of agents have been studied, antiviral chemotherapy has not been effective in the treatment of herpes labialis or herpes genitalis. Photoactive dyes such as proflavine or neutral red have been shown to inhibit the plaque-forming ability of HSV after photoirradiation of either infected cells or a suspension of dye and virus (4–7) by a process known as photodynamic inactivation (PDI). This technique not only inactivates HSV, but the resulting particles can transform hamster embryo fibroblasts (6). The molecular biologic targets critical for PDI by heterotricyclic compounds have not been elucidated, but Khan et al. (5) have suggested that irradiation of infected cells pretreated with proflavine interferes with viral production at a step subsequent to DNA synthesis. Conventional methods of PDI that employ photoactive dyes and light (λ) in the presence of ambient concentrations of O_2 have not proven to be effective antiviral therapies (8).

This laboratory has developed two modifications of PDI. Low concentrations of methylene blue (1 μM, MB, Sigma Chemical Co., St. Louis, Mo.) can be electrically reduced with small amounts of current, and, when irradiated in the presence of HSV, a dramatic fall in viral titer ensues (9). The antiviral effect is dependent on dye concentration and amperage. It is partially inhibited by superoxide dismutase, suggesting that the superoxide anion is an important intermediate (9). Alternatively, hematoporphyrin derivative (HPD),[2] a mixture of several photoactive tetrapyrrols, will completely inhibit the in vitro infectivity of HSV after illumination of a mixture of dye and virus. This effect is dependent upon the presence of oxygen as well as

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[1] *Abbreviations used in this paper*: Ad2, adenovirus 2; E, electricity; HPD, hematoporphyrin derivative; HSV, herpes simplex virus; λ, light; MB, methylene blue; PDI, photodynamic inactivation; PFU, plaque-forming units; SDS, sodium dodecyl sulfate.

[2] Lewin, A. A., C. S. Crumpacker, and L. E. Schnipper. Photodynamic inactivation of herpes simplex virus by hematophyrin derivative and light. Submitted for publication.

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carefully defined concentrations of HPD and intensity of λ.

In the current investigation, studies have been undertaken to compare PDI by employing either HPD plus λ or MB, λ and electricity (E). Their effects on viral DNA structure and synthesis, as well as on adsorption and penetration of HSV, reveal substantial differences. The data suggest that PDI can occur through a variety of mechanisms, one of which may inhibit photoirradiated particles from initiating infection of susceptible cells.

METHODS

Cells and media. Vero cells were obtained from the American Type Culture Collection (Rockville Md.) and propagated at 37°C in medium 199 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal bovine serum in the presence of penicillin (200 U/ml) and streptomycin (250 μg/ml). The cells and virus pools were negative for mycoplasma after growth in Hayflick's media (10).

Viruses. HSV cultured from patients with herpetic lesions was employed. All strains were shown to be HSV-1 or HSV-2 by the microneutralization technique (11). Virus pools were prepared by low multiplicity inoculation (0.01 plaque-forming units [PFU]/cell) of confluent Vero cells and were harvested from infected cultures at 72–96 h postinfection. Radiolabeled virions or infected-cell DNA were prepared by adding [*methyl*-^{3}H]Thymidine (56.9 Ci/mmol, New England Nuclear, Boston, Mass.) at 10 μCi/ml, from 2 h postinfection to the time of harvesting. Virions were assayed by the plaque-titration method on confluent Vero cells in six-well plastic plates (Flow Laboratories, Inc., Rockville, Md.). The cells were infected with dilutions of HSV, and after a 1-h adsorption period at 37°C-5% CO_2 they were overlaid with a 1:1 dilution of 2 × minimal essential medium – 4% fetal bovine serum and 2% methylcellulose. After 4 d of incubation at 37°C-5% CO_2 the cells were fixed with acetic acid-methanol, stained with crystal violet, and the plaques counted (6).

[^{14}C]Adenovirus was propagated by infecting KB cells in suspension in the presence of 0.05 μCi/ml [*methyl*-^{14}C]-thymidine, and purified by a modification of the method described by Burnett et al. (12). [^{14}C]SV40 and [^{3}H]SV40 were propagated in BSC-1 cells in the presence of 0.05 μCi/ml [^{14}C]thymidine ([*methyl*-^{3}H]thymidine) or 10 μCi/ml [^{3}H]-thymidine, respectively, as previously described (13).

Adsorption and penetration of susceptible cells. 24 h before infection confluent Vero cells were overlaid with growth media (M199-10% fetal bovine serum) containing 100 μg/ml cytosine arabinoside. At the time of viral inoculation the media was supplemented with unlabeled thymidine, 100 μg/ml. The cells were infected with [^{3}H]thymidine-labeled HSV-1 and incubated at 37°C in the dark. At various times postinfection cells were harvested by scraping, washed three times with virus buffer (0.02 M Tris, pH 7.4–0.15M NaCl) and a 0.05-ml aliquot precipitated with 20% TCA. Radioactivity representing adsorbed virions was determined in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

The remaining cells were diluted with reticulocyte standard buffer (0.05 M Tris-pH 7.5–0.04 M NaCl-0.002 M EDTA), permitted to swell at 4°C, and lysed with 1% NP-40 (Particle Data Laboratories, Ltd., Elmhurst, Ill.). Nuclei were separated from cytoplasm by centrifugation at 800 *g* for 10 min, and nuclear radioactivity was determined after precipitation in TCA.

Preparation of nucleocapsids. Purified nucleocapsids were prepared by a modification of the method of Kieff et al. (14). Roller bottles infected with 10 PFU/cell of HSV-1 were harvested 20 h after infection by trypsinization. The cells were pelleted in an International PR-6000 centrifuge for 10 min at 800 *g* and 4°C. The pellet was washed in phosphate-buffered saline and suspended in reticulocyte standard buffer (0.05 M Tris, pH 7.5, 0.04 M NaCl, 0.002 M EDTA). After 10 min at 4°C the suspension was made 1.0% with NP-40 and shaken vigorously. The nuclei were sedimented and the cytoplasm layered onto 37-ml linear 10-50% (wt/wt) neutral sucrose gradients prepared in virus buffer (0.02 M Tris, pH 7, 0.15 M NaCl) centrifuged for 1 h at 25,000 rpm in a Beckman SW 27 rotor at 5°C. The lower band of nucleocapsids was pelleted at 25,000 rpm at 4°C for 1 h and resuspended in virus buffer.

Purification of cell and viral DNA. High molecular weight cell DNA was purified by the method of Varmus et al. (15). Infected cells were lysed with 0.5% (sodium dodecyl sulfate) (SDS), incubated with nuclease-free Pronase (500 μg/ml, Calbiochem, La Jolla, Calif.) at 37°C for 1 h, and extracted twice with 0.1 M Tris, pH 7.4-saturated phenol. The DNA was then treated for 3 h at 37°C with 100 μg/ml of pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.; boiled for 10 min to inactivate DNase). After two additional phenol extractions, the DNA was dialyzed against 15 mM NaCl-1.5 mM Na-citrate with three changes over 24 h.

Radiolabeled adenovirus 2 (Ad2) and SV40 DNA were extracted from purified virions by lysis in 0.3% SDS-1 mg/ml nuclease-free Pronase, and extraction in Tris-saturated phenol, as previously described (12, 13).

Isopycnic centrifugation. Equilibrium density gradient centrifugation of DNA was performed in CsCl (optical grade, Harshaw Chemical Corp, Los Angeles, Calif.). Centrifugation was performed in an SW 50.1 rotor, at 30,000 rpm at 8°C for 68 h. Fractions were collected from the bottom and counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc.). Refractive indices were determined on an Abbe Refractometer (Bausch & Lomb Inc., Rochester, N. Y.).

Velocity sedimentation. Inactivated or untreated virions were suspended in 0.5% SDS and 2% sarkosyl, heated at 60°C for 2 min, and layered on 5-20% (wt/wt) neutral sucrose gradients. Alternatively, the lysed virions were denatured with 0.1 N NaOH and layered on 5-20% (wt/wt) alkaline sucrose gradients in 0.7 M NaCl, 0.15% sarkosyl, 0.1 N NaOH. Sedimentation was carried out in an SW 50.1 rotor for an appropriate time at 15°C. Fractions were collected and radioactivity determined in a liquid scintillation counter. Sedimentation constants were determined by the method of Burgi and Hershey (16), with SV40 or Ad2 DNA employed as a marker.

Photodynamic Inactivation of HSV-1

MB, λ, and electrical inactivation (9). All studies were performed in methyl methacrylate cells fitted with platinum electrodes and packed in ice. Cell dimensions were 3.5 (length) × 3.8 (height) × 0.8 (width) cm. A constant λ source was provided by Mobilite tensor lamp (Mobilite, Inc., Hauppauge, N. Y.) maintained at a fixed distance of 20 cm from the methyl methacrylate chamber. The spectral irradiance at this distance, as determined by an EEG radiometer 580-11 (EGG Co., Salem, Mass.), was 190 μW/cm^2. A Keithly 225 current source (DC) (Keith Instruments, Inc., Cleveland, Ohio) provided E, and the voltage across the sample was measured. MB was freshly prepared in water at appropriate concentrations (9).

Hematoporphyrin and λ. Hematoporphyrin derivative (HPD, Rouselle Corporation, Alco, Chicago, Ill.) was purified

according to the method of Gregorie et al. (17). A stock solution of 5 mg/ml was made by dissolving the pigment in 1 N NaOH, diluting it with 0.9% NaCl, and neutralizing it to pH 7.4 with HCl. The HPD was stored at 4°C in the dark and filtered (0.2 μm Nalgene filter; Nalgene Labware Div., Nalgene Co., Rochester, N. Y.) before use. A 300 W Kodak 760H slide projector lamp (Eastman Kodak Co., Rochester, N. Y.) was used to illuminate viral suspensions. Irradiance was determined with a calibrated YSI-Kettering model 65 radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). During illumination, the dye-virus mixture was maintained at 18°–21°C.

RESULTS

Single strand interruptions in herpes simplex viral DNA. HSV-1 or HSV-2 is rapidly and completely inactivated when a suspension of virions and HPD is irradiated with 26 mW/cm² of λ for 1 min (Fig. 1). In the absence of either λ or dye, no change in viral titer is observed. Similarly, a low concentration of MB, when electrically reduced and illuminated with λ in the presence of HSV types 1 or 2, inhibits viral infectivity to the limits of the assay (Fig. 2). Under these conditions neither MB alone, nor MB with E or λ results in inhibition of the viral particles.

To assess the introduction of single strand interruptions into viral nucleic acid, [³H]HSV-1 nucleocapsids were photodynamically treated by both techniques. The particles were then lysed at 60°C in the presence of sarkosyl (2%) and SDS (0.2%), denatured, and cosedimented with [¹⁴C]SV40 DNA through alkaline sucrose gradients. After photoirradiation by either method, a broad peak of slowly sedimenting DNA is observed having an average sedimentation constant of 4S. This is in marked contrast to the multiple species of higher molecular weight DNA derived from untreated nucleocapsids (Fig. 3) (14). Sedimentation of lysed, treated, or untreated nucleocapsids through 5–20% neutral sucrose gradients results in a peak of viral DNA of 56S when compared with a [¹⁴C]SV40 DNA marker (not shown). To confirm that the nucleocapsids contained viral DNA, untreated [³H]HSV-1 was lysed at 60°C in the presence of sarkosyl (2%) and SDS (0.2%), mixed with CsCl, and subjected to isopycnic centrifugation. A single sharp peak of [³H]DNA was observed at the expected *d* of 1.726 g/cm³. These findings are consistent with the introduction of single strand interruptions but not double strand breaks into viral DNA by either photodynamic technique.

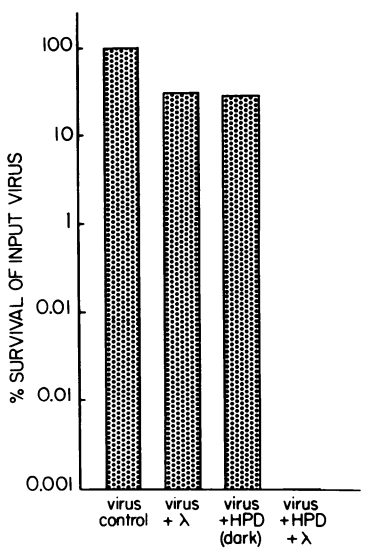

FIGURE 1 PDI of HSV-1 with HPD and λ. A suspension of HSV-1 (2 × 10⁷ PFU/ml) was mixed with HPD to a final concentration of 70 μM, and irradiated at 26 mW/cm² with visible λ for 1 min. Residual infectious virus was assayed by the plaque-titration method. In parallel, untreated virus, virus plus λ, and virus plus HPD in the dark were assayed.

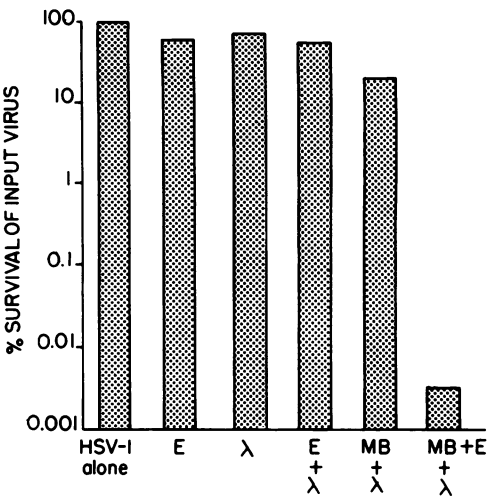

FIGURE 2 MB, λ, and E inactivation of HSV-1. HSV-1 (5 × 10⁵ PFU/ml) was mixed with MB to a final concentration of 1 μM and placed on ice. A 1-mA electrical current was applied, and simultaneously the suspension was irradiated with 190 μW/cm² visible λ. Infectivity was determined by plaque titration and compared with untreated virus, virions exposed to dye, dye plus λ, dye plus E, or E alone.

The susceptibility of a non-herpesvirus DNA to photodynamic damage was determined by treating [¹⁴C]thymidine-labeled Ad2, and by subjecting lysed virions to velocity sedimentation. After exposure to HPD and λ, viral DNA sediments through 5-20% neutral sucrose gradients as duplex molecules having an S value identical to untreated viral DNA (Fig. 4A and B) or DNA exposed to HPD in the dark (not shown). When denatured, treated Ad2 DNA migrates more slowly than untreated Ad2 DNA, forming multiple peaks between 9S and 32S in a 5–20% alkaline sucrose gradient (Fig. 4C and D). Similar findings are obtained after MB, λ, plus electrical treatment of Ad2 (not shown). These observations are consistent with single strand breaks in Ad2 DNA after either photodynamic technique.

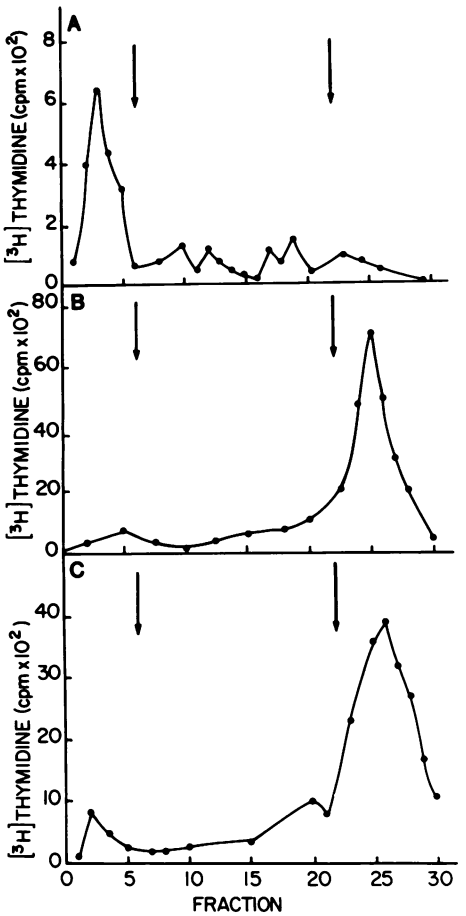

FIGURE 3 Single strand interruptions in HSV-1 DNA. [^{3}H]-Thymidine-labeled HSV-1 nucleocapsids were treated either with HPD plus λ or MB plus λ plus E as described in Figs. 1 and 2, lysed by heating at 60°C in the presence of 2% sarkosyl, 0.2% SDS, denatured with 0.1 N NaOH, and layered on 5–20% (wt/wt) alkaline sucrose gradients in the presence of a [^{14}C]SV40 DNA marker. After centrifugation at 47,000 rpm at 15°C for 100 min, fractions were collected from the bottom, neutralized, and radioactivity determined in a liquid scintillation counter. The arrows denote the positions of form I and form II SV40 DNA (53S and 18S), respectively. The direction of sedimentation is from right to left. (A) Untreated nucleocapsids. (B) Nucleocapsids treated with HPD and λ. (C) Nucleocapsids treated with MB, λ, and E.

Adsorption and penetration by inactivated virion. The initial stages in viral infection were investigated by determining the effects of PDI on adsorption and penetration of susceptible cells. Radiolabeled virus was prepared as described in Methods. To confirm that the label was in viral DNA, a large aliquot of the supernatant virus was pelleted at 100,000 *g*, lysed, and subjected to isopycnic centrifugation. A single sharp peak of [^{3}H]thymidine was seen at a *d* of 1.726 g/cm^3. 24 h before infection, Vero cells were overlaid with media containing cytosine arabinoside to inhibit cell DNA synthesis. At the time of viral inoculation the

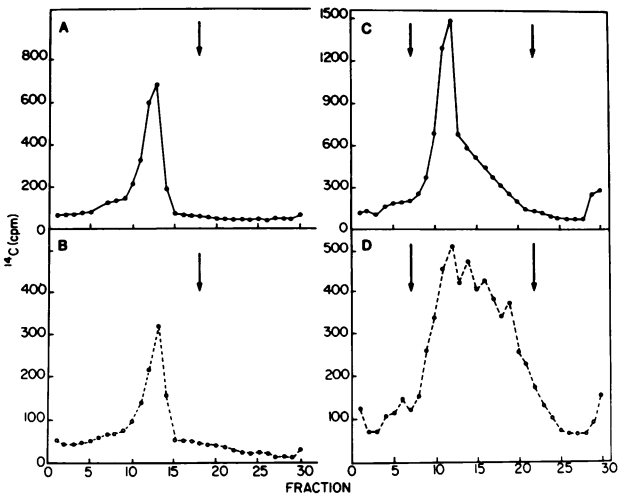

FIGURE 4 Effect of HPD and λ on Ad2 DNA. Purified [^{14}C]Ad2 virions (5×10^4 cpm) were either untreated, mixed with HPD to a final concentration of 70 μM in the dark, or exposed to HPD and irradiated with visible λ for 1 min. The virions were then lysed with Pronase, 1 mg/ml, and SDS, 0.1%, at 37°C for 1 h. Subsequently, aliquots were admixed with [^{3}H]SV40 DNA and layered on 5–20% neutral sucrose gradients, or denatured in 0.1 N NaOH and layered on 5–20% alkaline sucrose gradients. Sedimentation was carried out in an SW 50.1 rotor at 47,000 rpm for 110 min at 15°C. Fractions were collected from the bottom, neutralized, and radioactivity determined. The arrows denote the position of the marker DNA. The direction of sedimentation is from right to left. (A) Neutral sucrose gradient of untreated lysed [^{14}C]Ad2 virions. (B) Neutral sucrose gradient of [^{14}C]Ad2 virions exposed to HPD and λ. (C) Alkaline sucrose gradient of untreated [^{14}C]Ad2 virions. (D) Alkaline sucrose gradient of [^{14}C]Ad2 virions exposed to HPD and λ.

thymidine pool was expanded with addition of unlabeled base. The cells were infected with either untreated [^{3}H]thymidine-labeled HSV-1, or virions completely inactivated by HPD plus λ, or MB, λ plus E, at a preinactivation multiplicity of 10. At 0, 0.5, 1, 3, and 5 h postinfection, radioactivity adsorbed to intact cells was determined (Fig. 5A). Nuclei were then separated from the cytoplasm of remaining cells and nuclear penetration by [^{3}H]HSV-1 DNA was quantitated (Fig. 5B).

Untreated HSV-1 rapidly adsorbs to Vero cells and viral DNA penetrates to the nucleus maximally 3 h postinfection. Virions rendered noninfectious with MB (1 μM), λ (190 μW/cm^2) plus E as in Fig. 2, follow the same time-course for adsorption. However, photoirradiation of a suspension of [^{3}H]HSV-1 and HPD (70 μM) at 26 mW/cm^2 results in particles that neither adsorb to nor penetrate susceptible cells.

DNA synthesis in cells infected with photoinactivated herpesviruses. The different effects on the initial steps in viral infection suggest the importance of studying viral replication subsequent to inactivation by either technique. Induction of viral and cellular

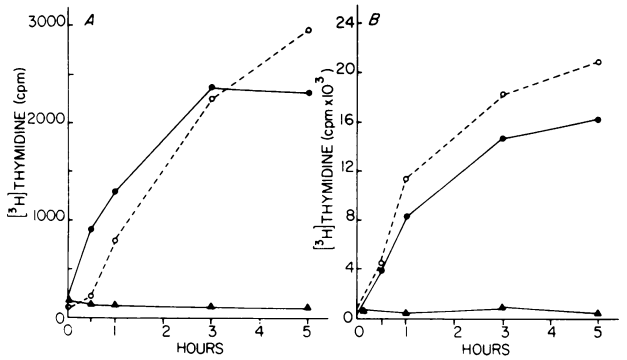

FIGURE 5 Adsorption and penetration of susceptible cells by inactivated virions. [³H]Thymidine-labeled HSV-1 was inactivated with MB, λ, and E or HPD plus λ, as described in Figs. 1 and 2. The equivalent of 10 PFU/cell of each was employed as an inoculum to infect confluent Vero cells, pretreated with 100 μg/ml cytosine arabinoside, and subsequently incubated in 100 μg/ml thymidine. Cells were harvested at 0, 0.5, 1, 3, and 5 h postinfection, and washed in virus buffer. Whole cell-associated radioactivity was determined by TCA precipitation of a 50-μl aliquot of intact cells. The remaining cells were then swollen in a hypotonic buffer (0.005 M Tris, pH 7.5, 0.04 M NaCl, 0.002 M EDTA) and lysed with 1% NP-40. Nuclei were separated from cytoplasm by centrifugation at 800 *g*, and nuclear radioactivity determined by precipitation in 20% TCA. (A) Adsorption to whole, infected cells. (B) Penetration of infected cell nuclei. (●) untreated [³H]HSV-1; (○) MB, λ plus E inactivated [³H]HSV-1; (▲) HPD plus λ-inactivated virions.

DNA synthesis by untreated HSV-1 or by photodynamically inactivated virions was determined by isopycnic centrifugation of purified infected-cell nucleic acid. Confluent Vero cells were infected with either 10 PFU/cell of untreated HSV-1 or the same amount of virus treated with MB-λ plus E, or HPD plus λ. The infectivity of inactivated virions was completely abolished, as determined by plaque titration. 2 h after infection, [³H]thymidine was added to the media to a final concentration of 10 μCi/ml and the cells harvested at 16 h. 10 μg whole DNA was mixed with CsCl and adjusted to a *d* of 1.700 g/cm³ before centrifugation.

DNA from the untreated HSV-1 infection reveals distinct peaks at 1.726 and 1.700 g/cm³ consistent with radiolabeled viral and cell DNA, respectively. (Fig. 6A). Virus inactivated with MB, λ, and E also induces viral DNA replication (Fig. 6B). However, the cells infected with HPD- and λ-treated virions demonstrate a single peak of labeled DNA at *d* 1.700 g/cm³, but none at 1.726 g/cm³ (Fig. 6C). This observation is consistent with a failure to induce replication of viral nucleic acid in cells infected with HPD plus λ inactivated HSV-1.

Differential effects of HPD and λ on virus and cells. To determine whether there is a selective effect of HPD plus λ on HSV, PDI of virus, cells, and virus-infected cells was undertaken. Inhibition of 99.99% of plaque formation by cell-free virus is observed at an

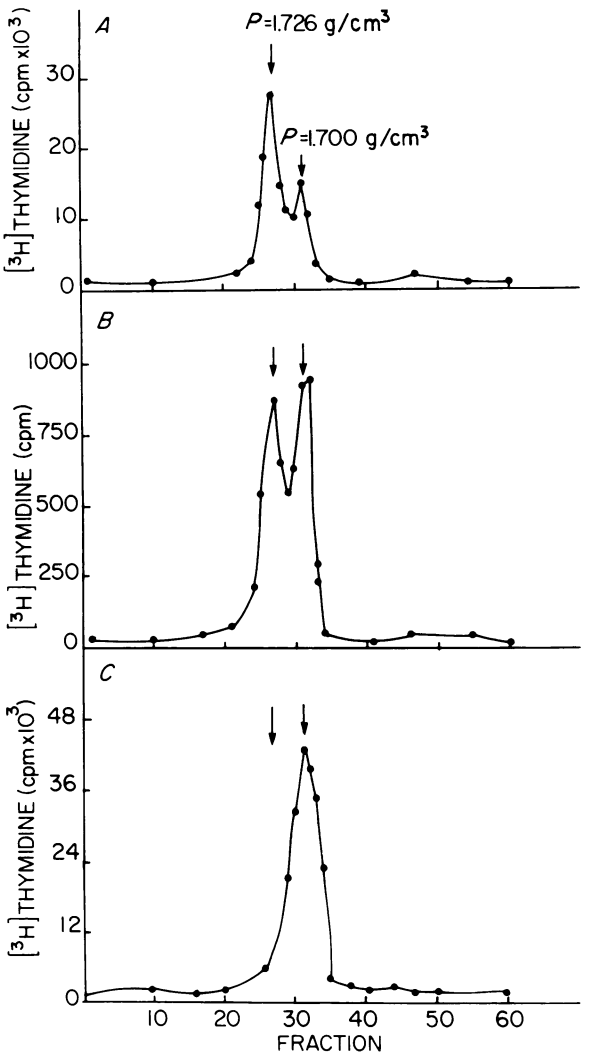

FIGURE 6 DNA synthesis in cells infected with inactivated virus. Confluent Vero cells were inoculated with the equivalent of 10 PFU/cell of virions inactivated by MB, λ plus E, or HPD plus λ. [³H]Thymidine was added from 2 to 16 h postinfection, and the cells harvested. Whole cell DNA was purified and 10 μg was mixed with CsCl-0.001 M EDTA to a final *d* of 1.700 g/cm³. Isopycnic centrifugation was performed in an SW 50.1 rotor at 30,000 rpm at 8°C for 68 h. Fractions were collected from the bottom, and radioactivity determined in a scintillation counter. The direction of centrifugation is from right to left. (A) After infection with untreated HSV-1. (B) After infection with MB, λ plus E treated HSV-1. (C) After infection with HPD plus λ treated HSV-1.

HPD concentration (1 μM) that diminishes cell survival by 35% (Table I). At the same dose level, cells that have been infected with 0.1 PFU/cell of HSV-1 and photodynamically treated after a 2-h adsorption period also demonstrate a greater reduction in viral production than in cell proliferation (Table I). This is observed at both the 1 and 6.5 μM dose levels, respectively. Cell survival after phototreatment of HSV-1-

TABLE I

Percent Survival of Virus and Cells after Photodynamic Inactivation by HPD and λ

HPD*	Survival of cell-free HSV-1‡	Survival of mock-infected cells§	Viral and cell survival in an HSV-1 infection‖
μM	%	%	%
0	100	100	100 (100)
1	0.01	65	8 (75)
6.5	0.04	5	0 (15)
10	0	0	0 (0)

* Conditions for preparing HPD and photoirradiation (26 mW/cm² × 30 s) are as described in Methods.

‡ HSV-1 (3×10^7 PFU/ml) was mixed with HPD, photoirradiated, and the surviving virus determined by plaque titration.

§ 1.5×10^6 Vero cells were mixed with the appropriate amount of HPD and exposed to 26 mW/cm² λ for 30 s.

‖ A suspension of 1.5×10^6 Vero cells were infected with 1 mM PFU/cell HSV-1 and photoirradiated after a 2-h adsorption period. The infected cells were then mixed with HPD and irradiated (26 mW/cm²) for 30 s. After 48 h the virus in the supernate was measured by plaque titration and viable cells determined by trypan blue exclusion. Expressed as percent of virus and cells (bracketed) present in the untreated infection.

infected cells is effected both by progress of the productive infection and by the cellular damage resulting from HPD and λ. The data suggest that propagation of virus is more vulnerable to this modification of PDI than is cell survival.

DISCUSSION

Photodynamic reactions mediated by reactive oxygen species, such as singlet oxygen ($^1O_2$), hydroxyl-free radicals, or superoxide anions (O_2^-), often have important biological consequences (18). They form the basis of the photosensitization that accompanies some of the porphyria syndromes, and of selected antiviral techniques (19, 20). Inactivation of herpes simplex viruses has been demonstrated after electrical reduction of MB concomitant with exposure of the virus-dye suspension to visible λ (9). Superoxide anion is an important intermediate in this reaction because the effect is partially inhibited by superoxide dismutase.

In a standard photodynamic reaction a heterotricyclic dye such as MB is believed to interact with the strands of duplex DNA and undergo an oxidation reaction after adsorbing sufficient λ energy in the presence of O_2 (21). This leads to loss of guanine residues, gaps in the base sequence, and single strand breaks (21). Although it has not been confirmed that the mechanism is the same, single strand interruptions are generated in viral DNA after MB plus λ plus E treatment of either HSV-1 or Ad2. Despite the introduction of structural changes into nucleic acid, these inactivated virions are capable of initiating the early stages of infection. In the current studies, the time-course for adsorption of treated and untreated virions to susceptible cells, and nuclear penetration by parental viral DNA, is similar to the findings of Hochberg and Becker (22) who employed a prototype strain of HSV-1. Isopycnic centrifugation of purified whole cell DNA after infection with either untreated or MB- plus λ- plus E-exposed virions demonstrates two distinct peaks of DNA synthesis, at 1.726 and 1.700 g/cm³. These represent induction of viral and cell DNA replication, respectively.

Similar findings emerged from a study of HSV-1-infected cells pretreated with low doses of proflavine and then irradiated (5). Despite a marked reduction in infectious progeny, viral and cell DNA synthesis were not inhibited. Thus, in both the proflavine and MB, λ plus E inactivation systems, the photodynamic effect appears to result from interference in the process of viral maturation subsequent to DNA replication. Confirmation of this finding awaits comparisons between viral proteins induced by treated and untreated virus after DNA synthesis.

Irradiation of HSV-1 in the presence of HPD inhibits plaque formation by a very different mechanism. The initial steps in viral infection are ineffective because radiolabeled virions neither adsorb to nor penetrate the nuclei of susceptible cells. This is consistent with the absence of a peak of newly synthesized viral DNA in a CsCl density gradient of radiolabeled infected cell nucleic acid and a failure to induce viral-specific antigens.[2] Although this photodynamic process can result in single strand interruptions in viral DNA, this does not appear to be the critical target for in vitro inactivation of suspensions of virus.

The defects in adsorption and penetration suggest an HPD-associated alteration in the viral surface. Porphyrins are known to form radicals in a lipid milieu that may lead to lipid peroxidation (23). This effect may be mediated in part by singlet oxygen or by direct interactions between virions and excited HPD (18). In either case, the lipid moiety within the envelopes of herpesvirions may be vulnerable to photochemical damage by this compound. Porphyrins can induce photodynamic damage to amino acids as well (24), sug gesting alternative or additional targets in the envelope that are relevant to HSV inactivation.

Virions inactivated by the heterotricyclic dye, neutral red, can transform cells in vitro. Although the relevance of this finding to humans is not clear, established methods of PDI may not be innocuous. The modification of PDI employing low concentrations of HPD plus λ may represent a promising alternative as an anti-HSV modality. In particular, the inability of HPD-treated HSV to penetrate a susceptible cell suggests

that the generation of transforming virus may be minimal. Comparative studies of the effects of HPD and λ on cells and virus indicate that at low concentrations of HPD, viral proliferation is more vulnerable to inhibition than is cell growth. This selective effect on HSV suggests the possibility that PDI with HPD will have a high therapeutic ratio in vivo. These observations provide an impetus for evaluating both the efficacy of the technique in a guinea pig model of mucocutaneous HSV infections, and the generation of transforming virus.

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