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Photodynamic Inactivation of Herpes Simplex Virus by Hematoporphyrin Derivative and Light (40727)

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Photodynamic inactivation (PDI) involves the dye-sensitized photooxidation of a substrate (1). This process has previously been utilized to inactivate bacterial viruses (2) and DNA-containing animal viruses (3). Herpes simplex viruses (HSV), a ubiquitous cause of human infections and possibly neoplasia (4), can be inactivated following exposure of virions to an appropriate dye and visible light (3, 5). The heterotricyclic acridine and phenazine dyes have been most intensively studied with HSV (3, 5). As structurally similar compounds, they are believed to intercalate in HSV DNA and undergo an oxidation reaction on irradiation with visible light (6), which preferentially destroys guanine residues (7, 9). Although the photodynamic effects of heterotricyclic dyes may inhibit viral replication, HSV inactivated by neutral red and light is still capable of transforming hamster embryo fibroblasts (10).

Porphyrins are a group of photoactive substances that are structurally different from heterotricyclic dyes, and whose photochemical properties are responsible for the photosensitivity reactions observed in the porphyrias (11, 12). Hematoporphyrin derivative (HPD), a mixture of naturally occurring tetrapyrroles, has been employed to induce photooxidative damage in neoplastic cells, *in vivo* and *in vitro* (13, 14). Following absorption of light energy these compounds are raised to an excited state, which reacts with gound-state triplet oxygen ($^3O_2$) to form the highly reactive singlet oxygen ($^1O_2$) and ground-state porphyrin (15). The interaction between porphyrins and lipids can generate free radicals, and may initiate a chain reaction resulting in lipid peroxidation (16). Herpes simplex viruses contain lipid in the envelopes of mature virions and therefore may be sensitive to the photodynamic effects of hematoporphyrins.

In the current study, rapid and complete photoinactivation of herpes simplex viruses with hematoporphyrin derivative (HPD) is described. This effect is dependent upon the presence of oxygen, HPD, and visible light, and appears to interfere with the initial stages of a viral infection.

Materials and methods. Cells and media. Vero cells were obtained from the American Type Culture Collection and propagated at 37°C in Medium 199 (Gibco), supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells and virus pools were negative for mycoplasma following growth on Hayflick's media (17).

Viruses. The KOS strain of HSV-1, and HSV cultured from patients with herpetic lesions were plaque purified prior to use in the study. All viral strains were shown to be HSV-1 by the microneutralization technique (18). Virus pools were prepared by low multiplicity inoculation (10^{-2} PFU/cell) of confluent Vero cells harvested from infected cultures at 72–96 hr postinfection, and clarified by low-speed centrifugation. Radiolabeled virions were prepared by adding [*methyl*-^{3}H] thymidine (56.9 Ci/mmole, New England Nuclear) at 10 μCi/ml, from 2 hr postinfection, to the time of harvesting. An aliquot of the supernatant containing [^{3}H]HSV-1 was pelleted at 100,000 *g* in a Beckman L3-50 preparative ultracentrifuge, and resuspended in 0.01 *M* Tris–HCl, pH7, prior to isopycnic centrifugation. When assayed by the plaque titration method, these pools had a titer of $4 \times 10^6 - 7 \times 10^8$ PFU/ml.

Virus assay. Confluent layers of Vero cells grown in six-well Linbro plates were

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inoculated with appropriate dilutions of herpes simplex virus. After virus adsorption at 37°C/5% CO_2 for 1 hr, the cell cultures were overlaid with a 1:1 dilution of 2 × MEM/4% FBS and 2% methycellulose. After 4 days of incubation at 37°C/5% CO_2, the cells were fixed with a 1:3 dilution of acetic acid–methanol and the plaques were counted (10).

Fluorescent antibody studies. Vero cells were grown to confluence on coverslips, and infected with untreated or inactivated virions at an m.o.i. of 10. At 18 hr postinfection the coverslips were removed and fixed in acetone for 10 min. They were then incubated with a 1:20 dilution of rabbit anti-HSV-1 (VR_3strain) antisera (CDC Biological Reagents Section, lot No. 1) at 37°C for 30 min. After three washes in phosphate-buffered saline (PBS) pH 7.4, they were incubated with a mixture of goat anti-rabbit IgG conjugated to fluoroscein (1:20, Hyland Laboratories, Inc.) and rhodamine-conjugated bovine albumin counterstain (1:5), at 37°C for 30 min. Following three washes in PBS, the coverslips were mounted on glass slides, examined with a Zeiss IIIR fluorescent microscope, and photomicrographs taken with an attached camera.

Isopycnic centrifugation. ^{3}H-Labeled HSV-1 prepared as described above, was mixed with SDS, 0.5%, nuclease-free Pronase B, 1 mg/ml (Calbiochem), and incubated at 37°C for 60 min. Following two extractions with Tris buffer (pH 7)-saturated phenol, the DNA was mixed with CsCl (Optical grade, Harshaw Corp.) in 0.01 *M* Tris–HCl, pH 7, 0.001 *M* EDTA, adjusted to a density of 1.728 g/cc, and centrifugation performed in an SW 50.1 rotor, at 30,000 rpm, 18°C for 72 hr. Fractions were collected from the bottom and counted in a Beckman LS-250 liquid scintillation counter. Refractive indices were determined on an Abbe Refractometer and density calculated from tables relating refractive index to density.

Adsorption and penetration of susceptible cells. Twenty-four hours prior to infection confluent Vero cells were overlaid with growth media (M199-10% FBS) 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 7.4–0.15 *M* NaCl) and a 0.05-ml aliquot precipitated with 20% trichloroacetic acid. Radioactivity representing adsorbed virions was determined in a Beckman LS-250 liquid scintillation counter.

The remaining cells were diluted with reticulocyte standard buffer (RSB; 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. Nuclei were separated from cytoplasm by centrifugation at 800 *g* for 10 min, and nuclear radioactivity determined following precipitation in TCA.

Reagents. Hematoporphyrin hydrochloride (Rousell Corporation) was purified with acetic-sulfuric acid according to the method of Gregorie (19). A stock solution of 5 mg/ml was made by dissolving the pigment in 1 *N* NaOH, diluting with 0.9% NaCl and neutralizing to pH 7.4 with HCL. The hematoporphyrin was stored at 4°C in the dark and filtered (0.2)μm Nalgene filter before use.

Illumination procedure. A 300-W Kodak 760H slide projector was used to illuminate viral solutions for varying time periods. Intensity was determined with a calibrated YSI-Kettering Model 65 Radiometer, and spectral irradiance was varied by adjusting the distance between light source and virus. During illumination viral solutions were maintained between 18 and 21°C.

Anaerobic treatment system. Oxygen-free gas was obtained by passing a mixture of gas (10% carbon dioxide and 80% prepurified argon, Med-Tech Gases) sequentially over a palladuim catalyst (Engelhard Industries) at room temperature and through a column of copper wire heated to 400°C.

Results. Inactivation of herpes simplex viruses by hematoporphyrin and light. A cloned clinical isolate of HSV-1 (2×10^7 PFU/ml) was mixed with a solution of hematoporphyrin derivative (HPD) to a

final concentration of 7×10^{-5} *M*, irradiated with white light (26 mW/cm^2) for 1 min and then assayed by the plaque titration method. Plaque tests were performed in parallel on untreated HSV-1, irradiated HSV-1, and a suspension of HPD and HSV-1 protected from light (Fig. 1). HPD does not inhibit the infectivity of HSV-1 for susceptible cells if the mixture is continuously shielded from light. A short period of photoirradiation results in complete loss of plaque-forming ability in the virus–HPD mixture. Two additional cloned isolates of HSV-1 are also completely inactivated under these same conditions. To establish that the effect observed was not due to porphyrin-mediated damage of susceptible cells during the plaque test, a solution of HPD in media (7×10^{-5} *M*) was irradiated at 26 mW/cm^2 for 1 min, and incubated with Vero cells for 2 hr in the dark. Viral titration was then performed in the absence of light and no reduction in plaque-forming ability was noted (not shown). This indicated that the plaque inhibition observed after photoirradiation of HSV is not due to an alteration in the susceptible cell, but is indeed, an antiviral effect.

Effect of hematoporphyrin concentration and intensity of light. To establish a dose–inactivation relationship for HPD, increasing concentrations of the porphyrin were mixed with HSV-1, and illuminated with a light source adjusted to an irradiance of 26 mW/cm^2. As shown in Fig. 2A, as the molarity of HPD increases there is an exponential decline in the percentage of surviving infectious particles. Viral inactivation did not occur at any dose level in the absence of light.

When the concentration of HPD is held constant at 7×10^{-5} *M*, the photoinactivation of HSV-1 occurs exponentially as a function of the intensity of white light (Fig. 2B). Even at the highest intensity

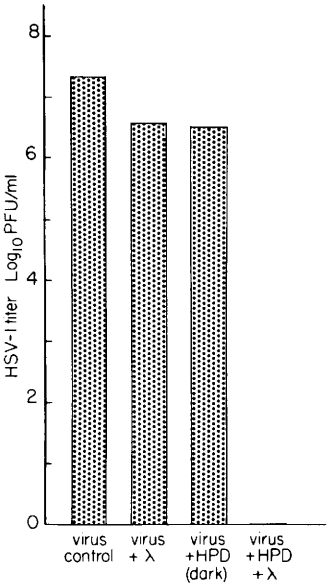

FIG. 1. Inactivation of HSV-1 with hematoporphyrin and light. A clinical isolate of HSV-1 (2×10^7 PFU/ml) was mixed with hematoporphyrin derivative (HPD, 7×10^{-5} *M* final concentration) and irradiated with white light (λ) at 26 mW/cm^2. The treated virus was then assayed in the dark by the plaque titration technique, as described under Materials and Methods. In parallel, untreated HSV-1, virus mixed with HPD in the dark, and virus exposed to light only at 26 mW/cm^2 were assayed for infectivity.

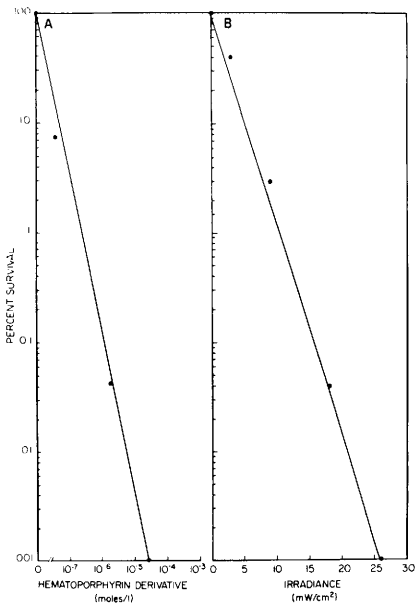

FIG. 2. Effect of hematoporphyrin concentration and light intensity on photodynamic inactivation. (A) 7×10^6 PFU/ml HSV-1 was mixed with increasing concentrations of HPD, and exposed to visible light at 26 mW/cm^2 spectral irradiance. (B) 10^6 PFU/ml HSV-1 was mixed with HPD to a final concentration of 6×10^{-5} *M*, and 1-ml aliquots were irradiated with visible light at different levels of spectral irradiance.

employed, no viral inhibition is observed in the absence of HPD. These observations emphasize the dependence of the HPD plus light inactivation of HSV on dye concentration and light intensity.

Oxygen enhancement of photodynamic inactivation of HSP-1 by HPD. The importance of molecular oxygen to photodynamic inactivation of HSV was investigated by irradiating a dye–virus mixture under anaerobic conditions. Suspensions of HPD (6×10^{-4} *M*) and HSV-1 (4.4×10^6 PFU/ml) were deoxygenated by bubbling a mixture of oxygen-free gases (10% CO_2–80% argon passed through a column of copper wire at 400°C) through a light-shielded solution of HPD and HSV-1 for 45 min. The solutions were then irradiated with 0.5, 3, 9, 18, and 26 mW/cm^2 of light for 1 min each while being continuously deoxygenated. Plaque-forming ability of treated virions was determined, and compared to photoinactivation in ambient air employing identical conditions of irradiation (Fig. 3). At all levels of illumination studied, viral inactivation was observed under anaerobic conditions. However, in the presence of ambient O_2, irradiation resulted in a more extensive antiviral effect.

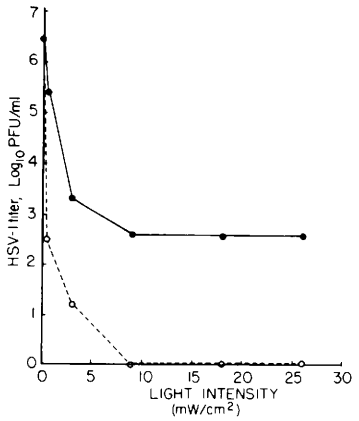

FIG. 3. Photodynamic inactivation under anaerobic and aerobic conditions. A clinical isolate of HSV-1 (4.4×10^6 PFU/ml) was suspended in a solution of hematoporphyrin derivative (HPD) to a final concentration of 6×10^{-4} *M*. Six 1-ml aliquots were deoxygenated by bubbling a mixture of oxygen-free gases through the light-shielded tubes for 45 min. The solutions were then irradiated with 0.5, 3, 9, 18, and 26 mW/cm^2 for 1 min. In parallel, six HPD–HSV-1 mixtures were irradiated at these wavelengths, under ambient air conditions. Viral titers were determined by plaque titration. ●, Deoxygenated virus-HPD suspension; ○, virus-HPD suspension in ambient air.

HSV-1 antigen expression in infected cells. When Vero cells are infected with HPD plus light-inactivated virions, they fail to demonstrate any cytopathic effects, and do not produce infectious progeny. To determine whether these cells express HSV-1-specific products, immunofluorescent studies of infected cells were undertaken. Untreated HSV-1 (5×10^6 PFU/ml) or virions completely inactivated with HPD (7×10^{-5} *M*) and light (26 mW/cm^2 for 60 sec) were employed to infect confluent Vero cells, at an m.o.i. of 10 (based upon preinactivation titer). Eighteen hours postinfection the infected cells were fixed in acetone, and an indirect fluorescent-antibody test performed using HSV-1 specific antisera. As shown in Fig. 4, cells infected with virions exposed to HPD without light, or those left untreated express cytoplasmic fluorescence consistent with the presence of HSV-1-induced antigens. Cells infected with photodynamically inactivated virus demonstrate no viral-specific fluorescence, and appear no different than uninfected cells.

Adsorption and penetration by inactivated virions. The inability of inactivated virions to induce virus-specific antigens suggests the possibility that treated particles are not capable of initiating an infection. The early stages of viral infection were investigated by determining the effects of photodynamically inactivated [^{3}H]-thymidine-labeled HSV-1 on adsorption and penetration of susceptible cells.

To establish that the tritium was actually labeling viral DNA, an aliquot of [^{3}H]HSV-1 was concentrated by centrifugation at 100,000 *g*, lysed and DNA subjected to isopycnic centrifugation for 72 hr. The resulting peak of [^{3}H]thymidine at a density of 1.728 g/cc confirmed that the label is in viral DNA (not shown).

Twenty-four hours prior to infection, Vero cells were incubated with cytosine arabinoside (100 μg/ml) in order to inhibit uptake of [^{3}H]thymidine by cells during DNA synthesis. At the time of viral inocu-

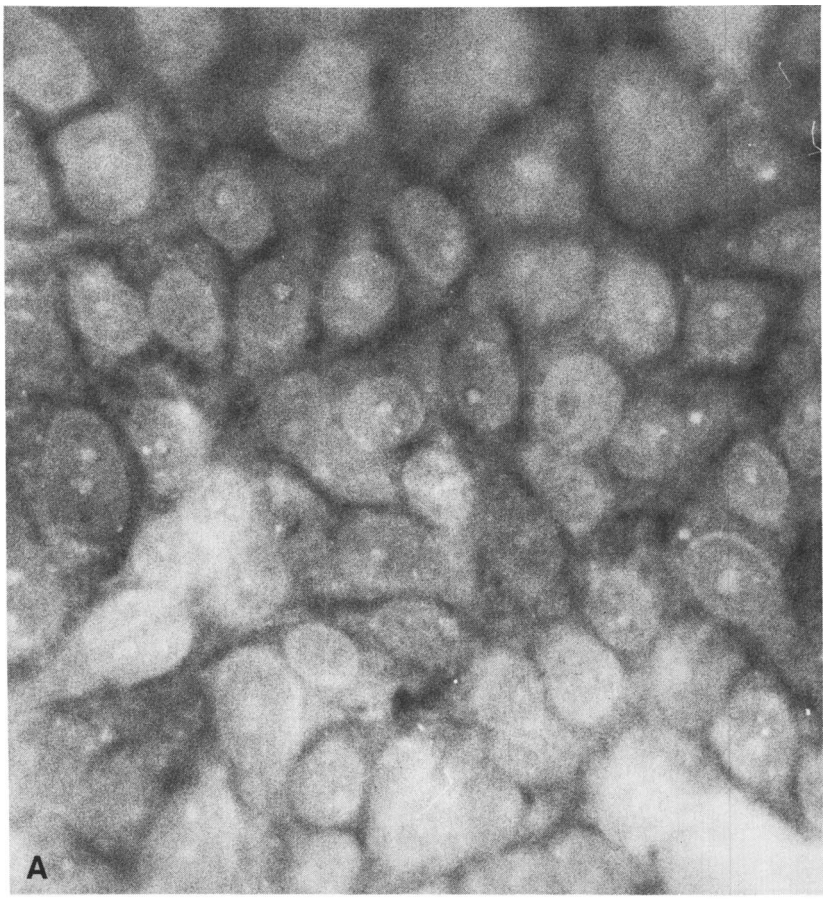

FIG. 4. HSV-1 antigen expression in infected cells. Confluent Vero cells were infected with HSV-1 and subjected to an indirect fluorescent-antibody test employing HSV-1-specific antisera. (A) Uninfected cells; (B) HSV-1, at an m.o.i. of 10; (C) HSV-1 + HPD (7×10^{-5} *M*) no light; (D) HSV-1 + HPD (7×10^{-5} *M*) plus light.

lation the thymidine pool was expanded with addition of unlabeled base. The cells were infected with either untreated [^{3}H]HSV-1, or virions completely inactivated by HPD + light, at a preinactivation m.o.i. of 10. At 0, 0.5, 1, 3, and 5 hr postinfection, radioactivity adsorbed to intact cells was determined. Nuclei were then separated from the cytoplasm of remaining cells and nuclear penetration by [^{3}H]HSV-1 DNA quantitated (Table 1).

Untreated HSV-1 rapidly adsorbs to Vero cells, and viral DNA penetrates to the nucleus maximally at 3 hr postinfection. Photodynamically inactivated virions (HPD, 7×10^{-5} *M*; 26 mW/cm^2 for 60 sec) neither adsorb to, nor penetrate susceptible cells.

Discussion. Photodynamic inactivation of bacterial and animal viruses has been most extensively studied using heterotricyclic dyes (2, 5). Although the mechanisms of the antiviral effect are not completely understood, methylene blue-associated inactivation of bacteriophage T4 is believed to interfere with the phage injection apparatus, and phage DNA replication (20). Evidence has been presented that herpes virions subjected to PDI using low doses of proflavine and light are inhibited at a step subsequent to viral DNA synthesis (21).

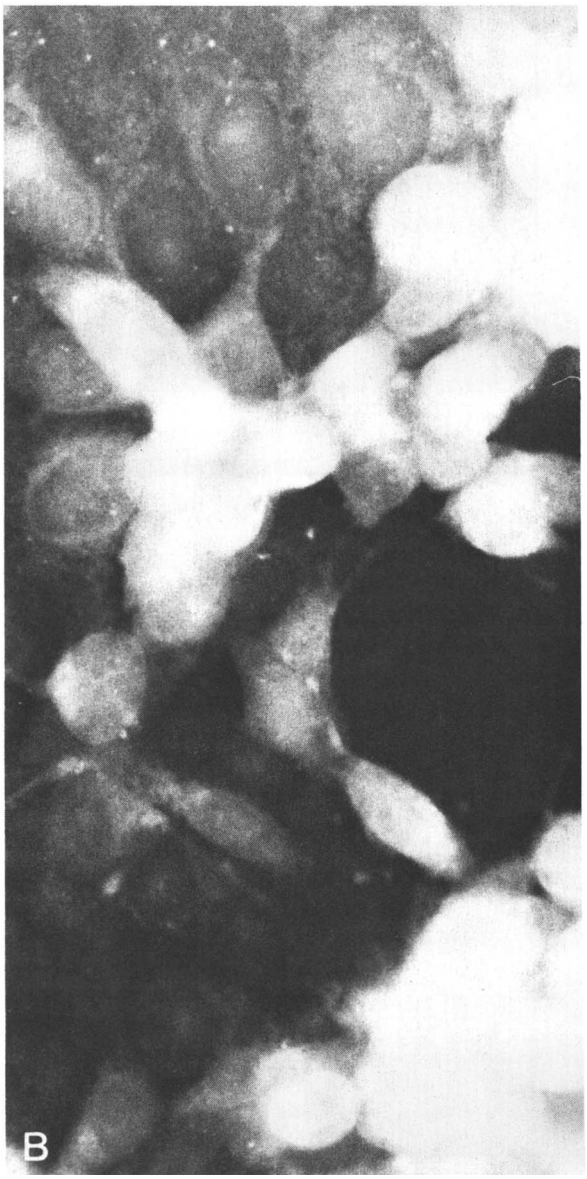

FIG. 4—*Continued.*

Photooxidative damage by porphyrins has been described in a variety of nonviral biological systems (22), but the precise molecular targets are not known. It has been suggested that porphyrins sensitize the photooxidation of small organic molecules, lipids, and certain amino acids under aerobic conditions, and produce singlet oxygen as a reactive intermediate (11, 12). In addition, hematoporphyrin derivative has been demonstrated to induce single-strand breaks in eukaryotic DNA following photoirradiation (23).

When 7×10^{-5} *M* hematoporphyrin derivative is mixed with HSV-1 and irradiated, the plaque-forming ability of the virus is completely abolished. The effect requires both HPD and light since omission of either one results in no reduction in infectivity. This is a specifically antiviral effect because

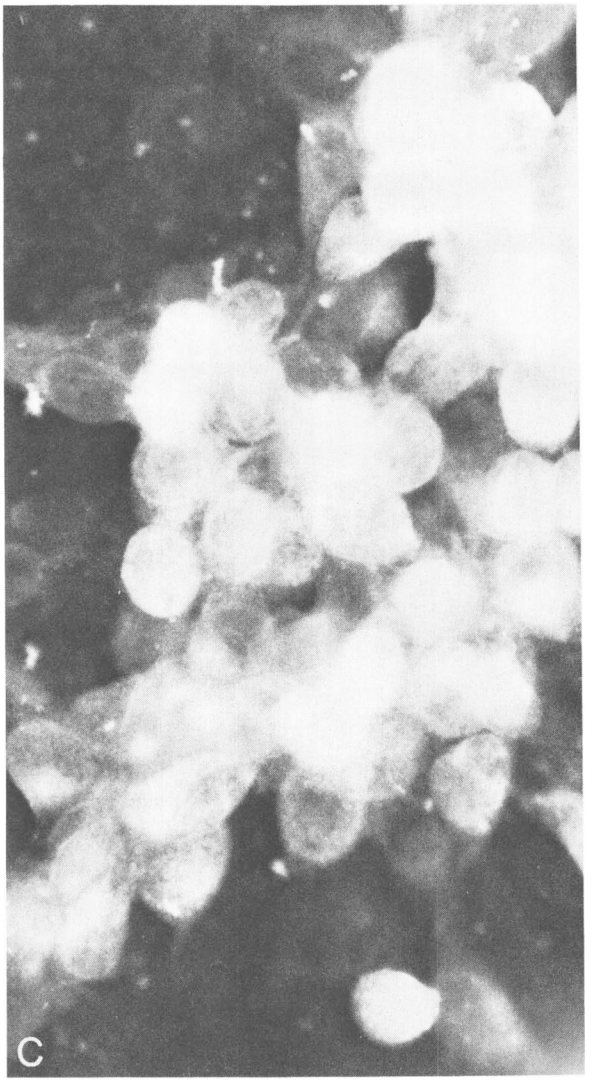

FIG. 4—*Continued.*

photoirradiation of a virus free solution of HPD which is subsequently incubated with light-shielded susceptible cells, does not inhibit HSV-1 plaque formation upon subsequent infection.

Photodynamic inactivation of HSV-1 varies with incremental concentrations of HPS. There appears to be an exponential reduction in surviving infectious particles as the molarity of HPD increases. Alternatively, at fixed levels of HPD concentration there is an exponential decline in viral infectivity with increasing light intensity until complete photodynamic inactivation occurs.

Another factor important to this antiviral effect is the presence of oxygen. In the presence of ambient O_2 concentrations, 100% of the plaque-forming ability of HSV is abolished. A substantial antiviral effect is also observed under anaerobic conditions, although a small fraction of input virus remains infectious despite the use of increasing light intensities. A possible explanation for the viral inactivation observed following deoxygenation is the presence of small

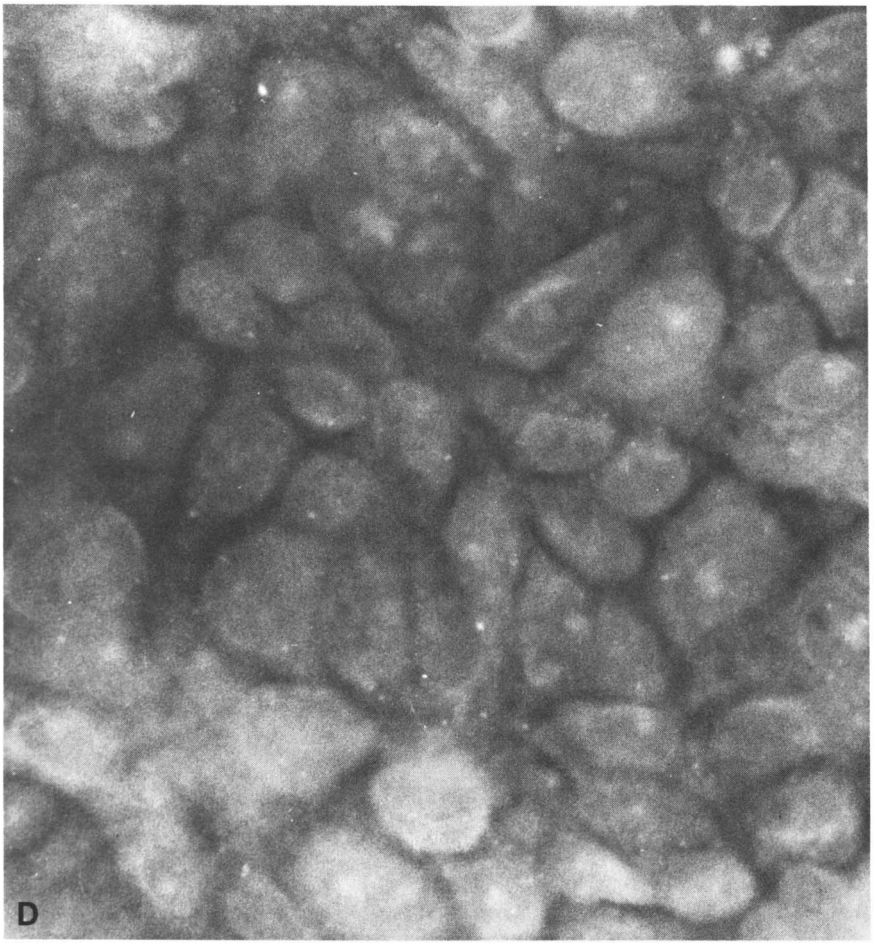

FIG. 4—*Continued.*

TABLE I. ADSORPTION AND PENETRATION OF RADIOLABELED HSV-1 FOLLOWING PDI

Input virus[a]	Time[b] (hr)	Adsorbed cpm[c]	Nuclear penetration[d] (cpm)
Untreated	0	200	73
	0.5	910	400
	1	1280	813
	3	2440	1438
	5	2390	1610
HPD + light inactivated	0	235	62
	0.5	211	40
	1	190	55
	3	220	70
	5	187	38

[a] Confluent Vero cells were grown in 100 μg/ml cytosine arabinoside and subsequently infected with untreated or inactivated [^{3}H]thymidine HSV-1, at a pretreatment m.o.i. of 10. Virions were inactivated as in Fig. 1.
[b] Hours postinfection.
[c] TCA-Precipitable [^{3}H]thymidine cpm associated with washed infected cells.
[d] TCA-Precipitable [^{3}H]thymidine cpm associated with nuclei of infected cells.

amounts of residual oxygen in the system. However, in a less rigorous anaerobic system, replacing O_2 with N_2 during studies on the photooxidation of NADPH by hematoporphyrin resulted in almost complete inhibition of the oxidative effect (15). An alternative possibility is that the photoreduced hematoporphyrin free radical can interact with virions in the absence of O_2, either directly or through radical intermediates, and thereby reduce infectivity (16). These studies do not identify the reactive intermediate(s) generated under aerobic or anaerobic conditions, which may specifically mediate the anti-HSV effect. However, in the presence of ambient O_2 singlet oxygen is the most likely candidate (15). Detailed studies of the photooxidation of NADPH by hematoporphyrin dichloride suggested this species as an important mediator of this biochemical process, and excluded hydroxyl free radicals, hydrogen peroxide and superoxide anion (15). Elucidation of the intermediate(s) essential to the HPD associated inactivation of HSV awaits studies employing specific inhibitors of various reactive species to protect the virus.

Virions that have been photodynamically inactivated with HPD do not induce cytopathic effects in susceptible cells. Immunofluorescent studies with specific viral antisera fail to demonstrate the induction of HSV-1 antigens following infection of cells with virions exposed to PDI. Virus mixed with HPD but shielded from light induces antigen expression identical to that of untreated virus. These findings suggest a viral target for HPD plus light that is important to the early stages of infection. Studies employing thymidine-labeled HSV-1 appear to confirm this, since inactivated particles fail to adsorb to, or penetrate, nuclei of susceptible cells.

Herpes virions contain lipid and protein in their envelope, and it is this structure that is believed to facilitate adsorption and penetration of infected cells (4). Porphyrins have been shown to form radicals in a lipid milieu, which leads to lipid peroxidation (16). Similarly, proteins and amino acids are vulnerable to photooxidation by these tetrapyrroles. Although these studies do not identify the envelope as a specific target for the inactivation of HSV with hematoporphyrin, they are consistent with the introduction of photochemical damage at the viral surface that interferes with initiation of infection.

This investigation does not address the possibility that HPD plus light is inducing viral DNA damage. Should such damage occur, it cannot explain the failure of treated virus to adsorb to cells, and therefore appears to be minimally important for the antiviral effect observed when treating cell-free virus. However, if structural changes in DNA are produced by PDI, it is possible that defective particles may be generated. Studies are currently underway to define the effects on viral nucleic acid of PDI of both cell-free virus and HSV-infected cells, as well as to determine whether HPD plus light treated virus demonstrates an enhanced transformation frequency.

Summary. A modification of photodynamic inactivation of herpes simplex viruses (HSV) has been developed employing hematoporphyrin derivative (HPD) and visible light. Isolates of HSV Type 1 rapidly and completely lose their plaque-forming ability when a mixture of virus and HPD is exposed to light. This effect is dependent upon optimal concentrations of the tetrapyrrole, light intensity, and the presence of molecular oxygen. Photodynamically inactivated virions do not cause viral cytopathic effects following infection of susceptible cells, and fail to induce viral antigens. The treated particles are unable to adsorb to or penetrate cells, and therefore cannot initiate an infection. These observations are consistent with photooxidative damage to the viral envelope occurring through the interaction of HPD plus light.

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