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Photodynamic inactivation of influenza and herpes viruses by hematoporphyrin

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Summary

Hematoporphyrin (HP), at concentrations as low as 0.5 μg/ml, was found to inhibit the in vitro replication of influenza A and herpes simplex viruses, but not of several other viruses. The effect required exposure of the viruses or cells to visible light and was demonstrable when HP was administered shortly before virus inoculation or during the infection.

In studies on the mechanism of action of HP, we found that in the presence of light, HP caused decomposition of GMP but not of various other nucleosides. It caused breakdown of yeast tRNA and inhibited polymerization of RNA and DNA by influenza virus and HSV-1-specific polymerases as well as some other polymerases isolated from bacterial and mammalian sources.

Protective effects of HP and light were demonstrable in embryonated eggs infected with the WSN and PR8 strains of influenza A virus and in mice infected with the WSN strain. HSV-1-induced keratitis in rabbits and HSV-2-induced dermatitis in mice were not responsive to HP treatment.

hematoporphyrin; influenza; herpes; nucleotides; polymerase; therapy

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Introduction

Herpes simplex virus (HSV) and varicella-zoster virus can be inactivated by neutral red or proflavin in the presence of light and this effect has been applied with relative success for the treatment of localized superficial lesions [3]. Similar photoinactivation of other animal viruses using other agents has also been reported [11]. In studies with acridine organe it was shown that the dye binds to the guanine-containing portions of the viral nucleic acid [10]. Exposure to light results in excision of these moieties, leaving single-stranded gaps [4].

Hematoporphyrin (HP) is a pigmented, iron-free natural breakdown product of hemoglobin. In the presence of light it has cytotoxic activity, which results from singlet oxygen generated by energy transfer from the light-excited pigment to molecular oxygen [2,12]. In the presence of light, HP also inhibits reverse transcription in vitro from an exogenous template by the RNA-dependent DNA polymerase of Moloney leukemia virus. The inhibition would result from interaction with the enzyme rather than with the template [9].

In this paper we report the photodynamic inactivation by HP of influenza and herpes simplex viruses.

Experimental

Primary chick embryo fibroblasts (CEF) were prepared from 11-day-old embryonated eggs by dispersion with 0.25% trypsin. Cells were grown in medium E-199, supplemented with 10% tryptose phosphate broth, 4% calf serum (CS), 1% chicken serum, and antibiotics (penicillin 100 units/ml, streptomycin 0.1 mg/ml, fungizone 2.5 μg/ml, and mycostatin 25 units/ml). Vero cells were received from Microbiological Associates and grown in minimum essential medium (MEM) supplemented with 10% FCS and antibiotics, as above. Influenza virus A strains WSN (H_0N_1), PR8 (H_0N_1), and Aichi (H_3N_2) were kindly supplied by Dr. Robert G. Webster, St. Jude Hospital, Memphis, Tennessee, U.S.A. Virus stocks were prepared in the allantoic sac of 11-day-old embryonated chicken eggs. Herpes simplex virus type 1, strain Patton, and Herpes simplex virus type 2, strain 333, were grown in primary rabbit kidney cells with MEM supplemented with 2% FCS.

Confluent CEF or Vero cells were grown, as described above for the plaque assay of influenza virus or herpes viruses, respectively. Growth medium was removed from the cells and 0.5 ml of the appropriate virus dilution in the presence of HP was added to duplicate plates. Following a 1-h adsorption period, a growth medium containing agarose (0.3%) or methylcellulose (0.5%) and HP was added and cells were incubated at 37°C for 2–3 days. The overlay was decanted, the cells were stained with 0.5% crystal violet and the virus-induced plaques counted. Significant inhibition of influenza virus and HSV-1 induced plaques (≥95%) was observed with HP concentrations as low as 0.5 and 1.0 μg/ml, respectively. Other viruses (vaccinia, poliovirus type 1, rhinovirus type 2, adenovirus type 12 and measles virus) were not inhibited at HP concentration of 10 μg/ml (data not shown).

The nature of the selectivity is unknown at present.

In order to determine the cytotoxicity of HP, primary CEF cells were seeded in 60-mm petri plates (1.5×10^6 cells/plate). After 3 days of incubation, fresh media containing various HP concentrations were added. Every 24 h cells were removed with trypsin (0.25%) and counted. No cytotoxicity could be detected with 5 μg/ml. Inhibition of 50% of cells was detected with 25 μg/ml within 24 h. No cell replication was detected at 50 μg/ml. Thus, a selective index of 100 for the antiviral activity of HP was determined by the ratio of the cytotoxic concentration (50 μg/ml) to the virus-inhibitory concentration (0.5 μg/ml).

In order to study the light dependency of virus inactivation by HP, influenza virus A/WSN or HSV-1 were incubated with or without 2.5 μg/ml of HP at room temperature. Half of each mixture was kept in the light (fluorescent lamp) and the second half was kept in the dark. Samples of virus were collected at the appropriate time intervals and assayed for plaque forming ability under light or dark

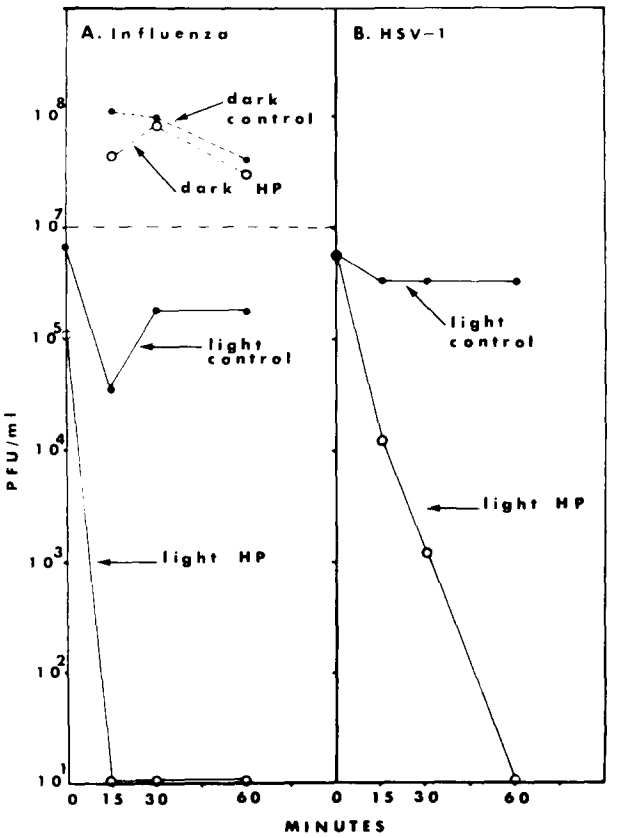

Fig. 1. Light-dependent inactivation of influenza virus and HSV by HP. Influenza virus A/WSN and HSV-1 (strain Patton) were incubated with or without HP (2.5 μg/ml) at room temperature under light and dark conditions. Virus samples were collected at selected time intervals and assayed for their ability to form plaques in light or in dark conditions.

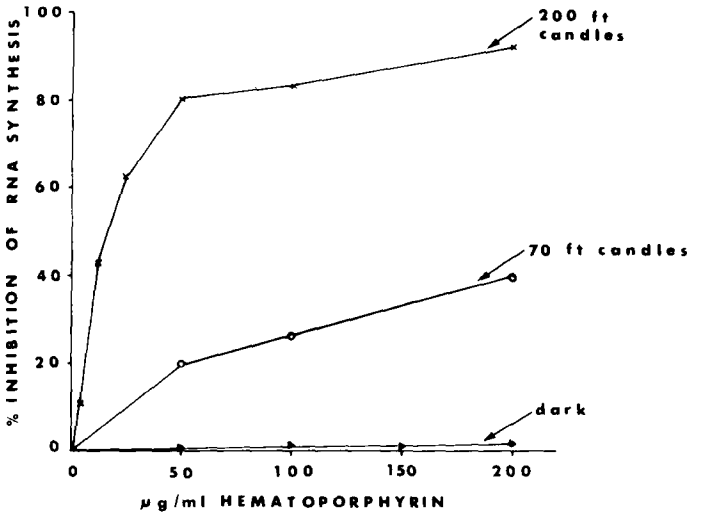

Fig. 2. Light-dependent inhibition of influenza virus RNA-dependent RNA polymerase by hematoporphyrin. Various amounts of HP were added in dark to the reaction mixtures. Samples were then exposed to various light intensities at 37°C. After 1 h, the radioactive, acid-precipitable material was collected and counted. The control value (no HP) of RNA synthesis was 19500 cpm.

conditions. As shown in Figure 1, influenza virus was completely inactivated within 15 min of incubation with HP in visible light. HSV-1 required 60 min for complete inactivation. Neither virus was inactivated when incubated with HP in the dark or without HP in the light. In addition, CEF cells were also treated with HP (10 μg/ml) for 1 h in the presence of light, prior to infection with influenza virus A/WSN. The HP was then removed, the cells were washed and the virus was added. Following virus adsorption, agar overlay without HP was added and the cultures were incubated at 37°C in light. The results showed that viral plaque formation was inhibited 99.9% under these conditions.

In order to investigate the mechanism of action of HP, the effect on influenza virus RNA polymerase was tested. Various amounts of HP were added in the dark to the reaction mixtures of 50 μl containing 50 mM Tris-HCl, pH 7.8, 1mM DTT, 10 mM KCl, 0.2% Triton, 5 mM $MgCl_2$, 0.5 mM each of ATP, CTP, and GTP, 1 μCi of [^{3}H]UTP, 0.1 mM ApG, and 10 μl of purified virus. Samples were kept in dark or exposed to various light intensities obtained from a 500 W light bulb placed at various distances and measured by light meter (Lunapro). After 1 h at 37°C, the radioactive material was precipitated by 10% TCA on a Millipore filter and counted in the liquid scintillation counter. The results in Figure 2 demonstrate that HP inhibited RNA sythesis when light was present. The degree of inhibition was related to light intensity. No inhibition was detected when samples were kept in the dark even at HP concentration as high as 200 μg/ml. We then studied the effect of HP on the nucleotides, AMP, CMP, GMP, UMP, and TTP. Each nucleotide (0.23 mM) was incubated with HP (0.5 mM) in 10 mM sodium phosphate buffer (pH

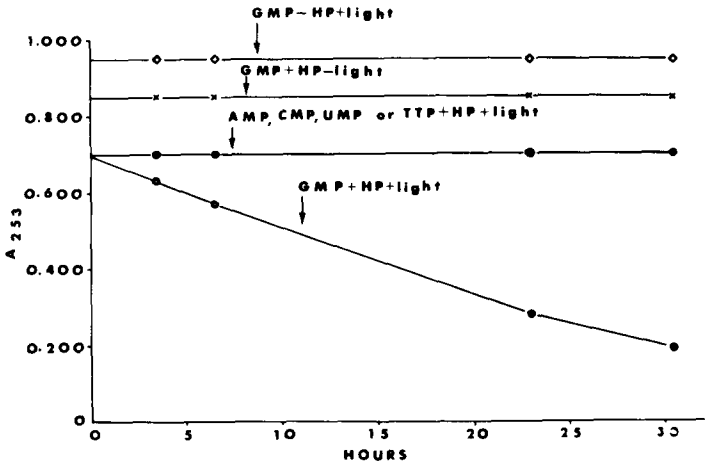

Fig. 3. Selective decomposition of GMP by hematoporphyrin and light. Various nucleotides (0.23 mM) were mixed with 0.5 mM HP in 10 mM sodium phosphate buffer, pH 7.2 and incubated at 21°C with light (200 foot candles) or in the dark. At time intervals, aliquots were taken and diluted 4-fold with 10 mM sodium phosphate buffer. The spectrum of each solution was scanned between 240 and 340 nm using a Cary 14 spectrophotometer with an appropriately diluted HP solution as the blank. Readings at wavelength of 253 nm were plotted against time.

7.2) at 21°C in the dark or with light (200 foot candles). At appropriate intervals, aliquots were collected and diluted 4-fold with the phosphate buffer. The spectrum of each solution was scanned between 240 and 340 nm. The changes at 253 nm are plotted and shown in Figure 3. GMP was the only nucleotide decomposed by HP and this decomposition was light dependent.

We also determined the effect of HP on intact RNA. The reaction mixture (0.2 ml) contained 1 mg of yeast tRNA and 0.5 mM HP in 10 mM sodium phosphate (pH 7.2). One sample was kept in the dark and the other was exposed to light (200 foot candle) for 24 h at 21°C. The reaction mixtures were layered on linear sucrose gradients (5–20% w/v) in 10 mM phosphate buffer and centrifuged at 40000 rpm in a SW41 rotor for 43 h at 20°C. Fractions were collected by bottom puncture and absorbance of 260 mM was determined. As shown in Figure 4, HP in the presence of light caused the breakdown of tRNA as expressed by the reduced amount in the peak and the shift of the peak to a lower density.

If the decomposition of guanosine is the mechanism of action, then HP should be a universal inhibitor for DNA as well as RNA synthesis. To determine this, the effect of HP on various RNA and DNA polymerases was tested. DNA polymerases from *Escherichia coli*, herpes simplex virus type I, calf liver, and WI-38 cells were assayed as described by Mao and Robishaw [8]. DNA polymerase of hepatitis B virus was assayed according to Kaplan et al. [6] and *E. coli* RNA polymerase was assayed by the method described by Burgess [1]. The 50% inhibition of reaction with both *E. coli* and influenza RNA polymerases and four DNA polymerases (herpes virus, WI-38 cells, calf liver DNA polymerases alpha and gamma) was detected by HP at concentrations less than 25 μg/ml. However, two DNA po-

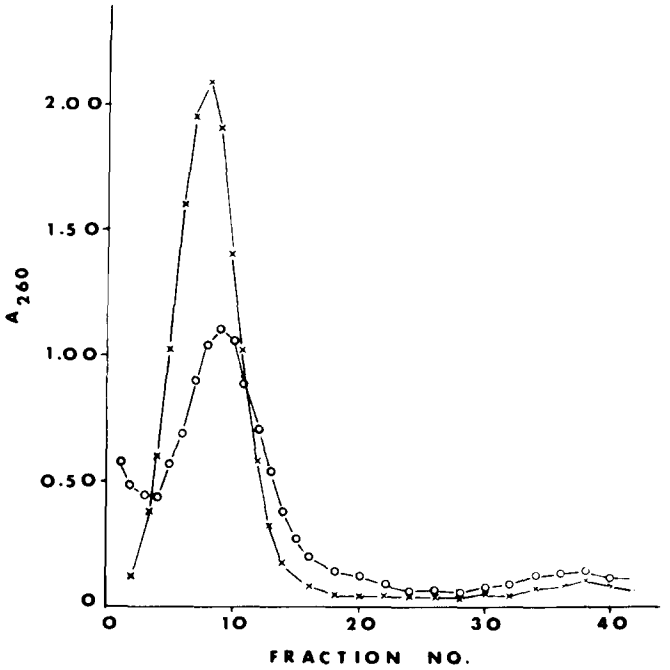

Fig. 4. Degradations of tRNA in the presence of hematoporphyrin and light. Reaction mixtures (0.2 ml) contained 1 mg of yeast tRNA and 0.5 mM HP in 10 mM sodium phosphate buffer, pH 7.2. One sample was kept in the dark (×—×). Another sample was exposed to light (200 foot candles) for 24 h (○—○). The reaction mixtures were layered onto linear sucrose gradients (5–20% w/v) in 10 mM sodium phosphate buffer, pH 7.2 and spun for 43 h at 20°C at 40000 rpm in the SW41 rotor. Fractions of 20 drops were collected from the bottom. Absorbance at 260 nm was read after dilution with 1.0 ml of 10 mM sodium phosphate buffer.

lymerases, hepatitis B virus polymerase, and *E. coli* polymerase were resistant to HP (100 or 200 μg/ml) in the presence of light. The nature of this resistance is not understood. However, at the present time we cannot conclude that decomposition of guanosine is the ultimate target in the biological activity of HP.

TABLE 1

Protection of chick embryos against influenza virus infection by hematoporphyrin[a]

Challenge virus	HP	Mortality	Virus yield (HA titer)
WSN	–	4/4	32, 64, 128, 128
(H_0N_1)	+	1/3	0
PR8	–	1/4	256, 0, 8, 32
(H_0N_1)	+	0/4	0

[a] Embryonated chicken eggs were inoculated with virus in the presence or absence of HP (1 mg/ml) and incubated at 37°C. At the time of death or after 9 days, the allantoic fluids were harvested and assayed in hemagglutination tests.

TABLE 2

Protection of mouse mortality induced by hematoporphyrin following influenza virus infection[a]

Treatment group	Mortality (%)	Average survival time (days)	Average lesion score	Average lung weight (mg)
Normal controls	0	>14	0.0	150
Infected controls	50	10.7	3.8	270
Amantadine				
200 mg/kg	10	13	3.33	180
100 mg/kg	0	>14	3.2	250
50 mg/kg	20	12.9	3.8	250
HP				
200 mg/kg	0	>14	1.4	240
100 mg/kg	10	13.5	1.2	230
50 mg/kg	70	10.1	3.6	230

[a] Groups of 10 mice were infected intranasally with influenza A/WSN virus (100 PFU). Daily doses of drugs were administered orally beginning one day prior to infection and continued for ten days after infection. The number of dead mice was recorded daily for 14 days after infection. On the 14th day surviving mice were killed and their lungs were analyzed for weight and lesion score (from 0 to 4). This is a representative of three experiments.

To determine the efficacy of the antiviral activity of HP in vivo, allantoic sacs of 9–11-day-old embryonated eggs were injected with 0.2 ml of influenza A virus (WSN or PR8) and 0.5 ml of HP (1 mg/ml). Controls without HP were also included. The eggs were incubated at 37°C for up to 9 days. Every 24 h after infection, the eggs were candled to detect mortality. At embryo death or after 9 days, allantoic fluids of all eggs were harvested. As shown in Table 1, HP inhibited death of the embryos and virus replication as determined by hemagglutination of 0.5% chick red blood cells. Also, groups of ten mice (strain CF-1, female) were infected intranasally with egg grown influenza virus A/WSN (100 PFU/mouse). HP solution (4 mg/ml, pH 7.2) was prepared in PBS. Amantadine (in 0.5% aqueous hydroxypropylmethyl cellulose) was used as the positive control. Uninfected and infected non-treated control mice were also included. A daily dose of 0.5 ml was administered orally beginning one day prior to infection and continuing until day 10 post-infection. Fourteen days after initiation of treatment, the surviving mice were killed and the lungs removed. The drug effect was evaluated by mortality, average day of death, lung lesion score (0 = no pathology apparent, 4 = all lobes consolidated), and average lung weight of surviving mice. As shown in Table 2, prophylactic administration of HP protected the infected mice from death as well as amantadine. In addition, the lung lesion score in HP-treated mice was reduced when compared to amantadine treated and virus-infected control animals, though no reduction in lung weight was detected with HP. However, when mice were infected with 1000 PFU/mouse and treated prophylactically with HP, or when mice were infected with 100 PFU/mouse but HP was administered therapeutically (1 or 2 days

after infection) no inhibition of mortality could be demonstrated (data are not shown).

Topical HP treatments against HSV-1-induced keratitis and HSV-2-induced dermatitis were applied as follows: The corneas of both eyes of anaesthetized New Zealand rabbits were abraded with a sterile swab and herpes simplex virus type 1 was applied. One eye was treated with 0.1 ml of 2.5% HP (pH 7.2) or 5% phosphonoacetate (PAA; Abbott). Four rabbits were used for each medication group. The drug treatments were started 2 h post-infection, and were repeated hourly for 4 h on the day of infection and for 8 h on each of 2 additional days. The eyes of the rabbits were examined each day for corneal lesions with fluorescein solution and a UV light and the size of the corneal lesion was used as an index of efficacy. HP treatment resulted in temporary improvement of lesions. However in contrast to PAA, no cure was obtained. HP was toxic to the eyes at a concentration of 5%.

Anaesthetized female CF mice had a 10 mm^2 area of their back plucked free of hair and herpes simplex virus type 2 (50 μl) was applied. The surface of the skin beneath the virus drop was pricked. The drug HP (2.5%) or PAA (2%) was applied to the site 2 h later and the skin was pricked again. The drugs were then applied twice daily for 5 consecutive days. Vesicles developed in 3 to 5 days followed by paralysis and death in 11 to 15 days. Mortality was used as a measure of the efficacy of the drug. 60% of the mice survived after treatment with PAA after 14 days of treatment. HP treatment delayed death by 2 days but eventually 90% of the mice died within 17 days.

All the in vitro biological activities of HP reported above were found to be light-dependent. In vivo, HP inhibited influenza virus replication in infected chick embryos. This was probably the result of direct inactivation of the virus when the embryonated eggs were exposed to visible light during the candling procedure. The compound was also effective in the mouse protection test, reducing the mortality and morbidity of influenza virus-infected mice. The protection could only be demonstrated when HP was administered orally and prior to infection with a low dose of influenza virus. No protection was obtained when HP was given after virus infection. Therefore it may be suggested that the observed protection was a result of viral inactivation in the upper respiratory tract during the brief exposure to light by HP which was available due to the prophylactic treatment. On the other hand, HSV-1-induced keratitis and HSV-2-induced dermatitis were not responsive to HP in spite of the fact that the virus-induced lesions in this study were readily accessible to visible light. One possible explanation for this negative result is that, as is known [5], HP penetrates normal skin and epithelial cells poorly. The drug was added topically 2 hours post infection. By that time the virus had already penetrated the cells. Herpes virus was less sensitive to direct inactivation by HP than influenza. Complete inactivation required 60 min of incubation of the virus with the drug in the light. It is possible that the combination of low cell penetration of the drug, the rate of clearance of the drug form the lesion and the slow inactivation of virus may have contributed to the low efficacy. Studies with prophylactic administration of the drug were not done. Also, systemic routes of administration of the drug may improve the accessibility of the drug to the virus and may thereby increase efficacy.

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