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Inactivation of Herpes Simplex Virus with Methylene Blue, Light and Electricity (40521)

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The use of photoactive dyes and light energy to inactivate living organisms has been known since 1900 when Rabb inactivated paramecia with low concentrations of acridine dyes and visible light (1). This process, called photodynamic inactivation, was applied to viruses in 1933, when methylene blue, light and oxygen were shown to inactivate vaccinia, herpes simplex, and fowl-plaque viruses (2–5). In addition, bacteriophage and other DNA-containing animal viruses have been rendered noninfectious by photodynamic treatment (6). The use of photosensitive compounds to inactivate herpes viruses has been intensively studied by Melnick and co-workers (7–9). The substances employed include the heterotricyclic acridines, proflavine or acridine orange, or phenazine dyes such as neutral red. When herpes viruses are exposed to visible light in the presence of these photo-sensitizing dyes and molecular oxygen, they lose their plaque forming ability. In addition to inhibiting the infectivity of herpes viruses, Rapp and co-workers have shown that either photodynamic inactivation with the heterotricyclic dye neutral red, or with ultraviolet light, facilitates herpes simplex virus-induced transformation of mammalian cells (10).

The precise mechanism leading to herpesvirus inactivation is not completely understood, but in virus infected fibroblasts treated with proflavine and light, Kahn *et al.*, have shown that a marked decrease in virion particle production occurs in the absence of appreciable inhibition of viral or cellular DNA synthesis (11). Some dyes, such as methylene blue or toluidine blue, when illuminated appear to preferentially destroy guanine residues during photo-autoxidation (12, 13). Alternatively, the acridines have been demonstrated to intercalate between the base pairs of DNA (14) and, following absorption of light energy, the dye-DNA complex produces an oxidation reaction. When raised to excited energy states by light, the sensitizer can interact with molecular oxygen to form singlet oxygen, which can then react with various acceptors in solution (15). Methylene blue (MB) is believed to form singlet oxygen when electronically excited and reduced methylene blue has been demonstrated to generate superoxide anion (16). Superoxide anion can then generate products such as hydrogen peroxide and hydroxyl free radicals (OH) which are likely to be mediators of biological damage (17–19). The purpose of this communication is to report that the simultaneous application of an electrical current and visible light to a solution of herpes simplex viruses (HSV) and methylene blue results in rapid viral inactivation. This effect is significantly inhibited by superoxide dismutase and therefore appears to be partially dependent on superoxide anion.

Materials and methods. Reagents. Methylene blue, reagent grade, was obtained from Sigma Chemical Co., and stored in the dark until ready for use. Superoxide dismutase was obtained from Sigma, and stored in the lyophilized state at −20°. Immediately prior to its use, an aqueous solution having an activity of 1595 units/ml was prepared. In the assay employed (20), one unit is defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome C by 50%.

Cells and media. Vero and CV-1 cells were obtained from the American Type Culture Collection and propagated in Medium M199 (Gibco) and supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) 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 Hayflicks' media (21).

Viruses. The McIntyre strain of HSV-1, Strain 333 of HSV-2 (a gift from Dr. F. Rapp), and isolates from patients with herpes

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labialis and herpes genitalis were employed. All virus strains were shown to be either HSV-1 or HSV-2 by microneutralization techniques (22). Virus pools were prepared by low multiplicity inoculation (10^{-2} PFU/cell) of confluent Vero cells and were harvested from infected cultures at 72–96 hr postinfection. When assayed by the plaque titration method these pools had initial titers of 4×10^6 PFU/ml.

All plaque titrations were carried out in 25 ml Falcon flasks containing a confluent monolayer of CV-1 cells. Serial 10-fold dilutions of virus samples were made in M199, the media removed from the flasks, and each was inoculated with 0.5 ml of a viral dilution. The virus was adsorbed for 2 hr, and 5 ml of M199 containing 2% FBS and 7% methylcellulose were added as an overlay to each flask. After 5 days of incubation at 37° and 5% Co_2, the cells were fixed in 2 ml of acetic acid methanol (1:3 acetic acid:methanol) solution, and the plaques counted (10).

Dye, light and electrical inactivation of HSV. All inactivation studies were performed in methyl methacrylate cells fitted with platinum electrodes and packed in ice. Cell dimensions were 3.5 (*l*) × 3.8 (*h*) × 0.8 (*w*) centimeters (Fig. 1). The cells were sterilized by autoclaving prior to use. A constant light source was provided by a Mobilite incandescent tensor lamp maintained at a fixed distance of 20 cm from the methyl methacrylate chamber. The lamp has a measured spectral power output (measured by EEG 580-11 Radiometer) of 197 microwatts/cm^2 over the 350–800 nanometer band with 51 microwatts/cm^2 in the 600–700 nm band overlapping the action spectrum of methylene blue.

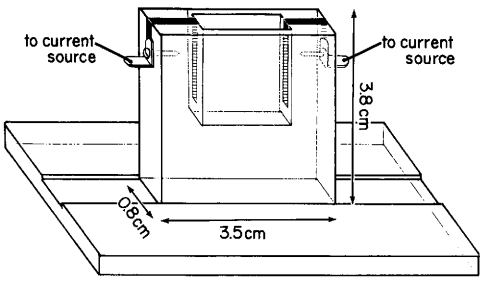

FIG. 1. Methyl methacrylate cell employed in viral inactivation. The actual dimensions and positions of platinum electrodes that attach to a Keithley 225 DC current source are shown.

For those studies involving electrical inactivation, a DC current source (Kiethly 225) was applied for varying intervals, such that the transsample voltage was 3 V.

In the studies employing the epinephrine - adrenochrome system, solutions of methylene blue (10^{-5} *M*) were prepared in matched quartz cuvettes in the presence of 2×10^{-4} *M* epinephrine, 1×10^{-4} *M* EDTA and phosphate buffered saline (PBS, pH = 7.4, 0.05 *M* phosphate). Electrical current was applied through platinum foil electrodes, and absorbance at 480 nm determined in a Gilford spectrophotometer.

Results. Inactivation of HSV by methylene blue, light and electricity. One ml of the McIntyre strain of HSV-1 (7×10^6 PFU/ml) was added to a methylmethacrylate cell and adjusted to a final concentration of 10^{-5} moles/liter of methylene blue. The electricity applied was 0.001 amperes for 3 min at 3 V and under these conditions the conductance of the solutions was stable at 8×10^{-2} mho's. Concomitant with the application of current, the cell was irradiated with a tensor lamp. The inactivation cell was packed in ice, and the temperature maintained constant at 8–9°C during the procedure. After 180 sec, an aliquot of virus was removed, appropriate dilutions made, and a plaque titration performed. Electricity or light, when employed alone or together resulted in no significant reduction in viral titer. Methylene blue (10^{-5} *M*) plus light resulted in a modest reduction in infectious virus (60%), whereas the application of a 2 mA current to a mixture of HSV-1, MB (10^{-5} *M*) and light reduced the titer of input virions by greater than 99.99% (Fig. 2). Similar studies using two clinical isolates of HSV-1 and the 333 strain of HSV-2 demonstrated the same enhancement of viral inactivation by MB, electricity and light as compared with dye plus light alone (data not shown).

Virus-free media exposed to methylene blue (10^{-5} *M*), light and electricity under identical inactivation conditions does not reduce the susceptibility of Vero cells to infection by untreated HSV-1. Under these conditions a subsequent virus infection results in a titer equivalent to that of the untreated control (data not shown).

Effect of coulombs (amperes × seconds) delivered and concentration of methylene blue.

The antiviral activity in this system is a function of both the concentration of dye employed and the electrical current delivered (Fig. 3). In the presence of a constant light source, and a fixed level of 1.0×10^{-4} coulombs transferred, the survival of infectious HSV-1 (McIntyre) falls as the concentration of MB is increased from 0, to 10 μM and then to 2 mM. For each concentration of methylene blue employed, there is an incremental

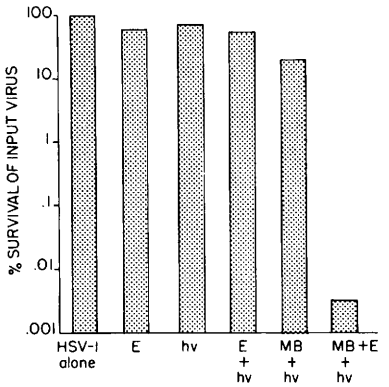

FIG. 2. Inactivation of HSV-1 (McIntyre) with methylene blue (MB), light and electricity. Input virus at a titer of 7×10^6 PFU/ml was transferred to a methylmethacrylate cell fitted with platinum electrodes and when appropriate, MB added to a final concentration of 10^{-5} M. Solutions of virus ± MB were irradiated with visible light at 197 $\mu W/cm^2$ (hv) and/or a 0.001 ampere current was applied, as described in Methods. Untreated or inactivated virus was assayed by the plaque titration method. Electricity = E; light = hv.

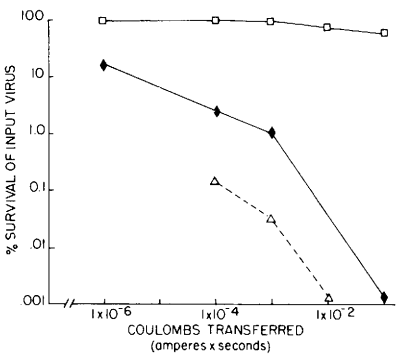

FIG. 3. Effect of varying the input of electricity and methylene blue on virus inactivation. The McIntyre strain of HSV-1 (7×10^6 PFU/ml) was employed in the inactivation system as described in Figure 1 and Methods. Electricity plus light, □—□; MB (10 μM), light plus electricity, ♦—♦; MB (2 mM), light, plus electricity, △-----△.

inactivation of HSV-1 as the coulombs transferred are increased. No antiviral effect occurs at either concentration in the absence of light and electricity (data not shown). At the maximal electrical input for each concentration of MB used, no infectious virus was observed at the limit of detection of the plaque assays employed. Fewer than 10 plaque forming units would not be detected in this assay; therefore 10 PFU/ml is taken as the lower limit of HSV present. This corresponds to more than 99.99% loss of infectivity. The inactivation of two clinical isolates of HSV-1 derived from cold sores shows the same dependence on dye concentration and electrical input (not shown) as does the prototype laboratory strain of virus.

Generation of superoxide anion by the interaction of methylene blue, light and electricity in virus-free solutions. Electrical reduction of MB in the presence of light and oxygen results in the formation of superoxide anion (O_2^-). To assess the generation of this reactive species and its dismutation with superoxide dimutase, epinephrine was employed to trap O_2^-, and in the process undergo oxidation to adrenochrome. The latter adsorbs light at a 480 nm wave length and is conveniently measured with a spectrophotometer (16).

When methylene blue and epinephrine are diluted with 10^{-4} M EDTA and phosphate buffered saline to final concentrations of 10^{-5} M and 2×10^{-4} M respectively, there is a minimal accumulation of adrenochrome. As shown in Fig. 4A, oxidation of epinephrine occurs after application of 3 V of electricity. Irradiation for 3 min with white light (198 $\mu W/cm^2$) results in a larger conversion of epinephrine, while light plus electricity substantially increases the production of adrenochrome.

To assess the generation of superoxide anions in these reactions, the epinephrine–methylene blue solution was mixed with an excess of superoxide dismutase (116 units/ml), and adrenochrome measured following exposure to light, electricity or both simultaneously. As demonstrated in Fig. 4B there is slight inhibition of conversion to adrenochrome when irradiation or current is applied independently. The oxidation of epinephrine is significantly inhibited by the dismutase when light and electricity are employed to-

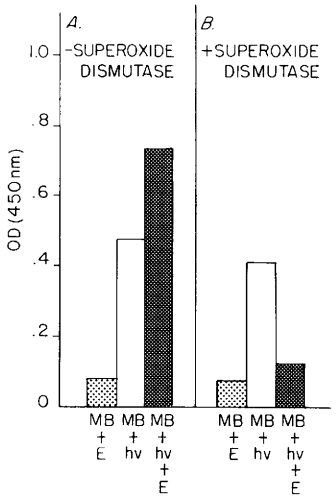

FIG. 4. Generation of superoxide anion (O_2^-) by methylene blue, light and electricity. A 10^{-5} *M* solution of MB in EDTA (10^{-4} *M*) and epinephrine (2×10^{-4} *M*) was exposed for 3 minutes to light alone, electricity without light or light plus electricity, in the presence or absence of superoxide dismutase (116 units/ml). At the conclusion of the treatment period the conversion of epinephrine to adrenochrome was measured by determining the optical density at 480 nm. MB + electricity (E), ▨; MB + light (hv), □; MB + light (hv) + electricity (E), ▩.

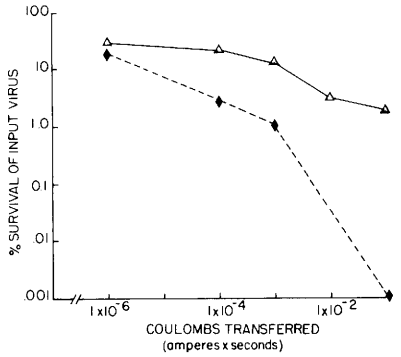

FIG. 5. Inhibition of viral inactivation by superoxide dismutase. Employing the inactivation conditions described in Fig. 1 and Methods, 7×10^6 PFU/ml of HSV-1 were adjusted to 10^{-5} *M* MB, and exposed to light and electricity in the presence or absence of superoxide dismutase, 116 units/ml. Methylene blue, light and electricity, ◆-----◆; methylene blue, light, electricity and superoxide dismutase, △—△.

gether. It is apparent that electrical reduction of methylene blue in the presence of light results in generation of superoxide anions more efficiently than either modality alone.

Role of superoxide anion in HSV inactivation. To determine the biological importance of superoxide anions for the inactivation of herpes simplex virus, the McIntyre strain of HSV-1 (7×10^6 PFU/m) was exposed to methylene blue, light and electricity (3 V) in the presence of an excess of superoxide dismutase (Fig. 5). An identical input of infectious virus was inactivated in the absence of this enzyme, and the percent survival in each plotted against the coulombs transferred. The loss of viral infectivity is again demonstrated to be a function of the amount of electricity applied. When superoxide dismutase is present a striking reduction in inactivation occurs at each level of electrical energy transferred. This suggests that the generation of superoxide anions is an important intermediate in viral inactivation secondary to the interaction between MB, light, electricity and HSV.

Discussion. In the current study, a modification of photoinactivation of herpes simplex viruses employing low concentrations of methylene blue, visible light and electricity is demonstrated to be an efficient method for abolishing viral infectivity. This is believed to be a direct antiviral effect and not the result of a toxic effect on cells making them resistant to viral infection because cells initially incubated with virus-free media exposed to dye, light, and electricity, were as susceptible to HSV infection as control cells. The combination of MB plus virus, at either concentration employed, did not have an antiviral effect in the absence of light and electricity. When compared with dye plus light alone under identical conditions of MB concentration, oxygenation and spectral irradiance, the simultaneous addition of transsample current for a brief time converts a 60% reduction in viral titer to 99.99%. Although all three modalities are necessary for viral inhibition, these studies do not exclude the possibility that prior treatment of herpes virions with methylene blue and light sufficiently sensitizes them, such that a subsequent dose of electricity results in a significant antiviral effect. Viral inactivation is directly dependent on electricity and more virus is inactivated as more coulombs (amperes × seconds) are applied. A similar dose-response relationship holds for the concentration of methylene blue at constant electrical input. The fall in viral

titer is not linear as the electrical input increases (Fig. 5). One interpretation of these findings may be the existence of several populations of virions varying in susceptibility to this type of inactivation.

When methylene blue is chemically reduced in the process of electron transfer from certain oxidative enzymes, it has the potential to interact with molecular oxygen and form superoxide anion (O_2^-) (16). Superoxide anion can also be produced electrolytically at a platinum cathode in buffered aqueous solutions following application of an electrical current (23), and detected either by its ability to oxidize epinephrine to adrenochrome, or its dismutation by superoxide dismutase (16, 24). When electricity is applied to an illuminated aerobic solution of methylene blue, there is not only the formation of significant quantities of O_2^-, but this is associated with a dramatic antiviral effect. Less O_2^- is produced as a result of the interaction of dye and light, and neither this technique nor electricity plus light without MB significantly reduce HSV plaque forming ability. The precise photochemistry defining the synergistic anti-HSV properties of MB, light and electricity have not been worked out. However, their interaction conforms to a type I photooxidation mechanism, as described by Foote (15). In this model, the sensitizer is raised to an electronically excited state by the absorption of light, and is then electrically reduced. Following reduction of dye, interaction with light no longer needs to occur as the reduced dye interacts with molecular oxygen to produce superoxide anions. Several possible reaction pathways for superoxide anions have been described including interaction with hydrogen peroxide to form hydroxyl free radicals, or with another O_2^- and 2 protons, to form H_2O_2 (23). These intermediates have the potential to induce single strand breaks in viral DNA, and inhibit the biological activity of either ϕx174 DNA or intact R17 bacteriophage (17–19). The partial inhibition of HSV inactivation by superoxide dismutase suggests that superoxide anion is central to the antiviral effect of MB, light and electricity.

The molecular sites in the herpesvirion vulnerable to this approach are unknown. Photodynamic inactivation of bacteriophage T4 with methylene blue and visible light results in damage to both the phage injection apparatus and DNA (25). This dye is capable of photoautoxidation of DNA, during which there is a specific and preferential destruction of guanine residues (12). Numerous sensitizers exert similar effects with nucleic acids from a variety of sources (13). The precise target(s) in herpes simplex virions that are vulnerable to the effects of MB, light and electrical treatment remain to be elucidated. Studies to define the role of reactive species in addition to superoxide anions, as well as to determine the biological and structural defects associated with loss of HSV infectivity are in progress.

Summary. A modification of photodynamic inactivation for herpes simplex viruses has been developed. Low concentrations of methylene blue, when electrically reduced in the presence of HSV, and irradiated with visible light, result in an efficient loss of plaque-forming ability. The antiviral effect is dependent upon the amount of electricity applied and the concentration of dye. Superoxide anion is an important intermediate since the photodynamic inactivation is partially inhibited by superoxide dismutase.

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