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Photodynamic Inactivation of Herpesvirus Hominis by Methylene Blue (38524)

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Genital and other infections caused by herpesvirus hominis have become increasingly common during recent years. The incidence of genital involvement by this agent among 12,647 cases seen in the Skin Clinic of the Tufts-New England Medical Center during 1972–73 was 0.8% (1). Therapy has presented considerable difficulty not only in eradicating primary mucocutaneous infection but also in decreasing their occurrence to an appreciable degree. Without simultaneous application of dimethyl sulfoxide, topical treatment with idoxuridine application has produced poor clinical results (2–4). A controlled study of the effect of locally applied neutral red plus light in patients with superficial herpetic lesions has indicated that the clinical course is shortened and the frequency of relapse reduced (5). Proflavine, a derivative of acridine, has produced similar results; however, a controlled study of the use of this agent has not been carried out. Methylene blue, a compound structurally resembling acridine, has been shown to produce photodynamic killing of a number of bacterial genera and species (6). It is not known, however, whether this dye acts on viruses in a similar manner.

The purposes of the present investigation were to (a) examine the photodynamic activity of methylene blue against herpesvirus hominis, (b) determine the sensitivity of clinical isolates of the virus to photoinactivation, and (c) study the development of resistance to photoinactivation by dyes and light. Because methylene blue, proflavine, and neutral red are all tricyclic compounds, the effects of these were also investigated.

Materials and Methods. In vitro sensitivity to photoinactivation. Thirty-seven fresh isolates of herpesvirus hominis, 29 recovered from genital and eight from oral lesions, were examined for sensitivity to proflavine, neutral red and methylene blue in the presence of light by the method described by Wallis and Melnick (7). The dye was dissolved in triple distilled water to make a 10^{-3} *M* solution, and sterilized by boiling for 15 min. Boric acid buffer (pH 9.0) was added in a volume to yield a dye concentration of 10^{-5} *M*. Dowex cation exchange resin, 50W-X4, 50–100 mesh hydrogen form was repeatedly washed with 0.85% NaCl until a pH of 6–6.5 was reached. It was then packed into 13 × 100 mm tubes to a height of 35 mm, sterilized by boiling for 15 min and washed repeatedly with sterile boric acid buffer at pH9 until pH of the supernatant was constant at 9.0. The fluid was then removed from the tubes.

The photosensitizing procedure was carried out in a room lighted by red lights. 4.5 ml of 10^{-5} *M* dye solution (pH9) was placed in a tube shielded by aluminum foil. One-half milliliter of virus was then added and the mixture incubated at 37° for 1 hr. A virus control was treated in similar fashion.

One-half of the supernatant (virus-dye complex) was stored in the dark, while the other half was exposed to a daylight fluorescent lamp (2 15-W tubes by Westinghouse) at a distance of 15 cm for 5 min.

The virus from both the exposed and non-exposed tubes was then titrated in primary human amnion cell cultures maintained in Eagle's minimal essential medium containing 2% fetal calf serum. All the cultures were covered by aluminum foil and incubated for 7 days before reading.

The development of resistance to photoinactivation. Twenty strains of the virus were tested for the development of resistance *in vitro* by repeated passage in tissue culture in the presence of neutral red, methylene blue or both. Dye–virus mixtures were studied in duplicate. One set was kept in the dark, while the other was exposed to 15-W fluorescent lamp at a distance of 15 cm for 10 min.

TABLE 1. *In Vitro* SENSITIVITY OF HERPESVIRUS TO METHYLENE BLUE, NEUTRAL RED AND PROFLAVINE.

Photosensitivity	Virus titer			Methylene blue	Neurtal red	Proflavine
	Control	dark	light			
High	6–8[a]	6–8	0	27[b]	26	2
Moderate	6–8	6–8	3–5	4	5	4
Low	6–8	6–8	5–7	6	6	2
Activity in dark	6–8	0–5	0–3	0	0	14
Total strains				37	37	22

[a] Range of TCD-50, No. of negative log.
[b] Number of strains of herpesvirus.

Excess dye was removed by cation exchange resin (Dowex 50-WX4). Tissue cultures containing virus alone served as controls. All three sets of cultures were titrated for infectivity in amnion cells after incubation at 37° for 7 days. Those that exhibited cytopathic changes after inoculation of a virus–dye mixture were passed once or twice in amnion cell cultures. After full infectivity was established, they were harvested and subjected to repeated study as outlined above until resistance to photoinactivation by neutral red, methylene blue or a mixture of both became apparent.

Results. In vitro photoinactivation of herpesvirus. The degree of photosensitivity of herpes virus to proflavine, neutral red and methylene blue was defined as follows: *Highly photosensitive*—no change of infectivity in the dark, but complete inactivation of virus in the presence of light. *Moderately sensitive* —greater than 100-fold reduction of viral infectivity after light exposure. *Least sensitive*—less than 100-fold reduction of viral titers in the light, as compared to the dark. Activity in dark was defined as a reduction of infectivity by more than 100-fold without exposure to light.

Thirty-seven strains of herpesvirus were examined for photosensitivity to methylene blue (Table I). Of these, 27 were highly and four moderately sensitive; six were least sensitive. Neutral red produced almost identical effects; two strains *moderately* sensitive to one dye were *highly* susceptible to the other. Cross-insensitivity was complete. Proflavine, on the other hand, behaved very differently. Unlike methylene blue or neutral red, it was active against 14 or 22 strains of the virus; 12 were completely inactivated. Two strains were very sensitive to proflavine; 2 were least sensitive to this dye but moderatelv sensitive to the others.

Photosensitivity to combined dyes. Twenty-eight strains of herpesvirus were examined for photosensitivity to a combination of neutral red and methylene blue as well as to each dye alone. The mixture of the dyes did not increase the sensitivity of most of the strains susceptible to the individual agents; however, four strains which were moderately sensitive to one or the other dyes alone became highly susceptible when treated with both simultaneously. Strains least sensitive to methylene blue or neutral red were no more susceptible when exposed to these agents at the same time.

Development of resistance. Of 20 photosensitive strains repeatedly cultured in the presence of methylene blue, neutral red or a combination of both *in vitro*, 16 became resistant after one and the rest after two passages. The combination of both dyes did not prevent the development of resistance.

Discussion. Methylene blue, tetramethylthionine is a phenothiazine derivative closely related chemically to neutral red, a phenozine compound, but differs from proflavine which is a congener of acridine. This may account for the similarity or difference in their photosensitizing activity.

Seventy-three percent of strains of herpesvirus were found to be totally inactivated by exposure *in vitro* to methylene blue and light in the present study; 16% were least sensitive. The antiviral effect was not produced in the dark, but was maximal when exposure to light was added. Almost identical results

were produced by neutral red. Proflavine, unlike the other dyes, was active in the dark for more than half of the strains studied. The incidence of insensitivity to this agent was less than that for methylene blue or neutral red. Strains with low sensitivity to proflavine were sensitive to the two other agents in the presence of light. Because the degree of photosensitivity produced by methylene blue and neutral red was approximately the same, it was not surprising to find that come bining them did not increase photosensitivity or prevent the development of resistance. Despite this, resistance to each agent alone or to a combination of them developed rapidly *in vitro*. These *in vitro* observations indicate that the clinical application of light-dye therapy with either methylene blue or neutral red will be most likely unsuccessful if the strains of virus involved are only slightly sensitive to these agents in the presence of light *in vitro*.

The mechanism of varying degrees of viral sensitivity and induced viral resistance to photoinactivation is not clear. It has been demonstrated that the dye combines with the guanine base portion of nucleic acid and that, on light exposure, deletion of the dye-guanine complex occurs (8, 9). Resistance may be associated with the failure of either complex formation or subsequent deletion.

Although methylene blue is not a vital dye, it enters living cells. (Unpublished observation). Methylene blue stains primarily the nucleus, but it does not persist in the cells when the medium of the cultured cells is kept free of the dye.

Conclusion. Methylene blue, in a concentraion of 10^{-5} *M* was virostatic in the presence of light but not in the dark for 31 of 37 strains of fresh isolates of herpesvirus hominis. Resistance to the dye developed during treatment. This photodynamic pattern was almost identical to that of neutral red which produced an identical effect. This was not true for proflavine which was active in the dark in many instances. Cross insensitivity between proflavine and methylene blue was not observed.

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