Tetracycline-mediated Photodynamic Inactivation of Animal Viruses

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SUMMARY

Demethylchlortetracycline (DMCT), doxycycline and, to a lesser extent, chlortetracycline were capable of mediating the in vitro photoinactivation of Venezuelan equine encephalitis (VEE) virus. Other tetracyclines tested were found to be inactive in this respect. However, no correlation between chemical structure and photosensitizing activity could be established. The photoinactivation of VEE virus by DMCT proceeds through a photodynamic mechanism as shown by the absolute requirement of O₂ for the inactivation to take place. The photoinactivating effect of DMCT was also exerted upon other animal viruses tested, i.e. vesicular stomatitis virus, herpes simplex virus and poliovirus, even when, in the case of poliovirus, the capsid seems to be impermeable to the tetracycline. The fact that the two most effective photosensitizing tetracyclines for VEE virus are also the drugs more frequently associated with drug-induced phototoxicity in humans, suggests that virus photoinactivation could be used as a screening procedure for potentially phototoxic drugs developed for human application.

INTRODUCTION

Viruses can be rendered sensitive to visible light when treated with dyes such as acridine orange, neutral red and others (Wallis & Melnick, 1965). The phenomenon of photosensitization has been described in a variety of biological systems, including drug-induced photosensitivity in humans (Harber et al. 1961). Treatment with the antibiotic demethylchlortetracycline (DMCT) results in a high frequency of sun-induced phototoxic reactions (Harber et al. 1961; Blank et al. 1968), and we have previously described how DMCT, and to a lesser extent chlortetracycline, were capable of mediating the in vitro photoinactivation of Venezuelan equine encephalitis (VEE) virus (Esparza et al. 1976). In this communication we extend that observation to other animal viruses and provide some evidence that the inactivation occurs through a photodynamic process.

METHODS

Viruses and virus assays. The following viruses were used in the present study: Venezuelan equine encephalitis (VEE) virus, TC-83 strain, subjected in our laboratory to four additional passages in Vero cells (Esparza et al. 1976); vesicular stomatitis virus (VSV), Indiana strain, carried in our laboratory for several years (Bergold & Munz, 1967; Lastra & Esparza, 1976); herpes simplex virus (HSV) type 1, KOS strain (Smith, 1964); and poliovirus type 2, a local strain obtained from R. Mazzali, from the Instituto Nacional de Higiene, in Caracas.

Virus stocks were prepared in Vero cells, as follows: monolayers of Vero cells, maintained in disposable tissue culture flasks (Corning Glass Works, Corning, N.Y.) were inoculated using a multiplicity of infection of 0·1. After 1 h adsorption at 37 °C, infected cultures were

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washed once with phosphate-buffered saline (PBS). Cells were maintained in Eagle's medium with a double concentration of vitamins and amino acids, 2% lactalbumin hydrolysate, 0.66% yeast extract, 0.16% sodium bicarbonate, 1.4% bovalbumin (fraction V), 100 units of penicillin per ml, 100 μg of streptomycin per ml and 2.5 μg of amphotericin B per ml (Bergold & Mazzali, 1968). Infected cultures were incubated at 37 °C for 1 to 3 days, when complete c.p.e. was evident. Viruses were harvested by one cycle of freezing and thawing, and clarified by centrifugation at 2000 rev/min for 15 min. Virus infectivity was assayed in Vero cells by a plaque method using an agarose-containing, serum-free overlay (Bergold & Mazzali, 1968).

Drugs. The following tetracyclines were used: demethylchlortetracycline and minocycline HCl, obtained from Cyanamid de Venezuela, Caracas; oxytetracycline and doxycycline, obtained from Pfizer S.A., Valencia, Venezuela; tetracycline crystalline and tetracycline hydrochloride were purchased from Sigma Chemical Company, St Louis, Mo. Neutral red HCl (Lot 1243500, BDH Chemicals Ltd., Poole, England) was used as a control. All drugs were prepared in PBS, and their final pH ranged between 6.6 and 7.1.

Light irradiation. In most experiments viruses were grown in the absence of tetracyclines and mixed with the drug immediately before exposure to light. In the experiments with poliovirus, the tetracycline was added to the maintenance medium during virus multiplication. After the addition of the drug, all handling of the cultures was done in the dark. For irradiation, samples were dispensed in 1 ml samples into glass test tubes and exposed 10 cm from three daylight-type fluorescent lighting tubes (15 W each), giving a total of 5000 lux of illumination (Esparza et al. 1976). All experiments were done at room temperature (22 °C). After light exposure, samples were immediately assayed for virus infectivity.

RESULTS

Photosensitization of VEE virus by different tetracyclines

When VEE virus was mixed with DMCT at a final concentration of 100 μg/ml, its exposure to light resulted in a rapid inactivation of the virus infectivity (Fig. 1). After 60 min of irradiation, a drop in the infectivity titre of more than 6 logs was observed, while less than 2 logs reduction occurred in control samples, which were irradiated in the absence of DMCT.
Table 1. Photoinactivation of Venezuelan equine encephalitis virus mediated by different tetracyclines

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inactivation velocity constant (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.013</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.022</td>
</tr>
<tr>
<td>Tetracycline-HCl</td>
<td>0.028</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.034</td>
</tr>
<tr>
<td>Tetracycline-crystalline</td>
<td>0.047</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>0.080</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.172</td>
</tr>
<tr>
<td>Demethylchlortetracycline</td>
<td>0.187</td>
</tr>
</tbody>
</table>

The observed drop in infectivity in control samples could have been due to photosensitizing agents present in tissue culture medium (Appleyard, 1967).

Although DMCT was the best drug to induce photosensitization of VEE virus, other tetracyclines were found to be active in that respect. Table 1 shows the velocity of photoinactivation of VEE virus mediated by different tetracyclines. The results are expressed as the inactivation velocity constant, calculated from the equation

$$K = \frac{(-2.3) \log P_0}{t},$$

where $P_0$ is the initial concentration of infectious virus, $P$ is the final concentration, $t$ is the time in min and $K$ is the inactivation constant as min⁻¹ (Zhdanov & Yershov, 1965). Doxycycline was almost as effective as DMCT mediating the in vitro photoinactivation of VEE virus. As previously reported, chlortetracycline was also effective, but to a lesser degree (Esparza et al. 1976). The other tetracyclines tested were found not to be efficient photosensitizing agents for VEE virus.

**Dose-response of DMCT-mediated photoinactivation of VEE virus**

The optimal concentration of DMCT for the photoinactivation of VEE virus was found to be 100 µg/ml. Fig. 2 shows that higher or lower concentrations resulted in a marked decrease in the photoinactivating effect. Pre-incubation of virus in DMCT did not change the results obtained.

**Oxygen requirement for the DMCT-mediated photoinactivation**

Because molecular oxygen is essential for photodynamic inactivation, we tested the effect of the dissolved O₂ on the photoinactivation of VEE virus by DMCT. Virus-drug mixtures were flushed with air or N₂ and then light irradiated, while kept in the same atmosphere. Fig. 3(a) clearly shows that the rate of photoinactivation is markedly decreased when the O₂ is replaced by N₂. Neutral red, a dye known to inactivate viruses through photodynamic effect (Hiatt, 1960; Wallis & Melnick, 1965) was used as a control (Fig. 3b).

**Reversibility of the DMCT-mediated virus photosensitivity**

The photosensitization of VEE virus by DMCT is a reversible phenomenon. In one experiment, VEE virus was mixed with DMCT at a final concentration of 100 µg/ml and incubated for 15 min at 37 °C. The virus-DMCT mixture was then diluted 1/10 in PBS, pelleted in the ultracentrifuge (55000 g for 4 h) and resuspended in either PBS or PBS containing 100 µg/ml of DMCT; samples were irradiated together with a control sample of virus which was not treated with the drug. The inactivation constant of the virus resuspended in PBS-DMCT was 0.302 min⁻¹, while the virus which was resuspended in PBS
Fig. 2. Dose–response curve of DMCT-mediated photoinactivation of VEE virus. Viruses were mixed with different concentrations of the drug and exposed to light for 60 min before titration.

Fig. 3. Effect of dissolved O₂ on the photoinactivation of VEE virus by (a) DMCT and (b) neutral red. ○—○, Virus–drug mixtures flushed with N₂; ●—●, virus–drug mixtures flushed with air.

alone had an inactivation constant of 0.064 min⁻¹, very similar to that of the control sample (0.070 min⁻¹).

**Photosensitization by DMCT of other animal viruses**

The photoinactivating effect of DMCT was also exerted with other animal viruses tested. Both vesicular stomatitis virus, a single-stranded RNA virus (Fig. 4a) and herpes simplex virus, a double-stranded DNA virus (Fig. 4b) were readily photoinactivated when mixed with DMCT. However, mixing of poliovirus with the drug does not result in photosensitization (Fig. 5a), while poliovirus grown in the presence of 100 µg/ml DMCT was found to be photosensitive (Fig. 5b).
Tetracycline-mediated virus photoinactivation

Photodynamic inactivation of viruses has been extensively studied using acridine related dyes as sensitizing agents (Hiatt, 1960; Wallis & Melnick, 1965; Chang & Weinstein, 1975). However, a number of other pharmacological agents such as tetracyclines, chloropromazine, furocumarins, sulphonamides, etc., are capable of producing photosensitivity reactions in man and laboratory animals (Baer & Harber, 1965). Drug-induced photosensitivity occurs through photoallergic or phototoxic reactions, the latter being in some cases of the photodynamic type (Harber & Baer, 1972). Our results indicate that some tetracyclines can mediate the inactivation of animal viruses by a photodynamic mechanism. This was clearly shown by the absolute requirement of O₂ for the photoinactivation to take place. Only three of the tetracyclines tested, DMCT, chlortetracycline and doxycycline (a non-chlorinated derivative of tetracycline) were found to be efficient photosensitizing agents for VEE virus. However, no correlation between chemical structure (Fig. 6) and photosensitizing activity was evident, neither as chromophores or by stereochemical requirements. The same difficulties have been found in relating tetracycline structure with antimicrobial activity (Hlavka & Boothe, 1973). The range of concentrations of DMCT capable of mediating photosensitization of VEE virus was found to be narrow. Concentrations of at least 50 μg/ml were needed,
with a very sharp optimum of 100 μg/ml. Higher concentrations of DMCT failed to photosensitize viruses, probably due to over-saturation and light-shielding effects. Any possible use of systemically administered DMCT for topical phototherapy in humans seems difficult, since the maximum serum concentrations obtained normally are of the order of 10 μg/ml (Frost et al. 1972).

The detailed mechanism(s) of action of the photodynamic inactivation of animal viruses by tetracyclines remains to be understood. The site of action of this drug seems to be located in the interior of the virus capsid, probably at the level of the nucleic acid. In support of this is the fact that polioviruses, whose capsid is usually impermeable to neutral red can be made photosensitive if the dye is allowed to be 'encapsidated' when added at the time of virus multiplication (Schaffer, 1962). The same behaviour of poliovirus was observed when DMCT was used as the sensitizing agent. No attempt was made to study the possible interference of organic components on the photosensitization of poliovirus by DMCT (Wallis & Melnick, 1963). In addition, the haemagglutinating ability of VEE virus was not decreased by DMCT-mediated photoinactivation (data not shown), indicating that virus surface glycoproteins were not grossly altered. Similar observations have been made in a number of viruses inactivated by acridine dyes (Frank & Foster, 1967; Wallis et al. 1967).

Preliminary experiments failed to show any change on the sedimentation coefficient of genomic RNA in DMCT-photoinactivated VEE virus as compared with untreated controls. Similarly, neutral red-mediated photoinactivation of poliovirus does not result in gross change in the structure of the virus RNA (Wilson & Cooper, 1965), an observation also made with tobacco mosaic virus (Murphy, 1975). Thus, damage induced by DMCT-mediated photodynamic inactivation does not seem to occur through RNA chain breaks; it is conceivable that the photo-oxidation of virus RNA results in mutagenesis by introducing changes in selected nucleotide residues (Singer & Fraenkel-Conrat, 1966).
The demonstration of tetracycline mediated virus photosensitization indicates that this biologically simple system could be useful in the study of the mechanism of drug-induced phototoxicity. It should be pointed out that the two more effective photosensitizing tetracyclines for VEE virus (DMCT and doxycycline) are also the drugs which have been shown to be associated more frequently with drug-induced phototoxicity in humans (Blank et al. 1968; Frost et al. 1972). These results suggest that virus photoinactivation could be used as a screening procedure for potentially phototoxic drugs developed for human application.

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